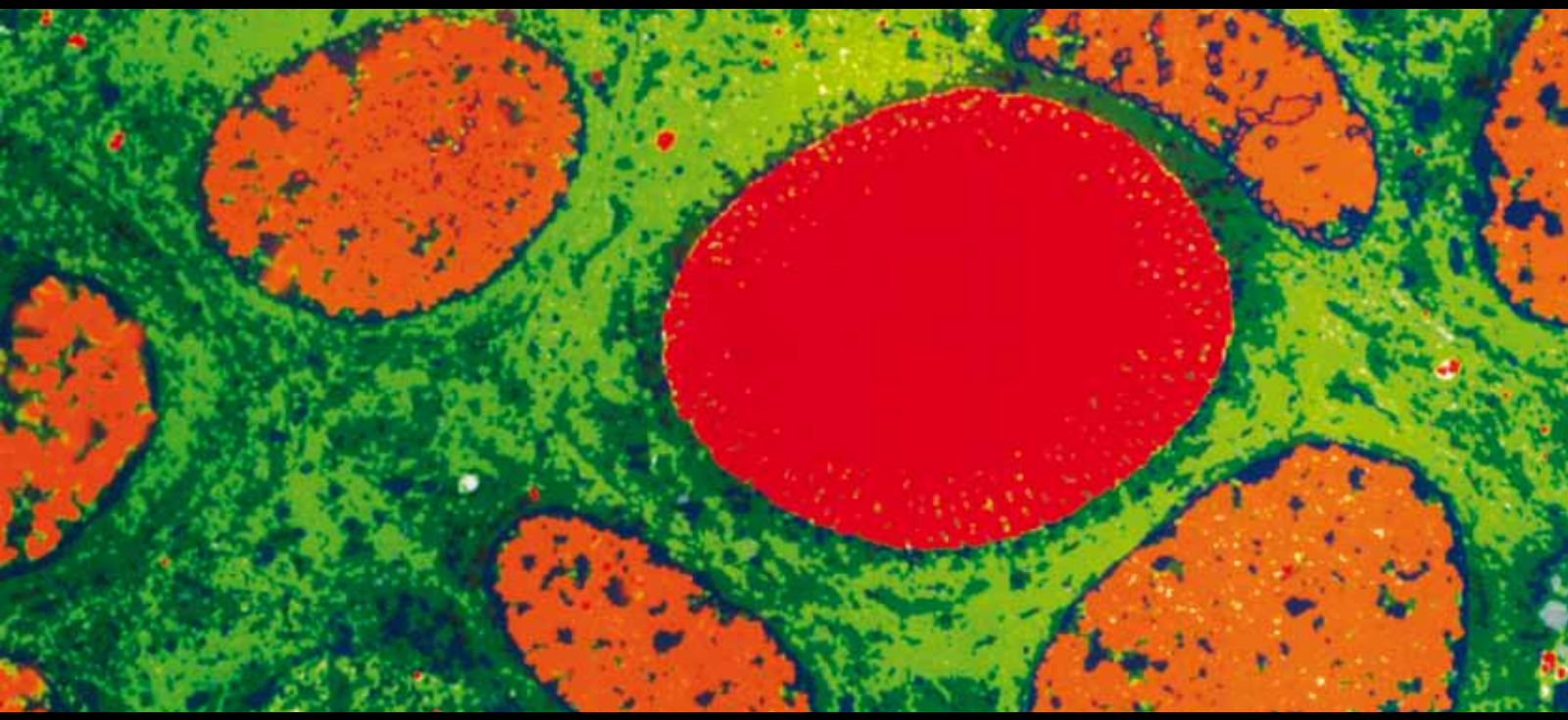
International Journal of Breast Cancer

Breast Axillary Lymph Node Metastasis

Guest Editors: Luciane R. Cavalli, Rachel E. Ellsworth, Christoph Klein, and Giuseppe Viale



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Editorial Board

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Editorial

Breast Axillary Lymph Node Metastasis

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The surgical management of breast cancer has evolved over the years from extensive radical mastectomy to breast conservation surgery. Until the introduction of the sentinel lymph node biopsy (SLNB), all patients with invasive breast cancer would undergo complete axillary lymph node dissection (ALND) and thus be at risk of suffering from its associated high morbidity. SLNB has become the standard of care and represented significant progress toward reducing the invasive procedures for the management of the axilla. The most recent clinical trials (NSABP-B32 and ACOSOG Z0011) performed in patients that underwent SLNB support this procedure as an accurate predictor of the risk of further axillary node involvement and of breast cancer recurrence. Additionally, the ACOSOG Z0011 trial challenged the standard of practice in the management of the axilla, in which ALND is mandated for all the patients with a positive sentinel node. A similar outcome was demonstrated for a selected group of patients (treated with breast conserving surgery and radiotherapy) with positive SLNB then followed by ALND or SLNB only. In light of these results, the role of ALND in the current management of breast cancer is being reevaluated for specific patient subpopulations. In this special issue, this timely subject is provocatively reviewed in addition to other relevant topics, such as the controversial meaning of the presence of micrometastasis and isolated tumor cells in the SLN in relation to local recurrence and overall survival and the feasibility of performing SLNB after neoadjuvant treatment and transaxillary breast augmentation.

Despite the advances in the lymphatic mapping and in the intraoperative methods for SLN analysis, the accurate identification of tumor cells in this node continues to be a challenge in clinical practice, and significant false-negative results in SLNB are still observed. In a research study published in this issue, a preferential cellular distribution of the malignant cells in the SLN is reported, suggesting that the pathological analysis directed to this area may contribute to a more precise identification of nodal metastasis. Additional progress in this direction involves the development of molecular markers, which would tackle not only the misdiagnosed SLN-negative patients but also the ones with low risk of recurrence that are unnecessarily submitted to SLNB. In this sense, the Bayesian-based nomogram developed by Westover et al. is proposed to be particularly useful, especially in cases where the SLNB assessment is predicted to be less sensitive. This nomogram would also lead to the identification of high-risk individuals for recurrence, based on the calculation of residual axillary disease risk, despite a negative SLNB.

Several new reports, mostly based on gene expression profiling, have suggested that the different rates of recurrence can be due to the distinct molecular types of breast cancer. The development of a genomic signature that effectively discriminates patients by lymph node status, as the one proposed by Ellsworth et al., could stratify patients based on their need of surgical evaluation of the lymph nodes, sparing the ones in which disease will probably be limited to its primary site. The assessment of specific tumor markers in the

lymph nodes is also discussed in this special issue, including a review of the prognostic and/or predictive implications of lymph node metastasis in tumors with elevated levels of CXCR4 (a protein chemokine receptor) and VEGF-C (a vascular endothelial cellular growth factor).

From the Halsted radical mastectomy to the commercial gene expression profiling tests, axillary lymph node management and recurrence prediction are still evolving topics for patients with breast cancer. The continued improvement of molecular tumor profiling and bioinformatics from larger and better defined patient cohorts will certainly provide answers to many challenging questions regarding the axillary metastatic process. The clarification of this complex molecular mechanism and the identification of novel and integrative molecular markers that can reliably predict lymph node involvement that will affect risk of recurrence and survival will continue to form the basis of the contemporary approach for breast cancer management, where an early prediction of axillary metastasis and a personalized cancer treatment can be achieved.

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Research Article

Lymphangiogenesis and Axillary Lymph Node Metastases Correlated with VEGF-C Expression in Two Immunocompetent Mouse Mammary Carcinoma Models

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Lymphangiogenesis and the expression of vascular endothelial cell growth factor C (VEGF-C) in tumors have been considered to be causally promoting lymphatic metastasis. There are only a few studies on lymphatic metastasis in immunocompetent allograft mouse models. To study the relationship between VEGF-C-mediated lymphangiogenesis and axillary lymph node metastasis, we used two mouse mammary carcinoma cell lines; the BJMC338 has a low metastatic propensity, whereas the BJMC3879 has a high metastatic propensity although it originated from the former cell line. Each cell line was injected separately into two groups of female BALB/*c* mice creating *in vivo* mammary cancer models. The expression level of VEGF-C in BJMC3879 was higher than BJMC338. As the parent cell line, BJMC3879-derived tumors showed higher expression of VEGF-C compared to BJMC338-derived tumors. This higher expression of VEGF-C in BJMC3879-derived tumors was associated with marked increase in infiltrating macrophages and enhanced expression of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) reflecting increased tumoral lymphatic density and subsequent induction of axillary lymph node metastasis. Our mouse mammary carcinoma models are allotransplanted tumors showing the same axillary lymph node metastatic spectrum as human breast cancers. Therefore, our mouse models are ideal for exploring the various molecular mechanisms of cancer metastasis.

1. Introduction

Based on clinical and pathological observations in human mammary carcinomas, the metastatic spread of mammary carcinoma cells is responsible for the majority of cancer deaths [1–5]. The common pathway of initial cancer dissemination is via lymphatics due to their characteristic endothelial structure with blind-ending capillaries [6]. In addition, metastasis to the regional lymph nodes through the lymphatic vessels is considered to be a common step in the progression of cancer and an important prognostic factor in many types of cancer including breast carcinomas. Lymphatic vessel density (LVD) in many types of solid cancer is associated with lymph node metastasis or poor prognosis, as has been reported in experimental and clinical studies [1–5, 7, 8]. Although mammary carcinoma is well known to have the character for lymph node metastasis, there are only a few mouse mammary carcinoma models showing extensive metastasis to lymph nodes. The chick embryo chorioallantoic membrane [9] and immunodeficient mice, SCID mice, or nude mice were used as xenotransplanted host animals to examine metastasis [10–12]. Recent studies have shown the important roles of tumor-associated macrophages (TAMs) expressing CD68 in cancer-mediated lymphangiogenesis [13–15]. Accordingly, the mouse immunocompetent model appears to be necessary for studying lymphangiogenesis and lymph node metastasis.

The vascular endothelial growth factor C (VEGF-C) is a major lymphangiogenic factor. There is some evidence that VEGF-C promotes lymphangiogenesis under several

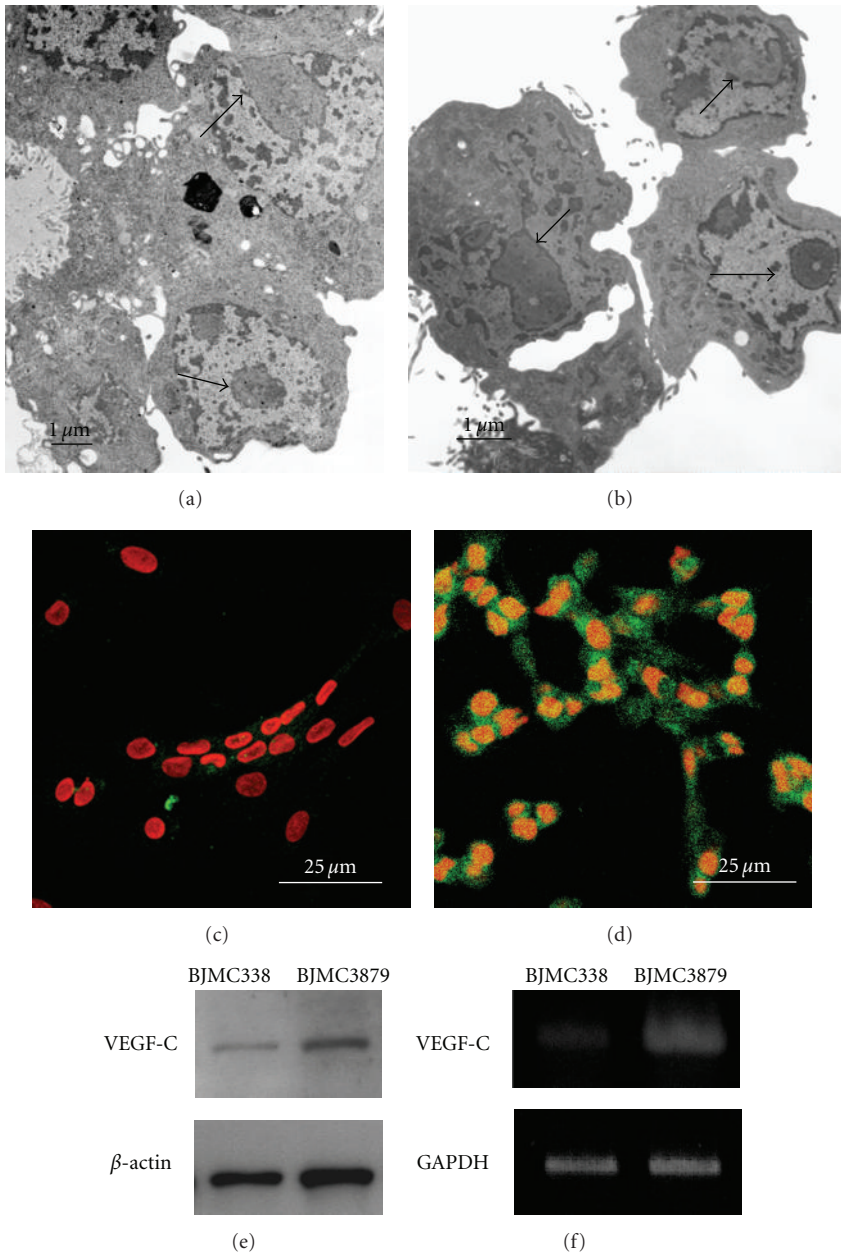

Figure 1: TEM micrographs (a and b), immunofluorecent staining (c and d), Western blot analysis (e), and RT-PCR analysis (f) of VEGF-C in the two-mouse mammary carcinoma cell lines. Both BJMC338 cells (a) and BJMC3879 cells (b) have the similar ultrastructure. BJMC338 cells (c) have minimally any activity of VEGF-C, whereas BJMC3879 cells (d) have moderate expression. Western blot analysis (e) demonstrates the same activity of VEGF-C as in (c and d). VEGF-C mRNA expression in BJMC3879 cells is higher than that of BJMC338 cells (f). Green fluorescence (FITC) indicates activity of VEGF-C, and red fluorescence (PI) shows nuclei of cells in (c and d).

normal and pathological conditions [16]. In VEGF-C-deficient mouse embryos, lymphatic vessels fail to develop from veins [17] resulting in prenatal death owing to fluid accumulation in the tissues of mouse embryos and edema in adults [18]. On the other hand, in VEGF-C transgenic mice, hyperplasia of lymphatic vasculature has been reported [19, 20].

To study the relationship between lymphangiogenesis mediated by VEGF-C and axillary lymph node metastasis, two-mouse mammary carcinoma cell lines with different metastatic properties were used in this study. Both cell lines were derived from the same BALB/*c* mouse, one of them, the BJMC338 cell line, an adenocarcinoma cell line, has a low metastatic propensity, whereas the other cell line, BJMC3879, has a high metastatic propensity, particularly to lymph nodes and lungs [21]. Each cell line was injected separately into two groups of adult female BALB/*c* mice creating *in vivo* mammary cancer models. In this inoculation study, we found that increased expression of tumor-derived VEGF-C correlates with LVD and axillary lymph node metastasis.

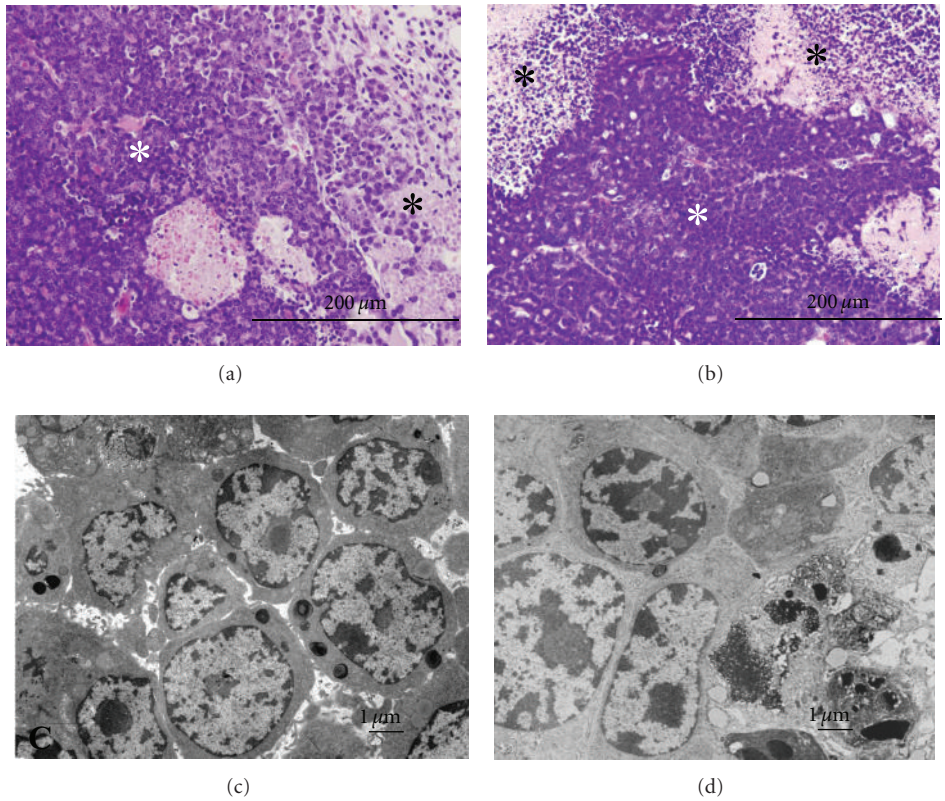

Figure 2: Histopathology of the inoculated tumors at 10 weeks postinoculation. H&E staining (a and b) show viable region (white ∗) and necrotic region (black ∗) in BJMC338 tumor (a) and BJMC3879 tumor (b). TEM micrographs (c and d) indicate tumor cells in the viable region of BJMC338 tumor (c) and BJMC3879 tumor (d).

2. Materials and Methods

2.1. In Vitro Studies

2.1.1. Cell Culture and Cell Preparation. Two-mouse mammary carcinoma cell lines were used in this study. The BJMC338 mammary adenocarcinoma cell line used in this study was derived from a female BALB/*c* mouse infected with mouse mammary tumor virus (MMTV) into the inguinal mammary glands and shows low metastatic property. The BJMC3879 mammary adenocarcinoma cell line was derived from foci within metastatic lymph node and lung of a female BALB/*c* mouse that had been injected with BJMC338 cell line into the right inguinal region. The BJMC3879 mammary cell line shows a high metastatic propensity, especially to lymph nodes and lungs [22]. Both cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS) with streptomycin/penicillin in an incubator under 5% CO_2. The cells were immediately rinsed with 0.01 M phosphate buffered saline (PBS), fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) for 1 h, for transmission electron microscopy (TEM). Cells were subsequently postfixed in 1% OsO_4 for 45 min at room temperature, dehydrated in a series of graded ethanol concentrations, cleared in propylene oxide, and embedded in an epoxy resin mixture. For immunofluorescent study, cells were fixed with 1% paraformaldehyde in PBS for 10 min.

Table 1: Number of mice which have lung and axially lymphnode metastases. Metastases were confirmed by H&E staining.

Lung	4W	6W	8W	10W
BJMC338	0	0	0	0
BJMC3879	0	0	5	5
Axially lymph node	4W	6W	8W	10W
BJMC338	0	0	0	0
BJMC3879	0	5	5	5

2.1.2. TEM. After preparation, ultrathin sections were prepared and stained with uranyl acetate and lead citrate. Sixty nm sections were examined by TEM using H-7100 and H-7650 (Hitachi, Tokyo, Japan).

2.1.3. Immunofluorescent Study. Following fixation, cells were transferred to PBS for 15 min, followed by exposure to 1% Block Ace (Dainippon Sumitomo Pharma Co., Ltd., Tokyo, Japan) for 20 min to block nonspecific antibody binding. Cells were then incubated with a primary rabbit anti-VEGF-C antibody (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, Calif, USA) for 1 h at room temperature (RT). After rinse in PBS for 15 min, cells were incubated with a secondary fluorescein-isothiocyanate- (FITC-) conjugated anti-rabbit antibody (Dako, Carpenteria, Calif, USA) for

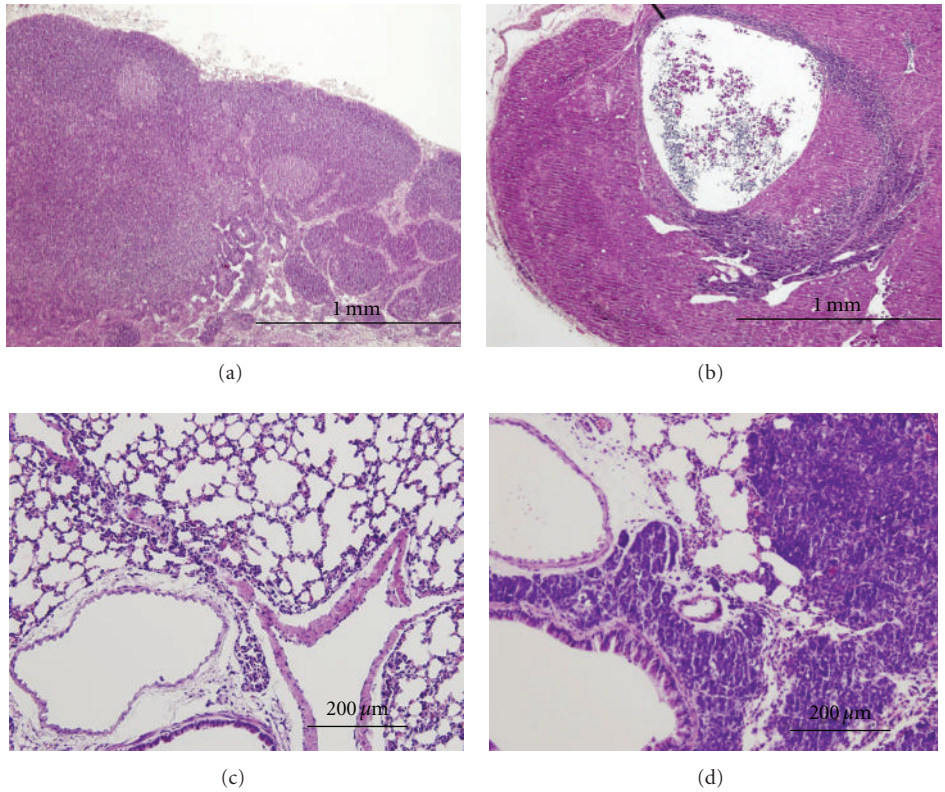

Figure 3: Histopathology by H&E staining of axially lymph nodes (a and b) and lungs (c and d) in mice at 10 weeks postinoculation. Axially lymph node and lung in BJMC338 tumor (a and c) show normal appearance, whereas those of BJMC3879 tumor (b and d) demonstrate metastatic foci.

30 min at RT. Samples were counterstained with propidium iodide (PI) for 15 min and observed under a model Radiance 2000 MP confocal scanning laser microscopy (Bio-Rad, Hercules, Calif, USA).

2.1.4. Western Blot Analysis for VEGF-C Protein. Samples containing 20 μg of protein from cultured cells and tumors were fractionated in 10% Tris-glycine gels under reducing conditions and transferred onto nitrocellulose membrane. Anti-VEGF-C antibody (Santa Cruz Biotechnology) was applied to membranes, incubated with appropriate horseradish peroxidase-conjugated secondary antibody, and visualized on X-ray films using enhanced chemiluminescence (Perkin Elmer Life Science, Inc., Boston, Mass, USA).

2.1.5. RNA Preparation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from cultured BJMC338 and BJMC3879 cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA Synthesis Kit (Roche Diagnostic Kit, Hilden, Germany) following the manufacturer's protocol, with the total mRNA concentration adjusted to 5 μg/μL in each sample. Primer sequences for VEGF-C were 5′-CCTTCTTTAAACCTCCATGTGT-3′ and 5′-GCAAAACTGATTGTGACTGGT-3′; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as internal control 5′-TGCACGGGAAGCTCACTGG-3′, 5′-TCCACCACCCTGTTGCTGTA-3′ (Nihon Gene Research Laboratories, Sendai, Japan). Products were amplified in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, Calif, USA) with preincubation at 98°C for 3 min followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s. The amplification was finished with a single-3 min incubation at 72°C. The PCR products were separated on 1.5% agarose gels, stained with 0.1 mg/mL ethidium bromide, visualized by UV transillumination, and documented on black and white instant films.

2.2. In Vivo Studies

2.2.1. Animals and Preparation of Tumors. A total of 40 female 6-week-old BALB/*c* mice (Japan SLC Inc., Hamamatsu, Japan) were used in this study. All manipulations of mice were performed in accordance with the procedures outlined in the Guide for Care and Use of Laboratory Animals in Osaka Medical College. Mice were divided into two groups (20 mice each), and BJMC338 and BJMC3879 cells (5×10^6 cells/0.3 mL in PBS) were inoculated subcutaneously into right inguinal region, respectively. Mice were sacrificed at 4, 6, 8, 10 weeks after inoculation under ether anesthesia. Tumor samples were frozen for Western blot assay and RT-PCR. Another one was fixed for histopathology and immunohistochemistry with 4 or 10% paraformaldehyde, and for electron microscopy in a same fixative as *in vitro* study. Lungs and right axillary lymph nodes of mice were

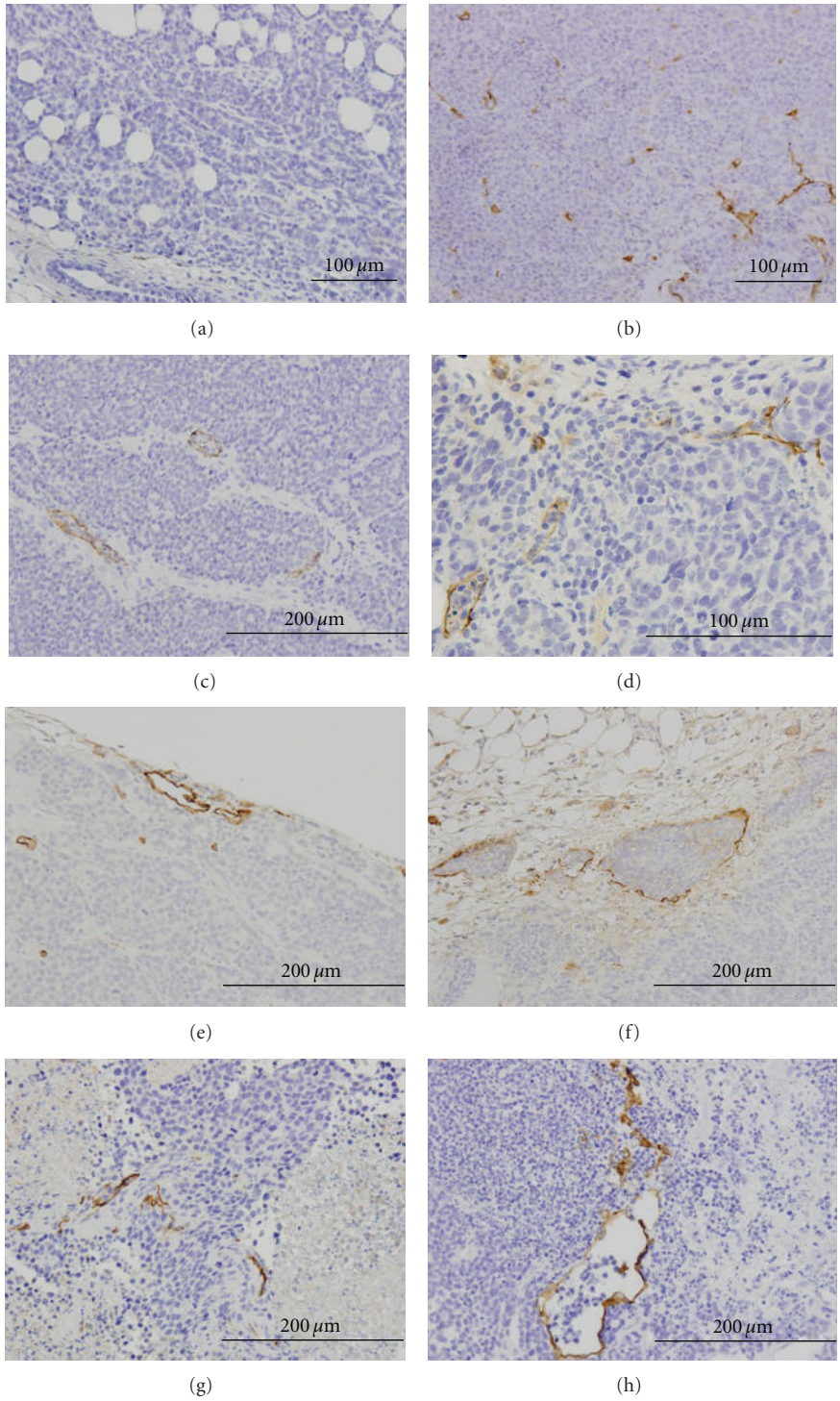

Figure 4: Immunohistochemistry of LYVE-1 in BJMC338 tumors (a, c, e, and g) and BJMC3879 tumors (b, d, f, and h) at 4 weeks (a and b), 6 weeks (c and d), 8 weeks (e and f), and 10 weeks postinoculation (g and h). Note the upregulation of LYVE-1 in BJMC3879 tumors.

removed and then fixed as same as tumors. Samples fixed with paraformaldehyde were processed through to paraffin embedding.

2.2.2. Histopathology and Immunohistochemistry. To examine histopathology and metastasis, tumors, lungs, and light axillary lymph nodes were stained with Hematoxylin and Eosin (H&E). Ultrastructural features of tumors were checked under TEM. The avidin-biotin complex method was used for immunohistochemistry. To visualize lymphatic vessels, tumors were stained with primary antibodies to the lymphatic-specific marker, lymphatic vessel endothelial

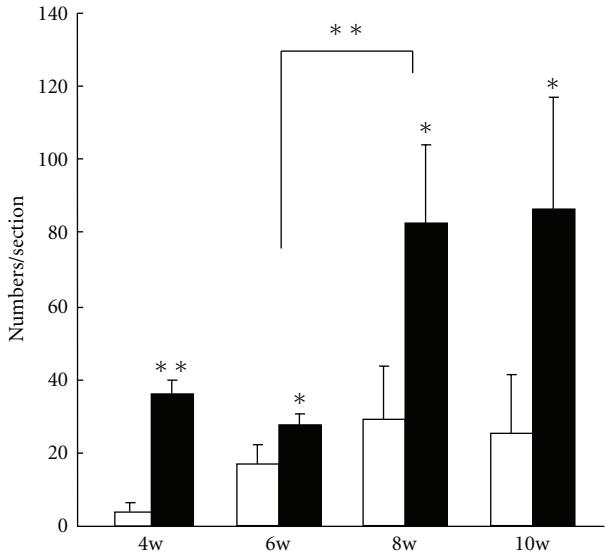

Figure 5: Density of lymphatic vessels (LVD) in tumors. The LVD in BJMC3879 tumors (black bars) is always significantly higher than that of BJMC338 tumors (white bars) ($^*P < 0.01$, $^{**}P < 0.001$).

hyaluronan receptor-1 (LYVE-1) (rabbit polyclonal, Acris Antibodies GmbH, Hiddenhausen, Germany). VEGF-C (Santa Cruz) was labeled on tumor sections, and activated macrophages were demonstrated by CD68 antibody (rat anti-mouse CD68, AbD Serotec, Oxford, UK).

2.2.3. Lymphatic Vessel Density (LVD) and Counting of Macrophage. The number of lymphatic vessels immunolabeled with anti-LYVE-1 antibody in tumor sections at 4 to 10 weeks postinoculation as well as the number of CD68-positive cells in tumor sections at 4 weeks postinoculation were counted under light microscopy at higher magnification (×200).

2.2.4. Statistical Analysis. The above-mentioned data were analyzed by Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. In Vitro Studies

3.1.1. Ultrastructure and Expression of VEGF-C. Ultrastructural differences between BJMC338 and BJMC3879 cells were investigated using TEM. Both cell lines showed the same morphology under TEM (Figures 1(a) and 1(b)). They had prominent nucleoli and dispersed small condensed chromatin in their nuclei. By immunofluorescent study, VEGF-C was barely expressed in BJMC338 cells, whereas moderately expressed in BJMC3879 cells (Figures 1(c) and 1(d)). The levels of VEGF-C protein in the two cell lines were determined by Western blot; the intensity of the bands was measured and corrected against β-actin intensity. A moderate increase in the VEGF-C protein level was detected in BJMC3879 cells (Figure 1(e)). RT-PCR analysis showed higher VEGF-C mRNA expression in BJMC3879 cells than in BJMC338 cells (Figure 1(f)).

3.2. In Vivo Studies

3.2.1. Histopathology and Metastasis. Histopathologically, the two types of inoculated mammary carcinoma (BJMC338 and BJMC3879 tumors) proved to be moderately differentiated adenocarcinomas. Both tumors were accompanied by a viable region, a central necrosis, and an inflammatory region (Figures 2(a) and 2(b)). The morphology of the tumor cells in the viable region was the same as that of the cultured cell lines (Figures 2(c) and 2(d)). Metastasis to axillary lymph nodes or lungs at 8 and 10 weeks postinoculation was validated by the observation of sections stained with H&E. At 8 and 10 weeks postinoculation, no metastasis was observed in the lymph nodes and lungs of mice that were inoculated with BJMC338 cells (Figures 3(a) and 3(c)). In contrast, all the mice inoculated with BJMC3879 cells showed distant metastasis to axillary lymph nodes and lungs at 8 and 10 weeks postinoculation (Table 1, Figures 3(b) and 3(d)).

3.2.2. Lymphangiogenesis in Tumor Mouse Models. The lymphatic vessels in the tumor were detected by immunohistochemistry using the antibody specific to lymphatic vessels, LYVE-1. At 4 weeks postinoculation, few lymphatic vessels were found in BJMC338 tumors (Figure 4(a)). At 6 weeks postinoculation, several lymphatic vessels were observed in intratumoral connective tissues and/or surrounding connective tissues (Figures 4(c), 4(e), and 4(g)). Conversely, large numbers of dilated lymphatic vessels with or without tumor cells were observed within and around BJMC3879 tumors at 4 to 10 weeks postinoculation (Figures 4(b), 4(d), 4(f), and 4(h)).

3.2.3. Lymphatic Vessel Density (LVD). The LVD of BJMC3879 tumors was always significantly higher than that of BJMC338 tumors (Figure 5). In BJMC338 tumors, the LVD at 6 weeks postinoculation was significantly higher than that at 4 weeks postinoculation ($P < 0.01$), after that, no significant difference in the LVD was observed (Figure 5). However, in BJMC3879 tumors, the difference in the LVD between 8 and 10 weeks postinoculation was not significant; a significant difference was detected between 6 and 8 weeks postinoculation ($P < 0.001$), namely, the LVD markedly increased at 8 weeks postinoculation (Figure 5).

3.2.4. TEM for Lymphatic Vessels. The status and ultrastructural features of lymphatic vessels in inoculated tumors were examined under TEM. Lymphatic capillaries were observed in the connective tissue surrounding and inside the tumors. They were distinguished from blood capillaries. The examination of lymphatic capillaries revealed that they had thin endothelium, abluminal protrusion of endothelial cells, no pericyte, no lamina densa, and overlapping junctions (Figures 6(a)–6(d)). Intraluminal tumor cells were observed in their lumen (Figure 6(a)). Interestingly, a leukocyte was shown to go into and/or out from the

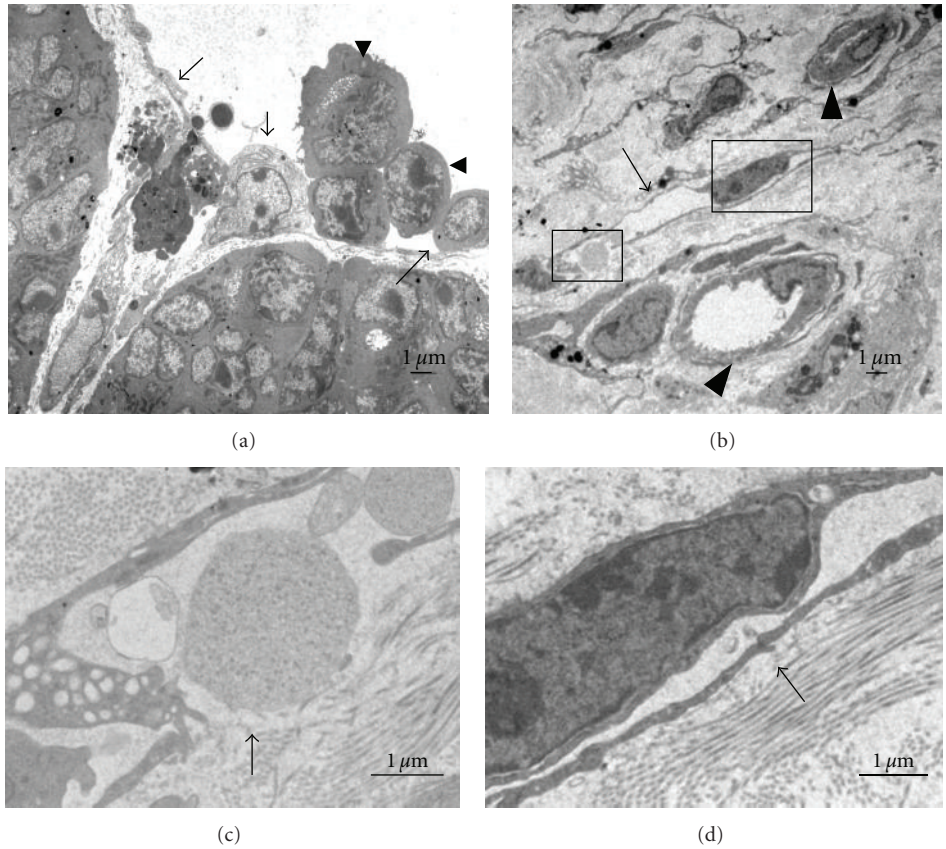

Figure 6: TEM micrographs of lymphatic vessels in BJMC3879 tumors at 10 weeks postinoculation (a) and 4 weeks postinoculation (b). The boxed areas in (b) are observed with high-power view (c and d). (a) A leukocyte (∗) is seen between endothelial cells (arrows), whereas tumor cells (arrowheads) are observed in the lumen. (b) Lymphatic capillary (arrow) and blood capillaries (arrowheads) are detected in the connective tissue surrounding tumors. Casein-like droplet (arrow) is located in the opening junction (c), and characteristic over-lapping junction (arrow) is showed (d).

lymphatic lumen (Figure 6(a)). Furthermore, the absorption of caseinlike droplets into the lymphatic lumen was observed (Figure 6(c)).

3.2.5. VEGF-C Expression in the Tumors. VEGF-C-positive cells were localized mainly in the peripheral viable regions of tumors. Immunohistochemical studies and Western blot analyses clearly demonstrated weak expression of VEGF-C in BJMC338 tumors (Figures 7(a) and 7(c)), relative to the strong expression in BJMC3879 tumors (Figures 7(b) and 7(c)).

3.2.6. Distribution of CD68-Positive Macrophages in the Tumors. CD68-positive macrophages were found mainly in the viable regions of both tumors (Figure 8). CD68-positve macrophages containing vacuoles in their cytoplasm aggregated in the tumors. The density of macrophages in BJMC3879 tumors was significantly higher than that in BJMC338 tumors ($P < 0.001$) (Figure 8).

4. Discussion

In this study, we found that mouse mammary tumor cells (BJMC3879) that have high metastatic propensity expressed a higher level of VEGF-C than the mouse mammary tumor cells (BJMC338) with low metastatic propensity, and the inoculated BJMC3879 tumors expressed VEGF-C equivalently to tumor cell lines. In highly metastatic mouse mammary tumors (BJMC3879), LVD and the VEGF-C expression level were higher than those in the poorly metastatic mouse mammary tumors (BJMC338). BJMC3879 tumor cell inoculation resulted in axillary lymph node and lung metastases, whereas no metastasis occurred after BJMC338 tumor cell inoculation.

There are some clinical surveys of human breast cancer to prove a causal relationship between LVD and malignancy, the VEGF-C expression, lymph node metastasis, and prognosis [1, 4, 5]. Increased LVD in breast cancer was correlated with lymph node metastasis and VEGF-C expression. It was concluded that a high LVD may be a significant unfavorable prognostic factor for long-term survival of breast cancer patient. Our results correlate with their reports. Contrary to the clinical importance of these LVDs and on the basis of clinicopathological studies including breast cancers, it is the hypothesized that intratumoral lymphatics have no function. None of the breast carcinoma was found to contain Ki-67-positive dividing endothelial cells of lymph vessels [23], and by experimental microlymphangiography assay, no

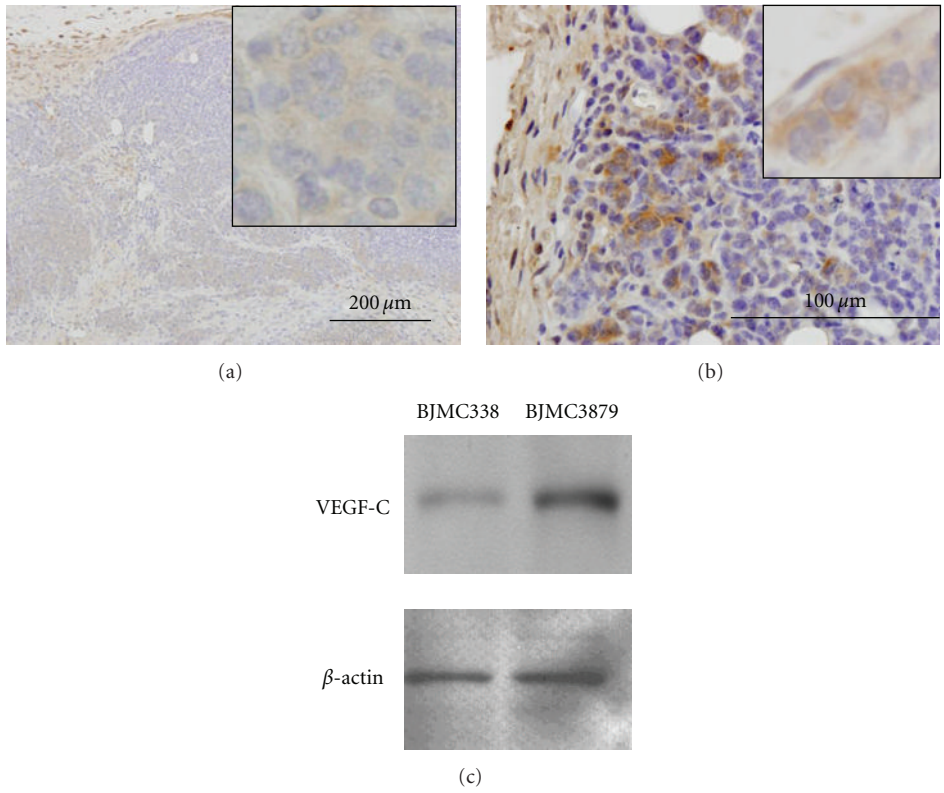

Figure 7: Immunohistochemistry (a and b) and Western blot analysis (c) of VEGF-C in BJMC338 tumor (a and c) and BJMC3879 tumor (b and c) at 8 weeks postinoculation.

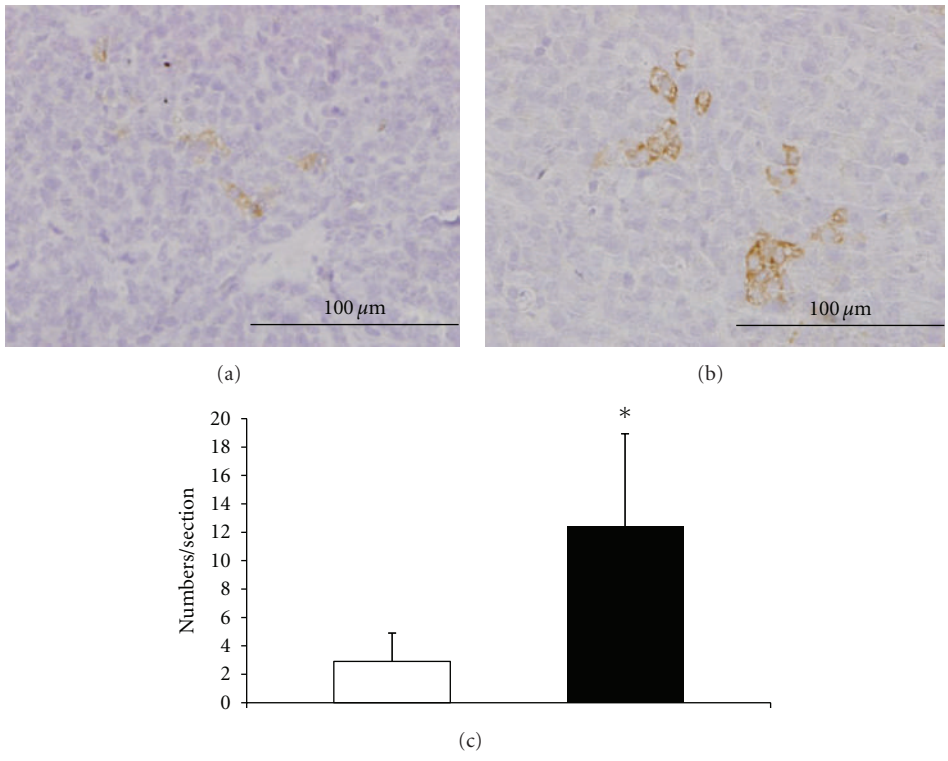

Figure 8: Immunohistochemistry of CD68 in BJMC338 tumor (a) and BJMC3879 tumor (b) at 4 weeks postinoculation. The density of macrophages in BJMC3879 tumors was significantly higher than that in BJMC338 tumors ($^*P < 0.001$) (c).

functional draining intratumoural lymphatics were found [24]. They also found that the functional lymphatics in the tumor margin alone were sufficient for lymphatic metastasis [24, 25]. It was reported that the degree of axillary lymph node metastasis increased in parallel with increasing LVD, patients with a high peritumoral LVD had only 58% 5-year distant disease-free survival as compared with 74% among those with a low peritumoral LVD. In addition, the presence of intratumoral lymph vessels was associated with neither axillary nodal status nor survival [24]. Moreover, not only LVD but also the size of peritumoral lymph vessels may be a significant consideration of lymph node metastasis [7]. As the enlarged lymphatics may collect interstitial fluid and cancer cells oozing from the tumor surface, it was suggested using mouse hybridoma cells and their syngenic mice that both peritumoral and intratumoral lymph vessels may play a crucial role in metastasis [26].

VEGF-C expression in breast cancer has been considered as a clinicopathological prognostic factor [8, 27–29]. However, a univariate study by Bando et al. revealed that high VEGF-C expression level was significantly associated with a favorable prognosis for disease-free survival and overall survival (e.g., high VEGF-C levels were associated with low-grade tumors and a smaller size). Furthermore, multivariate analysis confirmed the independent prognostic value of VEGF-C [30]. Watanabe et al. compared the expressions of CD44 variants and VEGF-C as associated factors with long-term prognosis, they concluded that there was no association between VEGF-C expression and clinicopathological prognostic factor [31]. A clinicopathological study using RT-PCR indicated that VEGF-C and VEGF-D were involved in lymphatic vessel invasion prior to lymph node metastasis, and their expression level decreased after the occurrence of lymph node metastasis [32]. In a retrospective study of 61 cases, it was reported that LVD may serve as a predictor of lymph node metastasis and a prognostic factor, whereas VEGF-C and VEGF-D may play important roles in lymphangiogenesis, making the carcinoma more aggressive and leading to a poor prognosis in breast cancer [8]. Because of controversial results showing that high VEGF-C levels are associated with low-grade tumors and a smaller size, Bando et al. suggested that the mechanism of VEGF-C protein processing in human cancer requires further study [30]. Because intratumoral VEGF-C protein level changes in response to intratumoral microenvironments, the expressions of VEGF-C and VEGF-D may be inadequate as clinicopathological prognostic factors by themselves.

To evaluate the effect of VEGF-C on lymphangiogenesis and lymph node metastasis, some human breast cancer cell lines were used *in vivo* and xenotransplanted to immunodeficient mice, SCID mice, or nude mice [11, 12]. Using human MCF-7 breast cancer cells, which are poorly invasive and estrogen dependent, Mattila et al. showed that tumor growth was stimulated *in vivo* in VEGF-C overexpressing MCF-7 cells xenotransplanted to nude mice. Furthermore, LVD in intra- and peritumoral lymphatic vessels was increased in tumors promoted by VEGF-C derived from xenotransplanted MCF-7 cells. While these reports, even though in xenotransplanted mice, support our results that tumoral VEGF-C expression plays an important role in lymphangiogenesis and lymph node metastasis of mouse mammary carcinoma, the role of immune cells including macrophages must be considered in tumor metastasis. Recent studies showed that VEGF-C is secreted by TAMs [13–15]. Our immunocompetent mouse models clearly showed the presence of CD68-positive TAMs in the inoculated tumors, and the density of TAMs in the high-metastatic mouse model was higher than that in the low-metastatic mouse model.

5. Conclusion

The LVD in mammary carcinoma strongly expressing VEGF-C was higher than that in carcinoma expressing a low VEGF-C level with the former showing axillary lymph node metastasis. Our inoculated mouse mammary carcinoma models in this study are allotransplanted and immunocompetent tumors which show the same lymph node metastatic spectrum as human breast cancers. Consequently, our mouse models are the most ideal for the study of lymph node metastasis.

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Research Article

Evaluation of Sentinel Node Biopsy in Locally Advanced Breast Cancer Patients Who Become Clinically Node-Negative after Neoadjuvant Chemotherapy: A Preliminary Study

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Introduction. Controversy continues over the appropriate timing of sentinel lymph node (SLN) biopsy in locally advanced breast cancer (LABC) patients receiving neoadjuvant chemotherapy. We evaluated the feasibility and accuracy of SLN biopsy in LABC patients with cytology-proven axillary nodal metastasis who become clinically node-negative after neoadjuvant chemotherapy. *Materials*. 30 consecutive patients with LABC, who had become clinically node-negative after 3 cycles of neoadjuvant chemotherapy, were included in the study. They were then subjected to SLN biopsy, axillary lymph node dissection, and breast surgery. *Results*. Sentinel nodes were successfully identified in 26 of the 30 patients, resulting in an identification rate of 86.67%, sensitivity of 83.33%, false negative rate of 20%, negative predictive value of 72.73%, and an overall accuracy of 88.46%. No complications were observed as a result of dye injection. *Conclusions*. SLN biopsy is feasible and safe in LABC patients with cytology-positive nodes who become clinically node-negative after neoadjuvant chemotherapy. Our accuracy rate, identification rate, and false negative rate are comparable to those in node-negative LABC patients. SLN biopsy as a therapeutic option in LABC after neoadjuvant chemotherapy is a promising option which should be further investigated.

1. Introduction

Prognosis in patients with breast cancer depends mainly on the extent of lymph node involvement, size of the tumor, and the histological grade of the tumor. Among these factors, axillary lymph node status is regarded as the single best marker of prognosis [1, 2].

For axillary nodal involvement, treatment in the form of level I and II axillary lymph node dissection (ALND) is considered optimum. But it is also associated with a number of complications including self-limiting complaints of numbness (70%), pain (33%), weakness (25%), swelling (24%), and stiffness (15%) which can interfere with daily living in upto 39% of cases. The risk of arm edema varies from 8% to 37% being related to the level of dissection and the number of nodes removed. Axillary vein thrombosis and injury to the motor nerves of axilla are extremely uncommon [2, 3].

Following the introduction of sentinel lymph node (SLN) biopsy for breast cancer, this technique has been widely adopted by cancer centers around the world for node-negative early breast cancer [2]. If the sentinel lymph node (SLN) is negative, the likelihood for other lymph nodes in the axilla to be negative ranges from 95 to 100%. So unnecessary ALND can be avoided in patients with negative axillae, and the associated morbidity of ALND can be reduced [4].

Neoadjuvant chemotherapy can reduce tumor size and downstage the primary tumor. Several studies could demonstrate a significant reduction in tumor size and a significant increase in breast-conserving surgery in operable breast cancer [5, 6]. Response to treatment is also an excellent indicator of chemotherapy effectiveness [5, 7–9]. In locally advanced breast cancer (LABC), treatment typically includes neoadjuvant chemotherapy, surgery, and radiation therapy [2].

Controversy continues over the appropriate timing of SLN biopsy in LABC patients receiving neoadjuvant chemotherapy as it could offer them the potential benefit of axillary downstaging and avoidance of axillary dissection. Recently, it has been shown that in locally advanced breast cancer, patients with a complete pathologic axillary response had a significantly higher overall survival than patients with residual disease [10]. Their study validated the prognostic stratification of patients with a complete pathological axillary response to neoadjuvant chemotherapy.

The aim of this study was to evaluate the feasibility and accuracy of SLN biopsy in locally advanced breast cancer patients with cytology-proven axillary nodal metastasis who become clinically node-negative after neoadjuvant chemotherapy.

2. Materials and Methods

The present prospective observational study was conducted by the Departments of Surgery and Pathology at Lady Hardinge Medical College, New Delhi.

A total of 30 consecutive patients (accrued over a period of 18 months) with cytology/biopsy-proven locally advanced breast cancer LABC (AJCC Stage III) and cytology-proven axillary nodal metastasis, who became clinically node-negative (on clinical examination) after neoadjuvant chemotherapy, were included in the study. Patients who had prior axillary surgery and those with inflammatory breast cancer were excluded from the study. The study was cleared by the Institutional Ethics Committee. Informed consent was taken from all the patients for the planned procedure.

All patients were subjected to a detailed clinical evaluation, routine investigations, and metastatic workup at the time of presentation. Investigations included a complete hemogram, blood sugar, liver function tests, kidney function tests, ECG, echocardiogram, chest X-ray, an ultrasound of the abdomen, and a bone scan. Breast investigations included a mammogram, breast ultrasound, cytology, and trucut biopsy for ER/PR/Her2 status. Breast ultrasound was used to define clinical response of the breast tumor to chemotherapy.

The patients were given three cycles of neoadjuvant chemotherapy (CAF: cyclophosphamide 600 mg/m^2, adriamycin 50 mg/m^2, 5-flurouracil 600 mg/m^2), and patients who satisfied the inclusion criteria were subjected to sentinel node biopsy, axillary lymph node dissection, and breast surgery. All patients were operated by the same consultant.

After the administration of anesthesia, the planned incision was marked by a marking pencil on the skin of the breast containing tumor. 2 to 5 mL of sterile 1% isosulphan blue (Patent Blue) dye was injected peritumourally (upper outer aspect of tumor) using a syringe and a 22G needle. This was followed by a breast massage for 10 minutes. Incision was made just below the axillary hairline. The incision was planned in such a way as to be included in the mastectomy incision in patients undergoing modified radical mastectomy. Dissection was rapidly done in the axilla to the clavipectoral fascia, and on reaching the fascia, the blue stained lymphatic(s) was carefully identified and traced up to the blue sentinel node(s). Sentinel node(s) was identified by its blue stain. Sentinel node(s) was then harvested before performing the breast surgery. Standard axillary dissection (ALND) was then performed removing level I and II axillary lymph nodes. Lymph nodes removed during ALND were labeled as nonsentinel nodes (NSN). Patients were observed for any immediate or late complications associated with dye injection.

For the purpose of histopathological examination (which included cytokeratin immunohistochemistry), sentinel node and nonsentinel nodes were submitted separately for histopathological analysis. In cases where more than one sentinel node was found, each node was analyzed as sentinel node.

A standardized set of data was abstracted from each patient. Data collection from all the patients was then analyzed using SPSS statistical software (version 9.0; SPSS Inc, Chicago, ILL, USA).

3. Observations and Results

The study group comprised of 30 consecutive patients of locally advanced breast cancer (AJCC Stage III) with cytology-proven axillary lymphadenopathy at presentation who became clinically node-negative (on clinical examination) after completion of three cycles of neoadjuvant chemotherapy. These patients were then subjected to sentinel lymph node biopsy followed by axillary lymph node dissection at the same operation. Out of these 30 patients, 29 patients underwent modified radical mastectomy while one patient had breast conserving surgery done.

In the present study, the mean age of the patients was 49.45 years (range from 25 to 65 years, median age 45 years). All the patients in the present study were females. Out of the 30 patients, 9 (30%) were premenopausal, while 21 (70%) were postmenopausal. None of the patients had a family history of breast, colon, ovary, or any other cancer. 60% (18/30) of patients had primary tumor in the left breast, while 40% (12/20) patients had tumor in the right breast. The size of the primary tumor varied from 3 cm to 7.5 cm (T2, T3, and T4b).

All the 30 patients had infiltrating ductal cancer, out of which 1 was well differentiated (grade 1), 21 were moderately differentiated (grade 2), and the remaining 8 were poorly differentiated (grade 3). 14 patients were estrogen receptor positive, 12 were progesterone receptor positive, and 11 patients had Her2 overexpression.

Out of these 30 patients, minimal response (less than 50% reduction in size) to neoadjuvant chemotherapy was seen in 14 patients (46.67%), partial response (more than 50% reduction in size) was seen in 14 patients (46.67%), and complete response was seen in the remaining 2 patients (6.67%). Breast ultrasound was used to define clinical response of the breast tumor to chemotherapy.

Out of the total of 30 patients who underwent SLN biopsy in the present study, the sentinel node was successfully identified in 26 patients. Sentinel node identification rate was 86.67%. The number of sentinel nodes removed per patient ranged from 1 to 4 ($1(n = 18), 2(n = 8), 3(n = 3), 4(n = 1)$). Average number of sentinel node identified per patient was

Table 1: Tumor metastasis in sentinel and nonsentinel lymph nodes in patients after neoadjuvant chemotherapy when sentinel nodes were successfully identified ($n = 26$).

	Non-sentinel node with metastasis	Non-sentinel node without metastasis	Total
Sentinel node with metastasis	12	3	15
Sentinel node without metastasis	3	8	11
Total	15	11	26

1.57. In most of the patients, sentinel node identified was 1 in number.

The number of nonsentinel nodes (NSN) identified ranged from 9 to 19. Average number of nonsentinel node identified after ALND was 13.5. Median number of NSN identified was 13.

Out of the total 26 cases in which a sentinel node was identified, the sentinel node was positive for tumor metastasis in 15 cases, the rest were negative on histopathology. In the 15 cases when the sentinel node was positive for metastasis, the nonsentinel nodes were positive for tumor metastasis in 12 cases. In the rest 3 cases in which the sentinel node was positive, the nonsentinel nodes were found to be negative for tumor metastasis on histopathology (Table 1). It appears that in these 3 patients, the sentinel nodes were the only involved nodes.

Out of the 11 cases when the sentinel node was negative for metastasis, in 8 cases the nonsentinel nodes were also negative for tumor metastasis on histopathology, while in the remaining 3 cases, the nonsentinel nodes were positive for tumor metastasis, thereby accounting for 3 false negatives in the study (Table 1). Out of these 3 patients, 2 had only one non-sentinel node positive for tumor, while the third had 2 non-sentinel nodes showing tumor. It is possible that fibrosis around the involved nodes could have resulted in the false negatives.

Thus in our study, sentinel node dissection was attempted in 30 patients, out of which sentinel nodes were successfully identified in 26 patients, with a sentinel node identification rate of 86.67%. We achieved a sensitivity of 83.33% (15/18), false negative rate of 20% (3/15), a negative predictive value of 72.73% (8/11), and an overall accuracy of 88.46% (23/26).

No complications were observed as a result of dye injection in any of the patients. All of the patients had a bluish green discoloration of the body (especially the face) and observed green-colored urine for 12 to 24 hours after surgery.

4. Discussion

Although the impact of resecting axillary lymph nodes on survival is currently a subject of controversy, accurate assessment of axillary nodal status provides the most important prognostic information for patients with primary breast cancer. It also directs selection of adjuvant systemic therapy and reduces the risk of regional recurrence of breast cancer in the axilla [2, 11, 12].

Following the introduction of sentinel lymph node (SLN) biopsy for early breast cancer, this technique has been widely adopted by cancer centers around the world. If the SLN is negative, the likelihood for other lymph nodes in the axilla to be negative ranges from 95 to 100%. So unnecessary axillary lymph node dissection (ALND) can be avoided, and its attendant morbidity can be reduced, in many patients with small breast cancers and negative axillae [4, 13–16].

Most of the reported experience with SLN biopsy includes patients with clinical stage T1-2 N0 [17]. Locally advanced breast cancer was also considered as one of the contraindications. However, recent studies have now shown that SLN biopsy can be considered if axillary lymph nodes are negative for metastases even in locally advanced breast cancer [10, 18, 19].

Neoadjuvant chemotherapy has become the standard of care for the treatment of patients with locally advanced breast cancer and has also been prospectively evaluated in patients with earlier-stage disease [20–23]. Neoadjuvant chemotherapy allows for individual *in vivo* assessment of primary tumor and metastatic lymph node response to chemotherapy. In addition, although chemotherapy is primarily thought of as important in eradicating occult distant disease, it can have a significant effect on locoregional disease as well. Tumor downstaging with neoadjuvant chemotherapy can convert inoperable disease to operable disease and can allow breast-conserving surgery in patients for whom mastectomy is initially the only option for control of locoregional disease [23–25].

During their study in locally advanced cases, Kuerer et al. concluded that neoadjuvant chemotherapy can completely clear the axilla of microscopic disease before surgery, and occult metastases (Isolated Tumor Cells-AJCC pN0(I+)) were found in only 10% of patients with a histologically negative axilla (AJCC pN0: No regional lymph node metastasis histologically). The results of their study have implications for the potential use of sentinel lymph node biopsy as an alternative to axillary dissection in patients treated with neoadjuvant chemotherapy. Their finding that only 10% of patients with complete axillary conversion (histologically negative axilla) have occult nodal metastases suggests that SLN biopsy may be appropriate in patients whose disease is downstaged with neoadjuvant chemotherapy [9].

Cox et al. [10] reported on a series of 89 patients with locally advanced breast cancer subjected to SLN biopsy before neoadjuvant chemotherapy. 27% of their patients had a complete pathologic axillary response; these patients had a significantly higher overall survival than patients with residual disease. Their study validated the prognostic stratification of patients with a complete pathological axillary response to neoadjuvant chemotherapy.

During the last few years, there have been a number of clinical trials on the effectiveness and role of SLN biopsy in patients after preoperative chemotherapy, mainly in early-stage breast cancer with negative nodes [26–28].

A retrospective analysis of 428 of 2,365 patients in the NSABP 27 trial who received chemotherapy followed by sentinel node biopsy and an axillary dissection was done [26]. 2,411 patients were randomly assigned to NSABP Protocol B-27. In the 2,365 patients (98.1%) for whom operative and pathology reports were available, there were 428 (18.1%) who had lymphatic mapping and for whom an attempt was made to identify and remove a sentinel node. There were significant differences in the distribution of some of the patient and tumor characteristics between the group of patients who had an SLN biopsy attempted and the group of 1,937 patients (81.9%) who did not. Patients in whom an SLN biopsy was attempted had smaller tumors and clinically uninvolved axillary nodes and are more likely to be lumpectomy candidates. Of the 428 patients in whom lymphatic mapping was attempted, at least one sentinel node was identified and removed in 363. Of the 363 patients in whom at least one sentinel node was identified and removed, 20 patients (5.5%) did not have the required axillary node dissection, leaving 343 patients in whom the accuracy of the sentinel node in correctly staging the axilla could be assessed. Because SLN biopsy was not mandated in the study, there was no predefined protocol dictating the method of lymphatic mapping or the approach to SLN biopsy. In the majority of the cases, nodal positivity was determined by hematoxylin and eosin staining only. However, in a handful of cases, additional immunohistochemical staining was performed to further evaluate the status of sentinel nodes. The analysis of these cases demonstrated an 85% sentinel node identification rate and a false-negative rate of 11%, which are similar to those observed in patients undergoing an initial sentinel node biopsy during the same period [26].

Xing et al. in 2006 [27] conducted a meta-analysis of twenty-one studies (total of 1273 patients) that examined the results of SLN biopsy after chemotherapy. The sensitivity of SLN biopsy in the individual studies ranged from 67 to 100 percent, the negative predictive value ranged from 56 to 100 percent, and the overall accuracy ranged from 77 to 100 percent. However, the majority of patients in these studies had stage II breast cancer with negative axillary nodes at presentation.

The ongoing ACOSOG Z1071 trial "A Phase II study of sentinel lymph node surgery and axillary lymph node dissection following neoadjuvant chemotherapy in women with stage II-IIIB node-positive breast cancer" attempts to determine the false negative rate for sentinel lymph node (SLN) surgery in women with node-positive breast cancer who have completed or plan to undergo neoadjuvant chemotherapy. However, this study includes both stage II and III breast cancer and also includes patients who are node positive after completing the chemotherapy. The trial is expected to be completed by end 2013 [28].

Studies on the feasibility and accuracy of SLN biopsy after preoperative chemotherapy in locally advanced breast cancer patients with documented axillary metastasis are few and the results are inconclusive.

Shen et al. [29] studied 69 patients with cytology-confirmed axillary metastasis who underwent SLN biopsy after chemotherapy. However, out of these, only 23 were LABC (AJCC stage III). The overall SLN identification rate was 92.8%, and a false negative rate of 25%. They concluded that the status of the SLN cannot be used as a reliable indicator of the presence or absence of residual disease in the axilla in this patient population. Newman et al. [30] evaluated 54 breast cancer patients with biopsy-proven axillary nodal metastasis. The SLN identification rate after delivery of neoadjuvant chemotherapy was 98%, with a false negative rate of 8.6%. They concluded that SLN biopsy after neoadjuvant chemotherapy in patients with documented nodal disease at presentation accurately identified cases that may have been downstaged to node-negative status and can spare this subset of patients from the morbidity of an ALND. Another recent study from Korea [31] concluded that SLN identification rate, but not accuracy, is significantly decreased after preoperative chemotherapy in axillary node-positive breast cancer patients. They also suggested that for patients who achieve complete axillary clearance by chemotherapy, SLN biopsy could replace ALND.

In the present study, although having a small sample size, we have showed that SLN biopsy is feasible and safe in locally advanced carcinoma breast who become clinically node-negative after neoadjuvant chemotherapy. Our accuracy rate, identification rate, and false negative rate are comparable to reports in the literature in node-negative LABC patients after chemotherapy. Blue dye method is a safe procedure and none of the patients developed any complications of the dye injection.

LABC consists of a heterogeneous group of patients falling in AJCC stage III. Having advanced stage disease, they have a poor prognosis. However, even in this group, the subgroup of LABC patients in whom there is a complete axillary response to neoadjuvant chemotherapy have a good prognosis—having shown to have a significantly higher overall survival than patients with residual disease [9]. Identification of this subgroup of LABC patients can help target treatment modalities for improved outcomes.

Most of the studies in the literature on SLN biopsy after neoadjuvant chemotherapy involved earlier stage disease, smaller tumors, with negative nodes at presentation. Our study focuses on the likely feasibility and role of SLN biopsy in this good prognosis subgroup of LABC patients with complete axillary response to neoadjuvant chemotherapy. Additional studies are needed to answer the on-going debate regarding optimal treatment of the axilla in LABC patients who are rendered clinically node-negative after neoadjuvant treatment. Following neoadjuvant therapy, accurate evaluation of the axilla by SLN biopsy is feasible in these patients. Sentinel lymph node biopsy as a therapeutic option in locally advanced breast cancer patients who become clinically node-negative after neoadjuvant chemotherapy is a promising option that can spare axillary dissection and its morbidity, and which should be further investigated.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Patterns of Cellular Distribution with the Sentinel Node Positive for Breast Cancer

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Background. Sentinel node biopsy (SNB) represents the standard of care in breast cancer axillary evaluation. Our study aims to characterize the patterns of malignant cell distribution within the sentinel nodes (SN). *Methods*. In a retrospective IRB-approved study, we examined the anatomic location of the nodal area with the highest radioactive signal or most intense blue staining (hot spot) and its distance from the metastatic foci. *Results*. 58 patients underwent SNB between January 2006 and February 2007. 12 patients with 19 positive SN were suitable for analysis. 4 (21%) metastases were located in the nodal hilum and 15 (79%) in the cortex. 6 (31%) metastases were found adjacent to the hotspot, and 9 (47%) within 4 mm of the hotspot. *Conclusions*. In our pilot series, SN metastases were within 4 mm of the hotspot in 78% of the cases. Pathologic analysis focused in that area may contribute to the more accurate identification of nodal metastases.

1. Introduction

The technique of sentinel node biopsy with lymphatic mapping has revolutionized breast cancer surgery, reducing the resultant risks of undesired consequences from more extensive axillary surgery and leading to the detection of increasingly smaller metastatic foci in the axilla. Surgeons strive to achieve a singular surgical intervention to the axilla by identifying those patients with positive sentinel nodes who would benefit from further nodal dissection. Several intraoperative techniques for lymph node evaluation are available including frozen section, touch imprint cytology, and RT-PCR breast lymph node (BLN) assay. These techniques have variable sensitivities and high specificity and involve different degrees of nodal tissue manipulation.

We hypothesize that a better understanding of the distribution of the metastatic foci within the sentinel lymph node may aid in their more accurate detection and better utilization of intraoperative evaluation techniques.

2. Methods

The study was reviewed and approved by the IRB. Patients with invasive breast cancer who underwent sentinel node biopsy at our institution by a single surgeon between January 2006 and February 2007 were eligible for the study. The sentinel nodes were identified after the injection of intradermal radioactive Tc99-labeled sulfur colloid and subareolar methylene blue.

The radioactive nodes were scanned with a gamma probe intraoperatively, and the area of highest radioactivity (hot spot) was identified and marked with a clip. Isotopically nonactive nodes were clipped at the site of maximum blue stain intensity. During subsequent routine pathologic examination, the hotspot was inked by the pathology technician, and the clip was removed. Data regarding the anatomic location of the hot spot and its distance from the metastatic foci in the positive sentinel nodes was collected. The charts were retrospectively reviewed.

3. Results

Fifty-eight patients with invasive breast cancer undergoing sentinel node biopsy for a clinically negative axilla at our institution by a single surgeon between January 2006 and February 2007 were identified, yielding a total of 127 removed sentinel nodes. Of these patients, 42 had a negative sentinel node biopsy and were excluded. In three cases,

the clip marking the hot spot was not present at the time of pathologic examination. One other patient had a positive sentinel node that was completely replaced with metastatic disease. These four patients were also excluded from the study. Patients who had been identified by axillary ultrasound-guided fine-needle aspiration as node positive were not included in the study group.

A total of 12 patients with 19 positive sentinel nodes fulfilled inclusion criteria and were analyzed. The patient and tumor characteristics are listed in Table 1.

The majority of the tumors were infiltrating ductal carcinomas (66.6%), and all were ER and/or PR positive. Of the 19 nodes evaluated, 3 (16%) were positive for isolated tumor cells (ITC), 8 (42%) contained micrometastases, and 8 (42%) macrometastases. The mean size of the examined positive lymph nodes was 10.9 mm, ranging from 5 mm to 21 mm and the mean size of the metastases 6.3 mm, ranging from ITC to 11 mm. The hotspot was located in the nodal cortex in 16 (85%) of the examined positive nodes. The 95 negative nodes in our cohort were also examined, and the hot spot was found in the cortex in 77 (81%) of nodes. In the nodes with tumor cells, the metastatic focus was located in the nodal cortex and adjacent medulla in 15 (79%) of the nodes (Figure 1) and in the nodal hilum in the remaining 4 (21%) nodes (Figure 2). Of the 4 nodes that had the metastatic focus in the hilum, one contained a micrometastasis and the other three macrometastases. The metastatic focus was located immediately adjacent to the marked hot spot in 6 (31%) nodes and within 4 mm of the hot spot in 9 (47%). Therefore, in 81% of the cases, the hotspot was located in the cortex, and in 78% of the cases the metastases were within 4 mm of the hotspot (Table 2).

Table 1: Patient and tumor characteristics.

	Study population N: 12 (100%)
Age (years)	
Mean	55
Range	44–79
Histologic type	
IDC	8 (66.6)
ILC	2 (16.6)
Mixed IDC/ILC	2 (16.6)
Tumor stage	
IIA	6 (50)
IIB	1 (8.3)
IIIA	5(41.6)
Tumor grade	
I	2 (16.6)
II	8 (66.6)
III	1(8.3)*
LVI	
Present	7 (58.3)
Absent	5(41.6)
ER/PR status	
ER and/or PR positive	12 (100)
ER/PR negative	0
Her2neu	1 (8.3)
Positive	11 (91.6)**
Negative	

* One patient had received neoadjuvant chemotherapy so tumor grade is not available.
** IHC was performed first and if intermediate FISH was done.
IDC: infiltrating ductal carcinoma, ILC: infiltrating lobular carcinoma, LVI: lymphovascular invasion, ER: estrogen receptor, and PR: progesterone receptor.

4. Discussion

Sentinel node biopsy represents the standard of care for axillary evaluation in patients with breast cancer. The NSABP B-32 phase III clinical trial comparing sentinel lymph node biopsy to conventional axillary lymph node dissection in clinically node negative patients showed equivalent outcomes in overall survival, disease-free survival, and regional control with decreased morbidity in the sentinel node group [1, 2]. More recent data on patient-reported outcomes for sentinel lymph node biopsy versus axillary lymph node dissection showed that in the first six to twelve months axillary node dissection patients reported ipsilateral arm and breast morbidity, impaired quality of life, restricted work, and social activity more than the sentinel lymph node resection group. By twelve to thirty-six months, less than 15% in each group had any residual symptoms [3].

In patients found to have positive nodes on sentinel node biopsy, the standard of care has been to perform a completion axillary node dissection either at the time of the initial surgery or at a later date. In evaluating outcomes based on timing of the dissection, the ACoSOG Z-0010 and Z-0011 trials demonstrated that patients who undergo immediate as opposed to delayed completion axillary node dissection experience more short-term morbidity, but long-term outcomes were the same in both groups [4]. However, clearly, the duration to recovery is significantly greater when reoperation is required.

In an effort to reduce reoperation, several techniques have been developed for intraoperative lymph node evaluation including frozen section, touch imprint cytology, and RT-PCR breast lymph node (BLN) assay. All techniques aim to accurately detect metastatic disease intraoperatively and allow the appropriate patient to proceed directly to axillary dissection. The sensitivity of touch imprint cytology hovers around 70% with a specificity of over 95% [5, 6]. High false negative rates have been noted in cases of micrometastases and invasive lobular carcinoma. Intraoperative frozen section, though reported to have similar sensitivity and specificity to touch imprint cytology, involves the undesirable loss of tissue for permanent section analysis, as well as longer turn around time and greater expense [7].

Several studies have been performed to evaluate genesearch real-time RT-PCR breast lymph node (BLN) assay in detecting intraoperative metastases in sentinel lymph nodes greater than 0.2 mm [8]. This method identifies mRNA from cytokeratin 19 (CK19) and mammaglobin(MG) genes expressed in epithelial cells but not present in lymphoid

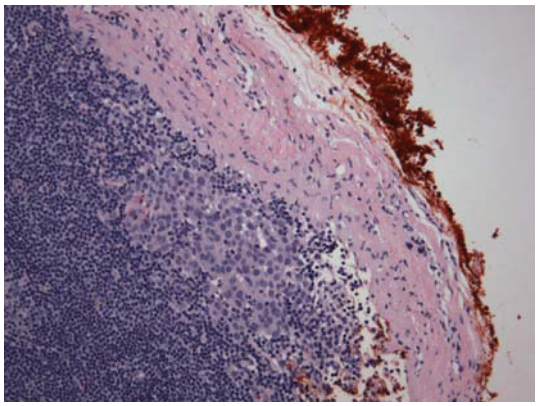

Figure 1: Micrometastases located in cortex close to hotspot.

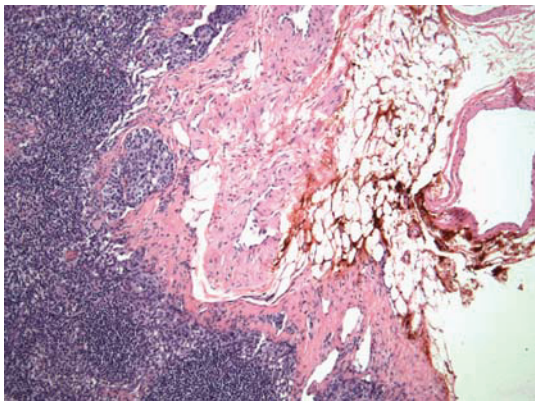

Figure 2: Micrometastases located in hilum close to hotspot.

Table 2: Results.

Total Sentinel Nodes	Cortex Hotspot	Hilum Hotspot
Positive (19)	15 (79%)	4 (21%)
Negative (95)	77 (81%)	18 (19%)

tissue. The nodal tissue is homogenized and using RT-PCR, and the mRNA is amplified and metastases identified. When compared to standard hematoxylin and eosin (H&E) staining, BLN achieves a sensitivity of 95.7% for macrometastases, 60.0% for micrometastases, and 55.6% for isolated tumor cells [9]. Though the accuracy of BLN assay is comparable to permanent section, it has not been rapidly adopted across the country with criticisms centered around the 30–40 minute turn around time, the specimen loss associated with tissue processing, the setup costs and the need for a skilled technician to perform the study.

At this point, the indications for the use of all of these techniques are under reevaluation in view of recently published data from ACoSOG Z-0011 regarding the impact of completion axillary node dissection in patients with 1–3 positive sentinel nodes [10, 11]. This trial evaluated the impact of axillary dissection on both locoregional recurrence and overall survival in patients undergoing breast conservation therapy who were found to have one to three positive sentinel nodes, by randomizing patients to axillary dissection or no further surgery. Both groups were treated with adjuvant systemic therapy and whole breast radiotherapy, and, after six years of follow-up, there was no difference in locoregional recurrence or survival. While these results suggest that simply identifying patients as node positive will provide adequate information for planning systemic therapy, the results remain inapplicable to patients who have undergone neoadjuvant chemotherapy, those with greater nodal burden than represented in the study, and those patients who will not be receiving radiation, due to planned omission, mastectomy, or contraindications to radiotherapy. In these scenarios, characterizing the sentinel node intraoperatively still merits attention.

Yet a separate question remains as to whether the type or distribution of metastases is important. Macrometastases by definition are those tumor foci greater than 2 mm. The seventh edition of American Joint Committee on Cancer (AJCC) staging for breast cancer defines micrometastases as tumor deposits greater than 0.2 mm but less than 2 mm and are designated pN1(mic) [12]. Isolated tumor cells (ITC) are defined as clusters of cells no greater than 0.2 mm and designated pN0(i+). The clinical significance of micrometastases and isolated tumor cells continues to be investigated. Recent studies have evaluated the prognostic impact of isolated tumor cells or micrometastases in breast cancer and have found that there was a statistically significant decrease in 5-year disease-free survival rate in women with favorable early-stage breast cancer who did not undergo adjuvant treatment compared to the adjuvant therapy group although the impact that this should exert on clinical decision making remains unclear [13, 14].

An understanding of tumor cell distribution and its relationship to lymphatic flow is important to highlight the significance of our findings. Unfiltered lymph fluid flows regionally through afferent lymph channels, traverses the outer capsule through the cortex, and flows through the paracortex to the medulla. The filtered fluid then exits in the lymph node through the efferent channel via the hilum. One study utilizing three-dimensional reconstruction to evaluate metastatic tumor cell distribution in sentinel lymph nodes found metastases to be located at the afferent pole in 17 of 19 tumor-involved sentinel nodes [15]. In seven nodes, metastases were confined to the afferent pole, with the balance containing metastases extending to the efferent pole. However, only two cases displayed metastases confined to the efferent pole. These findings correlate with our study results, showing that the afferent pole contained the majority of metastases. In addition, the hotspot was located in the afferent pole on the cortical surface of the node in the majority of both the positive and negative sentinel nodes for our study. We could, therefore, postulate that focusing in the area of the hotspot would improve the sensitivity of nodal analysis.

A limitation of our study is the small sample size. The study spanned just over a year, and the number of involved nodes is low. The standard use of axillary ultrasound paired with fine-needle aspiration biopsy (USFNA) as part of staging new breast cancers at our institution has decreased

the number of intraoperatively detected nodal metastases. Based on our previously published data, our institutional policy has been to recommend axillary USFNA for all invasive ductal carcinomas greater that 1.5 cm [16]. This strategy biases the study cohort towards low-volume axillary disease. Nonetheless, there are patients where USFNA is of limited success due to body habitus, patient tolerability, or the receipt of neoadjuvant chemotherapy.

Notably all patients in this study were hormone receptor positive. This also is likely related to the institutional interest in neoadjuvant chemotherapy trials. Given the documented role for systemic therapy in hormone receptor negative breast cancer, the majority of these patients are treated neoadjuvantly at our institution.

As the results of ACoSOG Z-0011 modify our current practices, intraoperative nodal analysis may become less prevalent, except in those groups where the trial results are not applicable, such as patients who will not receive whole breast radiotherapy, patients with more than 3 positive sentinel nodes, and patients undergoing mastectomy. As the mastectomy rate has been about 30–40% and is increasing nationally combined with the evolving strategies for less comprehensive breast radiotherapy, our results remain relevant to many patients [17]. Furthermore, understanding the patterns of disease spread may aid in reducing the false negative results in nodal evaluation and allow for more effective utilization of ever tightening resources.

5. Conclusion

In patients where the results of intraoperative assessment of the sentinel node will impact proceeding to axillary dissection, focusing the examination to the afferent pole and within 4 mm of the hot spot should enhance intraoperative detection of sentinel node metastasis.

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Review Article

Which Patients Need an Axillary Clearance after Sentinel Node Biopsy?

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Sentinel lymph node biopsy (SLNB) is a safe and accurate minimally invasive method for detecting axillary lymph node (ALN) involvement in the clinically negative axilla thereby reducing morbidity in patients who avoid unnecessary axillary lymph node dissection (ALND). Although current guidelines recommend completion ALND when macro- and micrometastatic diseases are identified by SLNB, the benefit of this surgical intervention is under debate. Additionally, the management of the axilla in the presence of isolated tumour cells (ITCs) in SLNB is questioned. Particularly controversial is the prognostic significance of minimal SLNB metastasis in relation to local recurrence and overall survival. Preliminary results of the recently published Z0011 trial suggest similar outcomes after SNB or ALND when the SN is positive, but this finding has to be interpreted with caution.

1. Introduction

For patients with operable breast cancer, the major prognostic determinant is whether there has or has not been spread to the axilla and the number of involved axillary nodes [1]. Several theories exist concerning the mechanism of breast cancer cell invasion and metastasis. Initially it was suggested that breast cancer first spreads locoregionally via lymphatics to the axillary lymph nodes and then metastasises more distantly. In accordance with this concept, Halsted developed radical mastectomy as the gold standard for breast cancer surgery [2].

Subsequently Fisher postulated that the extent of micrometastases at diagnosis of breast cancer is an indicator of outcome, with biological behaviour of cancer predetermining the likelihood of progression of the disease [3]. Nowadays gene expression profiling arrays can delineate tumour types with different prognoses [4]. The surgical approach for breast cancer treatment evolved from the extensive radical mastectomy and the Patey modified radical mastectomy [5] to breast conserving and minimally invasive techniques [6].

Traditionally the surgical management of breast cancer comprised wide local resection of the primary tumour and axillary lymph node dissection (ALND). Axillary status is the most important prognostic factor in breast cancer providing staging information and therefore largely defining treatment strategy [7]. Diagnostic imaging modalities such as ultrasound, magnetic resonance mammography, positron emission tomography, and 99 m Technetium (Tc) sestamibi scintimammography are not reliable for staging the axilla, particularly with lymph node metastases <0.5 cm [8, 9].

Clinical, pathological, and molecular features are inadequate for assessing ALN metastases. Clinically palpable lymph nodes prove to be false positive in 25–30% of patients [10] and about 40% have positive results after ultrasound with or without fine-needle aspiration node negativity [11]. Tumour size cannot serve as an accurate prognostic indicator for lymph node involvement. Studying 24,740 women with invasive breast cancer, Carter et al. showed that approximately 80% with tumour size <1 cm, 50% with up to 5 cm, and 30% with >5% had negative axilla, a fact suggesting that metastases do not occur exclusively via the axillary lymph nodes, but rather lymph node status serves as an indicator of the tumour's ability to spread [12]. Additionally, it has been recently shown that the molecular profile of the primary tumour is a more significant prognostic indicator in terms

of disease-free survival (DFS) and overall survival (OS) than lymph node metastases [4].

The combination of the introduction of population-based mammographic screening for breast cancer, modern imaging methods, and increased public awareness resulted in patients being diagnosed more often with smaller-size tumours and less likelihood of axillary lymph node metastases [13]. It is evident now that 60–70% of patients with early breast cancer are node negative at the time of diagnosis [14] and ALND puts them at significant risk of short- and long-term morbidities without benefit [15]. ALND is associated with acute complication rates of 20–30% including seroma formation, local swelling, numbness, impaired shoulder movement, neuropathy, infection, and chronic lymphoedema rates of 7–37% [16]. In a prospective study by Petrek et al. evaluating a cohort of 923 women with 20 years follow up, it was shown that breast-cancer-related lymphoedema following ALND occurred maximally in the first 3 years following surgery; however, up to 23% of patients may still develop arm swelling during the rest of their lives [17].

Several randomised studies have established that sentinel node biopsy (SNB) is a safe and accurate procedure for detecting tumour cells in SLN and predicting the status of the other axillary nodes (non-SLN). Although accuracy and appropriateness of SNB were disputed by the finding of 5–10% false-negative cases when SNB was followed by axillary dissection at high-risk patients for axillary nodal disease [13, 18], false-negative SNB results seem to have decreased with the increasing experience of surgeons, and it is expected that the utilisation of SNB in the future will be increased [19].

A meta-analysis of seven prospective randomised controlled trials by Kell et al. demonstrated that SNB is equivalent to ALND for the detection of lymph node metastasis with the additional advantage of reduction of up to 75% in morbidity in patients with early stage breast cancer. Furthermore, a trend towards an improved detection of LN metastases was shown when SNB is used [20]. Patients undergoing SNB have a 22% higher odds ratio of having a positive SLN, due to the more intensive pathological examination which utilises multiple sections and immunohistochemistry (IHC) [21]. In contrast, the false-negative cases seen after axillary dissection are probably due to the inability of the pathologist to perform serial sections and IHC on the 20–30 lymph nodes found in a complete axillary clearance specimen.

Studies have shown that SLN is the only positive lymph node in 38–67% of patients when ALNC followed [22]. Interestingly, it has been reported that only 4–8% of patients with negative ALNs have internal mammary lymph node involvement (IMN) whereas 25–50% of patients with affected ALNs have also IMN metastases [23, 24]. Dissection of IMN is not recommended because of the high morbidity and the uncertain benefit on survival [24].

The recently published outcomes of NSABP B-32 trial established the efficacy of SLN biopsy alone with no further ALND in 5611 breast cancer patients with clinically negative lymph nodes [25]. Women with invasive breast cancer who were randomly assigned to either SLN resection plus ALND (group 1) or to SLN resection alone with ALND only if the SLNs were positive (group 2), after 8 years of followup, showed statistically equivalent overall survival, disease-free survival, and regional control. Patient followup is still continuing for longer-term assessment of survival and regional control.

Moreover, a closer look into mature studies focused on axillary relapses and overall survival is in agreement with current findings favouring SLB. The National Surgical Adjuvant Breast and Bowel Project (NSABP) B04 randomised study compared breast cancer patients with clinically negative ALNs managed either by radical mastectomy, total mastectomy with axillary radiation, or total mastectomy alone. The results clearly defined that ALND decreases the risk of locoregional relaps; however, no significant differences in survival were found among the treatment groups [26]. This study has been criticised because of the variability of numbers of lymph nodes resected in the total mastectomy alone arm and the lack of statistical power to detect a small difference in outcome [27]. Indeed, a meta-analysis has suggested that inadequate axillary treatment may lead to not only an increased risk of local relapse but also a 5% reduction in survival [28].

As a result of increasing detection of early breast cancer and the high rate of micrometastases and ITCs (ITCs) found in the detailed pathological examination of SLN, a new debate has opened about the consequent necessity of ALND in these patients. This has arisen because of better understanding of breast cancer behaviour and improved efficacy of combined therapeutic modalities. In this paper we report the current guidelines concerning the management of the axilla after SLNB and review the different aspects arising from recent studies on the role of micrometastases and ITC clusters in SLN on decision making.

2. Current Guidelines

The American Society of Clinical Oncology (ASCO) Expert Panel conducted a systematic review of the literature available through February 2004 on the use of SNB in early-stage breast cancer in order to develop guidelines for the management of the axilla (http://jop.ascopubs.org/content/1/4/134) and these are similar to those of the National Institute of Health and Clinical Excellence (NICE) recommendations in UK (http://www.nice.org.uk/nicemedia/live/12132/43413/43413.pdf) [29].

SLNB is recommended for staging patients with clinically negative lymph nodes. ALND is the standard of care in those with a macrometastatic or micrometastatic positive SLN to maximise local control [30]. If the SLN is negative, a cALND (cALND) is not necessary. ITCs detected by IHC are of unknown clinical significance, and when identified, the SLN is regarded as negative and no further ALND is required. Although IHC is often used, it is not included in routine SLN evaluation for breast cancer at this time. ASCO and NICE recommendations for SLNB, ALND alone and managing of the axilla after SLNB are summarised in Table 1. In contrast the German guidelines do not recommend axillary clearance

for 1-2 SLN positive in patients with T1 and T2 tumours (http://www.ago-online.de).

It has been suggested that SLNB should be carried out by an experienced team in order to minimise false negativity and improve the predictive value of the procedure [30]. All suspicious palpable nodes should also be considered as SLNs.

3. Micrometastases and Isolated Tumour Cells (ITCs)

The American Joint Committee on Cancer (AJCC) in the sixth edition of the Cancer Staging Manual defined a lymph node metastatic tumour with maximum diameter > 2 mm as macrometastasis (pN1), when the diameter of deposit is 0.2–2 mm as micrometastasis (pNmi), and a lesion of single tumour cells or small cell clusters with diameter < 0.2 mm as ITCs [pN0(i+)] [31]. ITCs are not distinguishable by H&E staining but detected only with immunohistochemistry (IHC) or molecular methods. Moore et al. suggested that the presence of ITCs was unrelated to known prognostic variables and partly the result of instrumentation and manipulation of the tumour [32].

The management of patients with minimal SLN involvement is problematic [33]. In a meta-analysis of 25 studies of patients with SLN micrometastases, in approximately 20% there was nonsentinel node disease falling to 9% when the SLN involvement was detected by IHC [34]. Furthermore, the consequent effect on DFS and OS remains controversial, so the biological relevance and clinical significance is a matter of debate [35].

AMAROS investigates the benefit of a cALND in comparison to treatment with axillary radiotherapy (ART) in patients with SLN-positive breast cancer [36]. A recently published substudy evaluated the identification rate and the nodal involvement of the first 2,000 patients between 2001 and 2005 who entered from 26 European institutions [36]. The sentinel node identification rate was 97% which is high considering the relatively early days of this procedure. 34% were SLN positive of whom 63% had macrometastases, 25% had micrometastases, and 12% had ITCs. In the cALND arm non-SLN involvement was identified in 41% of patients with macrometastases and in 18% of patients with either micrometastases or ITCs.

Several studies have investigated the significance of occult metastases, such as micrometastases or small clusters of tumour cells in association with non-SLN involvement and the impact of cALND on disease-free survival and overall survival, and the larger ones are summarised in Table 2 [37–43]. Although the majority show no prognostic impact of ITCs in the sentinel node, a large Dutch investigation with 5-year followup indicated that women with ITCs who received adjuvant chemotherapy had a significantly better event-free survival compared with untreated cases with ITCs [39]. Furthermore, a Finnish study showed a worse 5-year breast-cancer-specific survival for those with ITC compared with node negative cases [42].

In the largest published multicenter retrospective study of 187 SLN-ITCs patients undergoing cALND, Houvenaeghel et al. reported an incidence of 16% non-SLN involvement [44]. The difference in the risk of non-SLN involvement between sentinel nodes with ITCs (16%) and those with micrometastases (14%) was not statistically significant. However it was not apparent whether the presence of non-SLN metastases should affect the therapeutic decision in these patients. The authors proposed that cALND could be avoided in patients with tubular, colloid, or medullary small primary tumours (pT1) with a risk of non-SLN involvement approximately ≤ 5%.

In contrast to the conclusions of the aforementioned studies come the MIRROR trial results [45]. MIRROR is a large Dutch cohort retrospective study which assesses the impact of SLN-ITCs and micrometastases on 5-year disease-free survival in patients with favourable primary tumour characteristics. According to recent published data, both patients with SNB micrometastases and those with ITCs who did not undergo cALND experienced a far higher 5-year axillary recurrence rate, 6% in comparison to 1% of SNB-negative patients who did not undergo cALND. Additionally, both patients with SLN micrometastases and ITCs had approximately 5-year disease-free survival improved by 10% with adjuvant systemic therapy. It is important to mention that micrometastases and ITCs had comparable prognostic impact [46]. MIRROR findings support an aggressive treatment approach in patients with either SLN micrometastases or ITCs.

4. Completion ALND and Micrometastases

Many investigators have studied the incidence of non-SLN involvement in patients with SLN micrometastases to define which patients may need further axillary treatment. Wada and Imoto. collected 22 studies from 1999 until 2006 referring to the frequency of SLN micrometastases in patients with breast cancer and the prevalence of non-SLN involvement in those patients after ALND [47]. The frequency of SLN micrometastases was 38% with non-SLN micrometastases ranging from 0 to 57%. Additionally, a wide range of non-SLN macrometastases was found (0–18%). Because the prevalence of non-SLN micrometastases was low, the prognostic impact was unclear. The wide range of results arose from the different numbers of patients involved, variations in number of pathological sections examined, and differences in tumour stage and grade.

Results of studies in which patients with micrometastases in SNB and who were not treated by completion axillary node clearance are summarised in Table 3 [38, 48–54]. Most of the studies had small numbers and relatively short followup and tended to conclude that there was no benefit from completion axillary node clearance. The largest study; however, found a significantly worse disease-free survival for women with micrometastases who did not undergo cALND. [38].

De Boer et al. conducted a systematic review of 58 studies conducted from 1977 to 2008 included 297,533 patients,

Table 1: Recommendations of SLNB, ALND, and treatment after SLNB.

SLNB	ALND	Post-SLNB	
T1, T2 tumour	T3, T4 tumours	SLNB +ve	ALND
Multicentric tumour	Inflammatory carcinoma	SLNB −ve	Observe
DCIS for mastectomy	Suspicious axillary node	Micromets	ALND
DCIS > 5 cm	Pregnancy	ITC	Observe
Older patient	Prior axillary surgery	SLNB +ve, 1-2 nodes, T1, T2	Observe*
Preneoadjuvant			

DCIS: ductal carcinoma-in-situ; SNB: sentinel lymph node biopsy; ALND: axillary lymph node dissection.
*Recommendation of the German guidelines.

Table 2: Incidence and prognostic impact of ITCs in sentinel node biopsies.

Author	Total	ITC (%)	Outcome
Herbert et al. [37]	514	16 (3%)	No effect
Reed et al. [38]	1255	25 (2%)	No effect
De Boer et al. [39]	2707	819	HR 1.5 (No adjuvant versus adjuvant)
Barbosa et al. [40]	1000	43 (4%)	No effect
Andersson et al. [41]	3369	107 (3%)	No effect
Leidenius et al. [42]	1390	63 (5%)	Reduced 5-year survival
Maaskant-Braat et al. [43]	6803	126 (2%)	No effect

aiming to define the prognostic relevance of micrometastases and ITCs in patients with breast cancer [55]. Using random-effect meta-analysis they showed that the presence of ALN metastases <2 mm in diameter detected on single-section examination was associated with poorer overall survival. Moreover the presence of occult metastases on retrospective examination of ALN-negative patients by step sectioning and/or immunohistochemistry ($n = 7740$ patients) was associated with poorer 5-year disease-free and overall survival. Outcomes from sentinel lymph node biopsy studies were not assessable due to small patient groups and short followup.

The International Breast Cancer Study Group trial IBCSG-23-01 is a randomised multicentre study designed to determine the significance of minimal LN metastasis in patients with breast cancer [56]. The trial was initiated in April 2001, and it compares survival between patients with SLN micrometastases who undergo SLNB alone with those who receive cALND.

5. Completion Axillary Lymph Node Dissection in SLN Macrometastases

It is known that the extent of macrometastases in SLN is strongly correlated with non-SLN involvement. The long-term effect of the residual axillary disease in the sentinel-lymph-node-positive patient on local and systemic recurrence has not been clearly defined for patients receiving modern radiotherapy and chemotherapy. Older studies of patients with symptomatic breast cancers have shown that inadequate axillary surgery does lead to reduced overall survival [57–60].

Recently, the first results of the multicentre Z0011 trial were published [61]. The study set out to randomise 1900 women with breast cancer and 1–5 involved SNLN to either cALND or observation. All had a lumpectomy and tangential breast irradiation, but systemic therapy was at the discretion of the treating centre. After a median followup of 6.3 years, the relapse-free survival for the ALND group was 82% compared with 84% for the observation group, and the overall survival was 92% in both groups. Unfortunately the trial stopped accrual after 891 cases had been entered which makes it underpowered to detect a 5% difference in outcome. An additional aspect to be considered is that the Guy's wide excision studies showed no difference between the wide excision group and the radical mastectomy group at 10 years whereas after 25 years there was a significantly worse relapse-free and overall survival in the wide excision group with inadequately treated axillae [58].

In a retrospective study, Takei et al. confirmed the importance of cALND in SLN-positive patients with high nuclear grade and hormone-negative breast cancer. It was noticed that of 459 patients with macrometastatic disease treated with cALND, after a median follow-up period of 34 months, the axillary recurrence rate was only 0.6% [62]. Bilimoria et al. studied a cohort of 403,167 patients with clinically node-negative breast cancer that underwent SLNB from the US National Cancer Data Base (1998–2005) [63]. Of the 97,314 (24%) patients identified with nodal metastases, 28% had no further surgical intervention in the axilla and 72% underwent cALND. After a median followup of 63 months, it was found that in all patients and separately in those with macroscopic and microscopic nodal disease, the unadjusted axillary recurrence rate and overall survival were comparable. After adjustment for clinicopathological differences, there was a trend towards a lower risk of axillary recurrence and death in patients with macroscopic nodal

Table 3: Studies of patients with micrometastases not treated by completion mastectomy.

Author	Total	Follow-up (months)	Outcome
Fan et al. [48]	27	17	1 recurrence
Nagashima et al. [49]	19	24	1 recurrence
Yegiyants et al. [50]	33	84	1 recurrence
Fournier et al. [51]	16	30	No recurrence
Langer et al. [52]	27	77	No recurrence
Meretoja et al. [53]	48	37	3 recurrences, 1 death
Pernas et al, [54]	45	60	1 recurrence
Reed et al. [38]	57	59	Significantly reduced disease-free interval

involvement undergoing cALND. For those with micrometastases, recurrence rates were similar in those undergoing either SLNB alone or cALND.

Of 26,986 patients with SLNB-positive breast cancer from the SEER database (surveillance, epidemiology, and end results), 16% had no further axillary treatment and 84% had cALND [64]. After a median followup of 50 months, although a higher rate of ipsilateral regional recurrence was noticed in patients who underwent SLNB alone, no statistically significant differences in overall survival (OS) between patients who underwent SLNB alone versus complete ALND were found. The investigators suggested that in patients with small, low-grade primary tumours, positive ER status, older age and who have received segmental mastectomy, cALND may be omitted [64].

Hwang et al. reviewed the outcome of 3,366 patients with invasive breast cancer who underwent SLNB from 1993 to 2005 [65]. Of 750 SLN-positive patients, 65%, 45.9%, and 34.2% were pN1, pN1mi, and pN0 (i+), respectively. Of these patients, 196 had no further axillary surgery due to clinician and patient preference. According to clinicopathological variables, adjuvant treatment was applied and locoregional and distant recurrence and survival were studied. After a median followup of 29.5 months, no patient had an axillary recurrence, one had supraclavicular lymph node recurrence, and three patients developed metastatic disease to the lung or bone. The median time to recurrence was 32 months. Notably the patients with distant metastases had T3 grade III invasive carcinoma. Despite the low axillary recurrence rate, authors suggested that it is not possible from these results to conclude definitively that cALND should be abandoned for these patients [65].

6. Predictive Models

Several factors correlated with the likelihood of additional non-SLN metastasis have been investigated in an effort to distinguish which patients could avoid extensive axillary surgery. Characteristics of the primary tumour, such as size [40, 66], grade [67], hormone receptor and HER2 profile [67], tumour type [67], multifocality, mean proliferative fraction, and lymphovascular invasion [68, 69], have all been studied. Additional features of the involved SLNs, such as size of metastases [40], number of positive SLNs [40], ratio of positive to resected SLNs, and the extracapsular spread have been also examined [21, 61, 70–72]. Particularly patients with minimal SLN metastases are at a significantly lower risk to have further non-SLN invasion than those with SLN macrometastases (13–24% versus 45–79%) [61]. However, none of these characteristics individually can determine a subset of patients for whom ALND is unnecessary. Molecular profiling of metastatic foci different from the primary tumour could be used as indicator for the selection of patients who might benefit of completion axillary dissection [73]. The most important prognostic factors for the presence of non-SLN metastases in patients with minimal SLN involvement are presented in Table 4.

Table 4: Major prognostic factors for non-SLN metastases in patients with minimal SLN metastases.

Feature	Author
Lymphovascular invasion	Mittendorf et al. [67], Van Deurzen et al. [66], Viale et al. [69], Jinno et al. [68]
Size of SLN metastases	Barbosa et al. [40], Van Deurzen et al. [66], Viale et al. [69]
Primary tumour size	Barbosa et al. [40], Van Deurzen et al. [66]
Lobular histology	Mittendorf et al. [67]
Number of positive SLN	Barbosa et al. [40], Viale et al. [69], Jinno et al. [68]

Several mathematical models have been developed to predict the risk of non-SLN involvement in patients with SLN-positive breast cancer [74]. These include four nomograms: the Memorial Sloan-Kettering Cancer Center (MSKCC) [75], (https://www.mskcc.org/mskcc/html/15938.cfm), Mayo [76] (http://www.mayoclinic.org/breast-cancer/sentinelbiopsy.html), Cambridge [77], and Stanford [70] (https://www3-hrpdcc.stanford.edu/nsln-calculator/). There are three scoring systems, the Tenon, MDA, and Saidi, and two recursive partitioning (RP) tools developed by Kohrt et al. [70].

The Institut Curie studied 588 consecutive patients with positive SLNs who underwent ALND to compare the actual

Table 5: Validation studies comparing OSNA with histopathology.

Author	Total	Concordance	Sensitivity	Specificity
Tsujimoto et al. [71]	101	98%	91%	100%
Schem et al. [72]	93	92%	98%	91%
Tamaki et al. [79]	185	93%	88%	94%
Snook et al. [80]	204	96%	92%	97%
Feldman et al. [81]	498	96%	78%	96%

rate of non-SLN metastases with those predicted by Breast Cancer Nomogram of Memorial Sloan-Kettering Cancer Center (MSKCC). While the predicted rate in non-SLN macrometastases was relatively accurate, when the nomogram was applied to the 213 SLNs that contained only micrometastases, the predicted rate 5–9% was far away from the actual rate 44% of non-SLN micrometastases detect-ed by IHC. Consequently, the authors concluded that a different predictive model should be created for patients with micrometastases.

Molecular tests based on technology such as Oncotype Dx (Genomic Health, Redwood City, Calif, USA) or other multigene arrays developed prognostic and predictive markers aiming to personalize surgical and adjuvant treatment of early breast cancer [4]. An accurate, intraoperative sentinel lymph node test probably could help in avoidance of delayed axillary dissections. Molecular tests may be proved more sensitive than current intraoperative tests but have not yet been validated.

7. One-Step Nucleic Acid Amplification (OSNA)

OSNA is an automated assay for the detection of cytokeratin message, CK19 mRNA, present in approximately 98% of breast cancers [78]. It provides an opportunity to make an intraoperative diagnosis of sentinel node involvement within 30 minutes, avoiding frozen section and allowing a one-stage procedure. Larger verification studies in which half of the bisected sentinel node was sent for histology and the other half homogenised for OSNA are shown in Table 5 [71, 72, 79–81]. The usual reason for discordance between histopathology and OSNA was an uneven distribution of nodal metastases (tissue allocation bias). This problem is abolished when the entire node is subjected to OSNA.

The studies indicate a good concordance between results of histopathology and OSNA. Indeed in a study conducted by Osaka, comparing OSNA with frozen section, the former was more sensitive, increasing the positive sentinel node rate by 30% [82]. It is likely that in time OSNA will replace histopathological examination of sentinel lymph nodes because of its ease, accuracy, and potential for enabling almost all patients to have a one-stage operation for early breast cancer.

8. Conclusions

Recent studies aiming to determine if cALND is beneficial in patients with SLN-positive breast cancer and even more in patients with minimal SLN involvement have reached contrary conclusions. Limitations in the published studies include the methods of pathological evaluation of lymph nodes and that the number of additional non-SLN-positive nodes is usually not known. Detection rate of micrometastases and ITCs depends on histopathological technique and protocol. Thus lymph node step sectioning and IHC lead to increased identification of minimal metastases upstaging 9% to 25% of patients who initially were considered node negative [83, 84] and did not have further axillary surgery.

The different rates of recurrence may be due to the molecular type of cancers and so that patients with SLN metastases may also have different risks of metastatic involvement [40]. Finally it is possible that not all minor tumour foci in axillary lymph nodes progress to local recurrences. According to Al-Hajj et al., only a minority of cancer cells potentially give metastases and most ITCs are not viable and do not have the ability to form new tumours [85]. There may be two different breast cancer cell populations, true stem cells that have the capacity to develop metastases and the nonstem cells that never grow and are finally destroyed [86].

At the level of everyday clinical practice, with both promising and disappointing results of the published studies, most breast surgeons will hardly ever take the risk of avoiding completion axillary dissection in breast cancer patients even with minimal sentinel lymph node metastases. Many are seeking to find a balance between the needs of the majority to have minimal axillary surgery with minimal postoperative morbidity against the possibility that a minority will suffer relapse, morbidity, and possible increased mortality from undertreatment. Results from ongoing phase III trials will perhaps provide new guidelines for the treatment of patients with micrometastases or ITCs. A predictive model which estimates accurately the likelihood of additional disease in the axilla might help tailor surgical therapy to the needs of the individual patient and identify those most likely to benefit from completion or ALND. Genetic assays defining prognostic markers and new intraoperative tests detecting accurately SLN involvement will help in early therapeutic decision making in the future. It is important that premature decisions to restrict axillary surgery are not made on a basis of early results from underpowered clinical trials.

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Review Article

CXCR4 and Axillary Lymph Nodes: Review of a Potential Biomarker for Breast Cancer Metastasis

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CXCR4 is a 7-transmembrane G-protein chemokine receptor that allows for migration of hematopoietic cells from the bone marrow to the peripheral lymph nodes. Research has shown CXCR4 to be implicated in the invasion and metastasis of several cancers, including carcinoma of the breast. CXCL12 is the ligand for CXCR4 and is highly expressed in areas common for breast cancer metastasis, including the axillary lymph nodes. Axillary lymph nodes positive for breast carcinoma have been an important component of breast cancer diagnosis, treatment, and subsequent research. The goal of this paper is to analyze the literature that has explained the pathways from CXCR4 expression to breast cancer metastasis of the lymph nodes and the prognostic and/or predictive implications of lymph node metastases in the presence of elevated CXCR4.

1. Introduction

Lymph nodes of the axilla have been studied in the context of breast cancer since before Halsted published his study proposing that this lymphatic drainage was a pathway for metastasis and recommending axillary node dissection (AND) [1]. The understanding of breast cancer metastasis has changed greatly over the past century. Sentinel lymph node biopsies (SNB) allowed for detection of smaller micrometastases that have previously gone undetected. Understanding the true benefit for the use of AND with SNB has been a topic of discussion, and a recent trial has suggested that there is no advantage of AND in patients with a negative SNB [2]. In contrast, an earlier report slightly favored disease-free survival and overall survival in AND patients over just SNB (albeit with a limited number of patients) [3]. This comes 10 years after data was published describing a 30-year followup of internal mammary node dissections that did not improve survival of breast cancer patients [4]. The questioning of the therapeutic value of axillary node dissection in certain patient populations has allowed for further exploration of the prognostic and/or predictive value of these organs. A clearer picture of the molecular mechanisms of lymph node metastases is the next step to designing optimal therapeutic options and to create new treatment modalities.

One such molecular avenue is chemokine receptor CXCR4, a seven transmembrane G protein-coupled receptor that has been implicated in the invasion and metastasis of several cancers, including breast. Over 20 chemokine receptors have been identified and are keys to pathways that include the body's response to allergy, inflammation, and metastasis. Although a great deal of research into CXCR4 began by focusing on its role in HIV entry of CD4+ cells [5, 6], Müller et al. discovered that CXCR4 was integral for the pathway that activates actin polymerization and pseudopodia formation in breast cancer cells [7]. CXC chemokine ligand-12 (CXCL12), also known as stromal-derived factor-1 (SDF-1), is the ligand for CXCR4. Müller's lab noted that CXCL12 was found in high concentrations at sites that were common for metastasis, such as brain and bone. For this paper, we will only use the term CXCL12.

A litany of studies have emerged in the last five years examining CXCR4 and axillary lymph nodes, covering multiple approaches of how CXCR4 in the setting of axillary lymph

nodes can impact our understanding of breast cancer. This review aims to coalesce several papers across the spectrum of this research to tie together molecular pathways and illustrate emerging trends to help direct future research into CXCR4 as a prognostic and/or predictive marker for breast cancer.

2. Biology of CXCR4 and Lymph Node Metastasis

CXCR4 was initially discovered as HUMSTR [8] and LESTR [9] and later renamed according to proper nomenclature for chemokine receptors. The action of CXCR4 leads to intracellular signaling cascades that are involved with trafficking, migration, and proliferation. One major site of involvement is in hematopoiesis, due to its expression on CD34+ cells in bone marrow. Evidence points to its role in maintaining hematopoietic progenitor cells [10]. It is also a factor in immune system cells, including monocytes, dendritic cells, NK cells, and naïve T cells. The proposed role of CXCR4 is to help the immune system migrate to sites of injury, but Müller et al. have shown it to be actively involved in metastasis at sites expressing its ligand, CXCL12, playing an important role in the tumor microenvironment [7].

The ligand CXCL12 has been further characterized by Crump et al. to understand how it binds to CXCR4 [11]. Their *in vitro* studies revealed CXCL12 to have two binding sites for CXCR4. The RFFESH loop on CXCL12 initially binds with an N-terminal segment of CXCR4. This allows for access to a second receptor site of CXCR4 for the N-terminal region of CXCL12 to bind, altering the conformation of the CXCR4 transmembrane helices and activating the G-protein signal pathway. That signal is able to affect multiple targets, including ERK1/2, MAPK, JNK, and AKT paths, with the end result being events such as chemotaxis, pseudopodia formation, and actin polymeriazation [12–14].

In addition to its role within the immune and hematopoietic systems, CXCR4 has also been implicated as a component in angiogenesis [15, 16]. A well-known component of angiogenesis in tumor cells is vascular endothelial growth factor (VEGF), which has also shown to be prognostic in colon and gastric cancer [17, 18]. CXCR4 has been connected with VEGF, as increased stimulation of CXCR4 leads to increased secretion of VEGF and ultimately angiogenesis and metastasis [19–21]. To achieve metastatic potential, the tumor cells must migrate away from the primary site. Tumor cells must break through the protective extracellular matrix (EM) in order to reach the lymph or blood vessels. Matrix metalloproteinases (MMPs) are essential for this component of invasion, as they cause degradation of EM. One MMP specifically, MMP-9, has been linked to VEGF [22].

In addition to CXCR4/CXCL12, VEGF, and MMPs, HIF-1α has also shown to be a component of this pathway. HIF-1α is a dimeric transcription factor that increases in deoxygenated environments. Hypoxic conditions are known to promote angiogenesis and have been connected to increased HIF-1α and VEGF [23]. Additionally, CXCR4 has proven to be increased in hypoxic conditions [24]. The connection between hypoxia, CXCR4, and invasive cancer has been outlined by Sun et al. in a series of experiments that illustrated the connection and molecular pathway between hypoxia in chondrosarcoma, CXCR4 expression, and matrix metalloproteinases. Hypoxic conditions that produced higher levels of HIF-1α were observed to subsequently increase CXCR4 expression through the binding of HIF-1α to the promoter region of CXCR4. Subsequent CXCR4/CXCL12 signaling via the ERK pathway increased MMP expression and activity [25]. While this pathway was worked out on cell lines for chondrosarcoma, HIF-1α increases in hypoxic conditions have also been shown to upregulate CXCR4 in carcinoma of the breast [26].

Another component that has been linked to CXCR4 upregulation is nitric oxide (NO). While already known to induce VEGF [27], Nakamura et al. have been able to show that NO also induces the lymphangiogenic factor VEGF-C [28]. In more recent work from their laboratory, MDA-MB-231 cell lines incubated with NO revealed an increased cytoplasmic CXCR4 staining. Cytoplasmic CXCR4 significantly correlated with nitrotyrosine levels, lymph node metastasis, and distant metastasis [29].

The location of CXCR4 staining is a common feature in several of the papers to be described in this review. The CXCR4 receptor resides on the membranes of cells, but IHC has helped reveal patterns between cytosolic and nuclear expression of CXCR4. Tarasova et al. [30] observed endocytosis of membranous CXCR4 receptors in the presence of CXCL12. Salvucci et al. [31] noted that cytoplasmic CXCR4 was seen more often in ductal carcinoma in situ (DCIS) patients when compared to nuclear staining. That group speculated that cytoplasmic CXCR4 staining could be indicative of "active CXCR4 functioning," as if the cancer cells are ready to leave the primary tumor. While nuclear staining of CXCR4 has been tested and observed in many studies, the reasons behind its difference in expression from cytoplasmic CXCR4 has not yet been uncovered.

A viable pathway illustrating the role of CXCR4 in breast carcinoma migration from the primary site does not explain how these cells are guided to axillary lymph nodes. Studies have shown CXCL12 to be expressed on the luminal surface of high endothelial venules (HEVs) in peripheral lymph nodes [32]. HEVs are postcapillary venules that enable circulating lymphocytes to enter lymph nodes. CXCL12 is a factor in hematopoietic precursors moving from the bone marrow into the circulation, and ultimately to peripheral tissues [10]. Blades et al. designed a study that showed that CXCL12-induced migration mediated by CXCR4 controlled the migration of human peripheral blood lymphocytes into lymph nodes previously transplanted into SCID mice [33]. Liu et al. performed a series of experiments exhibiting CXCL12 concentrations to be significantly higher in lymph node metastases compared to their primary breast cancer tumor [34]. The location-based chemotaxic ability of CXCL12, combined with its affect on CXCR4-expressed cells, creates a microenvironment for tumor migration (Figure 1).

Table 1: CXCR4 and lymph node metastasis in recent studies.

Study	Findings	*P* value
Hao et al. [35]	↑CXCR4 with ↑TNM stage	<0.037
	↑CXCR4 with +lymph node mets	<0.001
	↑CXCR4/VEGF w/+LN mets	0.007
	↑CXCR4/MMP-9 w/+LN mets	<0.001
Kang et al. [36]	↑CXCR4 in +LN tumors over −LN tumors	0.03
	↑CXCR w/OS or distant mets-not observed in this data	—
Parker et al. [37]	↑CXCR4 w/+LN had worse 5 yr OS	0.02
Cabioglu et al. [38]	↑CXCR4 w/+LN	0.113
Su et al. [39]	↑CXCR4 (cytoplasmic) with +LN mets	0.0325

CXCR4: CXC chemokine receptor 4, LN mets: lymph node metastases, MMP-9: matrix metalloproteinase-9, VEGF: vascular endothelial growth factor.

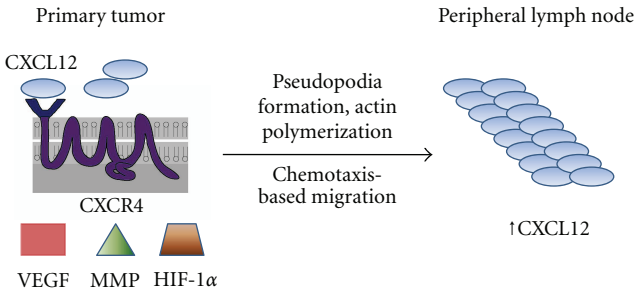

Figure 1: Depiction of a primary tumor overexpressing CXCR4 and the basic factors associated with its migration to peripheral lymph node sites. Increased concentrations of CXCL12 have been noted at lymph node metastasis sites when compared to the primary tumor.

3. CXCR4 Levels and Association to Lymph Node Status

With knowledge of the molecular connections between CXCR4, VEGF, and MMPs, Hao et al. asked if there was a any association between these three components of metastasis and lymph node status in breast cancer patients [35]. The location of CXCR4 staining was studied in breast cancer tissue samples, benign tissue samples that were adjacent to tumor tissue, and atypical hyperplasia samples. CXCR4 was found in both the cytoplasm and the nucleus of mammary cells. Benign tissue adjacent to tumor lesions exhibited weak cytoplasmic CXCR4 staining. The malignant samples had significantly higher rates of CXCR4, VEGF, and MMP-9 compared to the benign and nontumor (hyperplastic) samples ($P < 0.01$). Looking at clinicopathologic factors, statistical significance was found upon analyzing either CXCR4 or VEGF with the tumor's TNM stage. In addition, all three markers had significant association with advanced histologic grade.

When analyzing the primary tumors, increased expression levels of CXCR4, VEGF, and MMP-9 associated with the presence of lymph node positive breast cancer ($P < 0.001$). Results showed a lymph node metastasis rate of 79% with tumors expressing high levels of both CXCR4 and VEGF compared with a 45% rate when only one of these factors is high ($P = 0.007$). In contrast, a 6% rate was observed ($P < 0.001$) when neither factor was highly expressed. Any combination of two of the three markers, each highly expressed, had a significant increase in lymph node metastases. Finally, this study showed that high CXCR4 expression was positively associated with increased VEGF and MMP-9 expression (Table 1).

Kang et al. have shown that high CXCL12 expression in breast cancer tissue is linked to nodal and distant spread of breast cancer cells, as well as a link to overall survival [36]. To follow up this study, this group focused on CXCR4's relation to metastasis and survival [40], as opposed to its ligand. Using immunohistochemistry, CXCR4 was detected in both breast cancer cell lines as well as normal mammary tissue. It was shown that node-positive tumors had a significantly increased expression of CXCR4 when compared to node-negative tumors. CXCR4 expression was higher in the metastatic cohort compared to the nonmetastatic group, but significance was not achieved when looking at a relationship between elevated CXCR4 and presence of distant metastases or overall survival (Table 1).

Parker et al. evaluated a cohort of 185 node-positive breast cancer patients and found that CXCR4 overexpression level in primary tumors independently predicted a poor outcome for these patients [37]. The 5-year overall survival for patients with low and high CXCR4 overexpression was 69% and 57%, respectively, ($P = 0.02$, Table 1). These results suggest that even within this high risk group (i.e., node positive patients), CXCR4 can be employed to identify those patients who have a more aggressive disease course and therefore can be targeted for more intensive and/or novel therapy.

Not all studies have successfully concluded CXCR4 to definitely predict outcome. Cabioglu et al. studied CXCR4 as a predictive marker for lymph node metastasis along with CCR7 [38], another chemokine receptor that has been shown to be expressed in breast cancer cells [7]. The group attempted to reduce confounding effects that can accompany T2-4 lesions by limiting this study to only T1 lesions. Differences in CXCR4 and CCR7 staining location were tested, with node-positive tumors showing higher cytoplasmic CCR7 and HER2 staining than node-negative tumors. There was an increased rate of CXCR4 in node-positive patients (11.2% versus 5.1%), but this difference was not significant (Table 1). The authors theorized that CCR7 is associated

with lymph node metastasis, while CXCR4 expression aids in the reliability of CCR7 as a biomarker. When Liu et al. investigated the relationship between CXCR4 and CCR7, they found in their data set that CXCR4 and CCR7 each significantly associated with lymph node metastasis. A lower overall survival was noted via the Kaplan-Meier survival analysis of both CXCR4 and CCR 7 overexpression [34].

The difference between nuclear and cytoplasmic CXCR4 staining has emerged as an important factor in CXCR4's prognostic/predictive ability [41]. Su et al. examined the expression location of CXCR4 in breast cancer cells and tested for associations in marker staining and metastasis [39]. The study was designed to compare 3 groups: (1) patients with sentinel and nonsentinel lymph node metastasis, (2) patients with only sentinel lymph node metastasis, and (3) patients with neither sentinel or nonsentinel metastasis. Combining groups 2 and 3 together (they chose for sole sentinel node positivity to be equivalent to no metastasis) revealed that high cytoplasmic CXCR4 expression was associated with axillary lymph node status ($P = 0.0325$, Table 1). Their data did not show any other correlations with cytoplasmic or nuclear CXCR4 staining and any other clinicopathological factor. Essentially, these results show that elevated cytoplasmic CXCR4 are indicative of spread beyond the sentinel lymph node.

4. Autocrine versus Paracrine CXCL12

The knowledge that stromal cells express CXCL12 opens the theory that location, along with concentration, can be important in predicting outcome. Kang et al. found that when adding CXCL12 to the cell line MDA-MB-231, there was increased migration and invasion ability. That study showed an inverse relationship between CXCL12 expression levels and disease-free and overall survival in breast cancer patients. Increased CXCL12 caused an increased incidence of recurrence and lymph node metastasis [36].

Mirisola et al. conducted a series of experiments to help explain the autocrine/paracrine effect of CXCL12 [42]. When analyzing 100 breast cancer samples, IHC studies showed that groups highly expressing CXCL12 tended to be smaller tumors and lymph node negative ($P = 0.04$, $P = 0.002$). Also noted was a significant association between DFS and the expression pattern of CXCL12, specifically expression at the tumor periphery ($P = 0.002$). CXCR4 expression in this data was not significant for DFS or OS. Tumors expressing CXCL12 had a better clinical outcome than those that lost the chemokine. The authors' explanation is that when CXCL12 is produced by the tumor, the autocrine function of CXCL12 renders the tumor insensitive to its effects and cancels any metastatic potential. Tumor growth via the ERK pathways and VEGF are still in play, but metastatic potential is lost. Thus, it can be inferred that tumors not overly expressing CXCL12 show a paracrine effect and maintain metastatic potential.

The idea that CXCL12 can inhibit the effect of CXCR4 was further explored by Shim et al. [26]. First, they were able to produce results that showed breast cancer lymph node metastases were overexpressed at a lower rate than they were at the primary tumor. Next, they moved to explain how increased CXCL12 concentrations can affect CXCR4, a finding previously noted in Liu et al. [34]. After creating an "expression score" to quantify CXCR4 expression in their breast cancer and lymph node specimens, this group was able to show that primary breast cancer specimens exhibited a higher score than lymph node metastases ($P < 0.001$). Two immunostaining patterns were noticed. 58% of primary tumor samples had membranous staining of CXCR4 predominate, versus 80% of lymph node metastases having cytoplasmic staining predominate. In the reviewed papers Hao et al. [35], Su et al. [39], Yasuoka et al. [29], and Liu et al. [34] each of these observed cytoplasmic staining to associate with lymph node metastasis.

This group studied CXCL12 mRNA levels of 10 primary breast cancer tumors along with their matching lymph node metastases and observed CXCL12 levels to be higher in lymph node tumors than the primary breast tumor ($P < 0.001$). For a control, the MDA-MB-231 cell line was examined. CXCR4 staining was observed to be predominantly membranous in this line. However, after incubation with CXCL12 for 30 minutes, CXCR4 expression was found in the cytoplasm. Furthering this experiment, MDA-MB-231 cells were exposed to 100 and 200 ng/mL concentrations of CXCL12 for 48 hours. CXCR4 was then measured by Western blot, and expression as found to be significantly decreased. This group then tested chloroquine, known to inhibit proteolysis of lysosomes, with the MDA-MB-231 cell line and found CXCR4 expression returned while still in the presence of high concentrations of CXCL12. This suggests that high CXCL12 concentrations cause cellular degradation of CXCR4 receptors.

5. Alternate Research Pathways and Questions

It has been widely accepted that CXCL12 is the exclusive ligand of CXCR4. Burns et al. have altered this belief with a study documenting the ability of CXCL12 to bind to CXCR7 (RDC1, CCX CJR2), a novel receptor that was first characterized as a chemokine receptor in this paper [43]. They were able to show that CXCL12 binds to CXCR7 by transfecting a cell line lacking CXCR4 and CXCR7 with the RDC1 gene, resulting in high-affinity CXCL12 binding even in the presence of the CXCR4 inhibitor, AMD3100. In the following experiments, Burns et al. introduced CXCR7 into breast cancer cell line MDA MB435s and documented an increase in cell growth and increased adhesion to human umbilical vein endothelial cells. This lab's continued investigation of CXCR7 has since shown that it promotes breast and lung cancer in murine models [44]. Additionally, CXCR7 was undetectable or at low levels in normal human breast tissue from mammoplasties, but was clearly detected in over 30% of human breast cancer specimens. It was also detectable in 97% of blood vessel specimens from human breast cancer, versus being "undetectable or nearly undetectable" in normal blood vessels from normal breast tissue.

Searching for new biomarkers is more complex than locating factors with changed expressions from their benign

baseline, according to Ransohoff and Gourlay [45]. They state that several forms of bias might account for the fact that while many targets have been identified as biomarkers, very few have had "clinical value." This bias possibly occurs before a specimen arrives to a laboratory in the form of collection and storage. One example cited details a group investigating prostate cancer that questioned whether differences in storage time between the cancer and noncancer specimen groups affected the end results [46]. Ransohoff and Gourlay's assessment of bias concludes that subject selection is of utmost importance—"inequality of specimen groups" is a major source of bias in experiments where the outcomes are observations, not laboratory results. They believe that improving the attention given to specimens, both in selection and management once acquired, might improve the quality of results in biomarker identification research.

6. Conclusion

CXCR4 is an important factor in breast cancer metastasis. The molecular pathway for its action, along with associated factors, is continuing to be broken down piece by piece. A myriad of components appear to play important roles in the overexpression of CXCR4, from NO and VEGF to MMPs and HIF-1α. Differences have been noted in CXCR4 between primary tumors and lymph node metastases, specifically in the amount of overexpression: CXCR4 has been found to be more highly overexpressed at the primary tumor than at lymph node metastases. Repeated in many papers is the fact that cytoplasmic CXCR4 staining is noted to associate with lymph node metastasis while nuclear CXCR4 staining has not had significant results. Studies into the responsibilities of CXCR4's ligand, CXCL12, have revealed increased concentration of the ligand at distant sites, specifically the lymph nodes. Another important issue with CXCL12 is the idea that tumors that produce high amounts of the chemokine effectively downregulate CXCR4 receptors, while tumors without high CXCL12 expression maintain a prometastatic ability. The role of CXCR7 further clouds the picture when attempting to understand what is more important to target while grasping CXCL12's effect on both CXCR7 and CXCR4 in the same tumor microenvironment.

CXCR4 has been identified as a receptor for CXCL12, changes in expression location patterns of CXCR4 have been described, and positive associations with disease outcome have been derived. New discoveries concerning CXCR7, paracrine/autocrine CXCL12 effects, and more careful planning in discovery and examination of biomarkers will help shape the future directions of this research. Possibilities exist for collaboration and shared information; the results of which could alter the current understanding and treatment of breast cancer, both local and systemic.

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Research Article

Should a Sentinel Node Biopsy Be Performed in Patients with High-Risk Breast Cancer?

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A negative sentinel lymph node (SLN) biopsy spares many breast cancer patients the complications associated with lymph node irradiation or additional surgery. However, patients at high risk for nodal involvement based on clinical characteristics may remain at unacceptably high risk of axillary disease even after a negative SLN biopsy result. A Bayesian nomogram was designed to combine the probability of axillary disease prior to nodal biopsy with customized test characteristics for an SLN biopsy and provides the probability of axillary disease despite a negative SLN biopsy. Users may individualize the sensitivity of an SLN biopsy based on factors known to modify the sensitivity of the procedure. This tool may be useful in identifying patients who should have expanded upfront exploration of the axilla or comprehensive axillary irradiation.

1. Introduction

In breast cancer, metastases to the axilla are associated with an increased risk of distant micrometastatic disease [1–3]. Sentinel lymph node (SLN) biopsy has become standard practice for evaluating the axilla in patients without palpable lymph nodes [4]. This procedure involves injection of a tracer, usually a radioactive colloid, alone or in combination with dye, into the tissue surrounding a tumor. Lymph nodes with evidence of uptake are surgically removed. The SLN procedure typically yields 1–5 nodes for pathologic examination whereas full axillary lymph node dissection (ALND) can yield greater than 20 nodes when taken to completion. On the other hand, SLN biopsy is associated with less pain, lower rates of postsurgical lymphedema, and better arm mobility when compared to full ALND [4]. Many patients with a positive SLN biopsy go on to have ALND for both diagnostic and therapeutic purposes. However, there is growing evidence that axillary irradiation may be used instead of ALND in select cases with excellent results [5]. Indeed a recent randomized trial showed that ALND offered no benefit over SNL biopsy in terms of local control or survival to women with early clinical stage breast cancer who also received radiation therapy [6].

Like any diagnostic test, SLN biopsy can yield false-negative results. Several factors can affect the sensitivity of

Table 1: Factors influencing the sensitivity of SLN biopsy.

Factor	Sensitivity	Reference
T1	89.7–93.3%	[4, 7, 8]
T2-T3	82.0–92.6%	[7, 9]
Grade		[10]
1	95.7%	
3	90.4%	
Skill of surgeon	72.4–100%	[8]
Method		[11]
Combined dye and isotope	86.3–96.0%	
Dye	85.7–90.4%	
Isotope	86.3–97.8%	
Number of SLN removed		[10, 12]
1	82.3–89.1%	
3	93.1–98.9%	
5	99%	
Medial tumor	Decreases	[8]
Age > 50	Decreases	[8]
Obesity	Decreases	[10, 13]

an axillary SLN biopsy (Table 1). Large tumors have been associated with decreased sensitivity (equivalently, higher false-negative rates), perhaps because they access a greater number of local lymphatic pathways and therefore have the potential for spreading to a larger distribution of nodes [7, 9]. Age-related fatty changes in nodes may decrease the capacity for dye or isotope uptake [8]. Medially located tumors may drain more frequently to internal mammary nodes than tumors located centrally or in the lateral breast [8]. Finally, two prospective studies suggest that the sensitivity of SLN biopsy correlates with the number of nodes removed [10, 12]. It should be noted that as techniques and protocols have improved, the sensitivity of SLN biopsy has generally improved. Nevertheless, the procedure remains imperfect, and most recent studies demonstrate false-negative rates in the range of 5–10% for small tumors [10, 12].

Breast cancer risk calculators are being increasingly used to guide adjuvant systemic and local treatment [14–17]. Using such a calculator, the probability of axillary nodal involvement for a given patient can be estimated prior to SLN biopsy based on a number of prognostic factors including age, tumor size, and histopathological features of the breast cancer. One example of such a risk calculator was developed at Memorial Sloan Kettering (MSKCC) and is available for use online. This calculator was originally intended to spare *low-risk* patients an SLN biopsy when the probability of nodal involvement is low [14]. However, this calculated probability may also benefit *high-risk* patients when used in combination with estimates of the false-negative rate of SLN biopsy to calculate the risk of having residual nodal disease in the setting of a negative SLN. We developed a nomogram that combines this probability of axillary disease with estimates of the sensitivity of SLN biopsy, to calculate the risk of residual axillary disease despite a negative SLN biopsy.

2. Methods

Bayes' rule combines the pretest probability of a given diagnosis with results from a test with known sensitivity and specificity to yield a posttest probability of having the diagnosis. In this analysis, the pretest probability is the probability of having axillary disease prior to any nodal evaluation; the posttest probability is the probability of axillary disease given a negative SLN biopsy; the false-negative rate (1-sensitivity) of SLN biopsy can be estimated from Table 1. The specificity of SLN biopsy is by definition equal to one (equivalently, the probability of a positive SLN in the absence of lymph node involvement disease is zero).

In this setting, Bayes' rule takes the following form:

$$\text{Post} = \text{pre} * \frac{(1 - \text{sens})}{\text{pre} * (1 - \text{sens}) + (1 - \text{pre})(\text{spec})}. \quad (1)$$

With "post" and "pre" defined as posttest and pretest probabilities, respectively, "sens" defined as the sensitivity of SLN biopsy, and "spec" defined as the specificity of the procedure, which in our situation is 1.

Using this formula we can estimate the probability that a breast cancer patient has residual axillary disease despite a negative SLN biopsy. A Bayesian nomogram was constructed in MATLAB (MathWorks, v7.8) using the mathematical relationship above. We used a range of pretest probabilities from 5 to 85% and a 4 estimates of sensitivity (80%, 85%, 90%, and 95%) for the SLN biopsy procedure.

3. Results

We created a Bayesian nomogram for the probability of axillary nodal involvement despite a negative SLN biopsy (Figure 1). The nomogram was designed to be flexible in order to accommodate a variety of clinical scenarios. A range of sensitivity values are displayed along the middle axis as discrete points; the appropriate value for a given patient can be estimated using Table 1. A line drawn through a given pretest probability and sensitivity point will intersect with the appropriate posttest probability (probability of having residual axillary disease despite a negative SLN biopsy) on the right-hand axis.

For example, the nomogram can be used to calculate the risk of residual nodal disease in a 62-year-old woman who presents after a lumpectomy revealing a 1.5 cm, grade 2 invasive ductal carcinoma, hormone receptor-negative, with no lymphovascular invasion and an SLN biopsy yielding 3 negative nodes. According to the MSKCC model her risk of axillary disease prior to SLN biopsy is 19%. Assuming 95% for the sensitivity of SLN biopsy in this situation, the nomogram reveals that the probability of having residual axillary disease is 1.2%. Even if the sensitivity of SLN biopsy was assumed to be 85%, the posttest probability remains low at 3.4%.

Likewise, the nomogram can be used to calculate the probability of residual axillary disease despite a negative SLN biopsy in a woman at higher risk of axillary involvement. A 64-year-old patient with a 2 cm, grade 3, hormone receptor-positive, invasive ductal carcinoma with lymphovascular

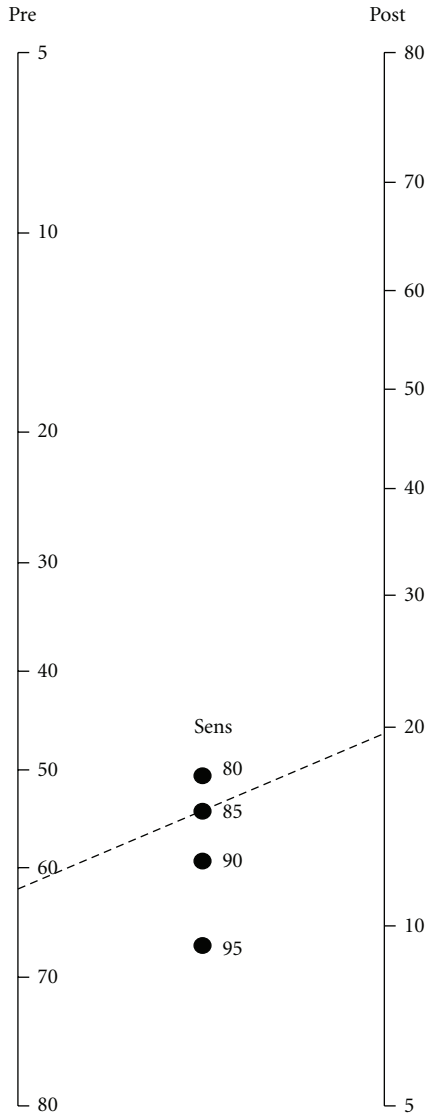

Figure 1: Bayesian nomogram for probability of metastatic disease as a function of pretest probability and negative SLN biopsy. The "pre" or pretest probability of axillary disease can be estimated using a risk calculator such as the one described [14]. The "sens" or sensitivity of SLN biopsy can be estimated using Table 1. Positions for the central dots are calculated assuming a SLN biopsy sensitivity of 80%, 85%, 90%, or 95%; specificity is assumed to be 100%. The calculation for the example patient is shown by the dotted line: if we assume sensitivity of 85% for SLN biopsy and a pretest probability of 62%, the posttest probability for axillary disease is 20% for this patient even in the presence of a negative SLN biopsy.

invasion has a 62% pretest probability of metastases to the axilla based on her pathology. Using a sensitivity of 85% for SLN biopsy, the nomogram reveals that the probability of residual axillary nodal involvement is 20%. In other words, despite a negative SLN biopsy, she still has a 20% probability of finding metastatic disease with completion ALND. If the SLN biopsy procedure has a 95% sensitivity the nomogram reveals that the risk of additional axillary disease after a negative SLN biopsy decreases to 7.5%.

Table 2: Calculated posttest probability of residual axillary disease despite negative SLN biopsy for a range of pretest probabilities for axillary disease with varying sensitivities of SLN biopsy.

	Posttest probability of axillary disease		
Pretest	Sens 0.85	Sens 0.90	Sens 0.95
0.05	0.008	0.005	0.003
0.1	0.016	0.011	0.006
0.2	0.036	0.024	0.012
0.3	0.060	0.041	0.021
0.4	0.091	0.063	0.032
0.5	0.130	0.091	0.048
0.6	0.184	0.130	0.070
0.7	0.259	0.189	0.104
0.8	0.375	0.286	0.167
0.9	0.574	0.474	0.310

Table 2 provides a summary of findings for the posttest probability of residual axillary disease despite negative SLN biopsy for a range of pretest probabilities of axillary disease prior to SLN biopsy at each of 3 different sensitivities for the SLN procedure.

4. Discussion

The presence of axillary disease is the most important prognostic factor in breast cancer. Disease in the axilla can indicate biological aggressiveness and extent of tumor involvement, often suggesting systemic spread and the need for additional therapy. In addition, the link between locoregional disease control and overall survival in breast cancer has been firmly established by meta-analyses of randomized data [18]. The potential importance of ALND in select patients is underscored by a recent analysis which suggested a survival benefit for women with macroscopic nodal disease that received ALND as compared to women with SLN biopsy alone [19]. Additionally the NCIC CTG MA.20 trial which randomized patients with high-risk breast cancer to postoperative whole-breast (WB) radiotherapy alone versus WB plus regional nodal irradiation showed improved locoregional control and an even greater improvement in distant disease control in the arm with regional nodal irradiation [20]. Therefore, the risks of more extensive axillary treatment versus the risks of missing occult disease must be carefully considered.

Our nomogram is intended to be flexible and enable increased personalization of cancer care. Specifically, our analysis is most applicable to two clinical scenarios and argues that

(1) for a patient who had a negative SLN biopsy, but still has a high posttest probability of axillary disease, comprehensive axillary radiation may be warranted;

(2) for a patient who has not yet undergone any axillary surgery, who has a high pretest probability of positive axillary nodes based on clinical features and who also has clinical characteristics that might decrease the

sensitivity of SLN biopsy (as in Table 1), expanded axillary assessment up front may be warranted.

These conclusions seek to limit overtreatment in the form of multiple surgeries (SLN biopsy followed by ALND) and undertreatment in the form of omission of axillary radiation in breast patients with high-risk disease.

We created a nomogram to estimate the risk of residual axillary disease despite a negative SLN biopsy as a function of the sensitivity of the SLN biopsy procedure and the pretest probability of axillary disease prior to axillary evaluation. Our nomogram reveals that for patients with a high pretest probability of axillary metastases and factors associated with a lower SLN biopsy sensitivity, the posttest probability of axillary disease often remains high despite a negative biopsy. While SLN biopsy is the appropriate test for most breast cancer patients, a preemptive expanded assessment of the axilla may be a better choice for high-risk patients or in cases where an SLN biopsy is predicted to be less sensitive (Table 1).

In contrast to other probability calculators which estimate the risk of nonsentinel axillary nodal disease in a woman with breast cancer only *after* a positive SLN, our decision tool estimates the probability of nonsentinel axillary nodal disease *without* prior pathologic assessment of the axilla [15–17, 21]. The posttest probability obtained from the nomogram presented can then be assessed to be acceptable or not based on the specific clinical scenario. For example, a predicted posttest probability greater than 20–25% may warrant consideration of a more thorough axillary assessment upfront. Similarly, in cases where a negative SLN biopsy has already been obtained, a posttest probability of greater than 10% may suggest the need for the addition of axillary radiation.

In patients identified by the nomogram to have an unacceptably high-risk of residual axillary disease despite negative SLN biopsy, more aggressive preemptive exploration of the axilla will necessarily mean a higher risk of lymphedema, nerve injury and general surgical complications compared to SLN biopsy; nevertheless, with modern ALND where only levels I-II are removed, these risks are less than observed historically [20]. Another option may be a less morbid lymph node sampling procedure as was reported in the UK, where at least 4 palpable lymph nodes are obtained by dissection starting at the axillary tail [22]. For patients where a negative SLN biopsy has already been obtained, but a high posttest probability of axillary disease remains, the addition of axillary radiation therapy could be considered in lieu of completion axillary dissection.

Use of this nomogram after a breast biopsy is not expected to result in excessive axillary treatment because it is likely to slightly underestimate rather than overestimate the probability of residual nodal disease. This is because the MSKCC risk calculator used above to obtain a pretest probability of having nodal disease was validated with information from complete pathologic specimens whereas, in practice, physicians are likely to substitute incomplete biopsy specimens such as findings from core needle biopsy, resulting in underdetection (due to undersampling) of certain negative prognostic factors, such as lymphovascular invasion, multifocality, and higher-grade tumor areas, which if found, increase the probability of having nodal disease. Therefore, use of only biopsy information would lead to underestimation of the pretest probability of having axillary involvement, which in turn would lead to underestimation of the posttest probability when using the nomogram. Another possible source of error associated with the use of incomplete biopsy specimens is that tumor size estimate entered into the MSKCC risk calculator must be estimated indirectly, based on imaging. However, assessment of tumor size by MRI, mammography and ultrasound does appear to correlate well with size as determined by pathologic exam [23, 24].

Although the recently published ACOSOG Z0011 and NSABP B-32 trials show similar rates of local control in both their SLN alone and ALND arms, the patients included in these trials were conservatively chosen and by definition had a low-risk profile [6, 25]. For the average patient in these trials, the risk of additional nodal disease after a negative SLN biopsy according to our nomogram would be under 6% even when using a low value of 85% as the sensitivity for SLN biopsy. Therefore our nomogram would not change the management of the average patient on these trials.

For high-risk patients systemic therapy recommendations are also unlikely to change based on the output of this nomogram, because many of the factors which prompt addition of adjuvant therapy are the same factors that increase the pretest (and therefore also posttest) probability of having axillary disease. However, with regard to local management of the axilla, using this nomogram may change management, especially in light of the NCIC CTG MA.20 trial which included high-risk patients and suggests that residual untreated nodal disease may affect distant disease even in the absence of a clinically evident nodal recurrence.

This nomogram is anticipated to apply to the small proportion of breast cancer patients who present with clinically high-risk disease. Randomized studies for this subset are difficult to perform because most breast cancer patients present with early-stage disease. Therefore, Bayesian estimation of risk based on a mathematically sound extrapolation from available data is especially useful in this clinical situation. Our nomogram is a particularly important tool for this group of high-risk patients because they may benefit from more extensive surgery or axillary radiation.

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Research Article

Differential Gene Expression in Primary Breast Tumors Associated with Lymph Node Metastasis

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Lymph node status remains one of the most useful prognostic indicators in breast cancer; however, current methods to assess nodal status disrupt the lymphatic system and may lead to secondary complications. Identification of molecular signatures discriminating lymph node-positive from lymph node-negative primary tumors would allow for stratification of patients requiring surgical assesment of lymph nodes. Primary breast tumors from women with negative ($n = 41$) and positive ($n = 35$) lymph node status matched for possible confounding factors were subjected to laser microdissection and gene expression data generated. Although ANOVA analysis ($P < .001$, fold-change >1.5) revealed 13 differentially expressed genes, hierarchical clustering classified 90% of node-negative but only 66% of node-positive tumors correctly. The inability to derive molecular profiles of metastasis in primary tumors may reflect tumor heterogeneity, paucity of cells within the primary tumor with metastatic potential, influence of the microenvironment, or inherited host susceptibility to metastasis.

1. Introduction

Breast cancer is the most common cancer in women from Western countries. In 2009, approximately 190,000 women in the United States were diagnosed with and more than 40,000 died from breast cancer [1]. Progression of malignant breast cancer from localized to systemic disease can lead to impaired organ function, widespread systemic failure, and eventually, death. Five-year survival rates differ dramatically between women with negative lymph nodes (>90%) compared to those with lymph node metastasis (<70%) [2]. Lymph node status is not only the most reliable predictor of survival but is also critical in developing treatment regimens [3].

Assessment of lymph node status was originally performed by axillary lymph node dissection (ALND); however, ALND is associated with significant morbidities and has not been associated with significant survival advantage [4, 5], thus alternate methods of evaluating lymph node status have been developed. Sentinel lymph node biopsy (SLNB) assesses lymph node status in the sentinel or first-draining nodes along the axillary lymph node chain; on average, two-three lymph nodes are removed and patients with negative lymph node status are spared complete axillary dissection. Recent results from the NSAPB 32 and ACOSOG Z0011 trials demonstrated that in patients with node-negative disease, SLNB is as effective as ALND, and in patients with positive nodes, despite the risk of axillary recurrence, SLNB performed without follow up ALND is reasonable for patients with early-stage breast cancer [6, 7].

Although SLNB is associated with lower morbidities, surgical disruption of the lymphatic system can result in serious

side effects, including numbness, decreased mobility and lymphedema, significantly impacting the quality of life of breast cancer patients. For example, lymphedema can result in pain, decreased functional ability, cosmetic deformities and psychological stress [8] and is estimated to affect 10–20% of breast cancer survivors [9]. In addition, SLNB is associated with a false negative rate of 8–10% [4, 10]. Development of a signature that effectively discriminates patients by lymph node status could stratify patients into those needing surgical evaluation of the lymph nodes for prognostic purposes from those at low risk of metastasis who may be spared possible serious side effects as well as identify those 8–10% of patients misdiagnosed with negative lymph node status after SLNB, who may in fact benefit from more aggressive treatment. In this study, microarray-based gene expression analysis was performed on primary breast tumors from patients with and without metastatic lymph nodes to identify molecular signatures associated with lymph node metastasis.

2. Materials and Methods

2.1. Tissue Samples. Tissue samples in the Clinical Breast Care Project (CBCP) tissue bank were collected with approval from the Walter Reed Army Medical Center Human Use Committee and Institutional Review Board. All subjects enrolled in the CBCP voluntarily agreed to participate and gave written informed consent. Clinical information was obtained for all CBCP samples using questionnaires designed by and administered under the auspices of the CBCP. The CBCP database was queried to identify all patients diagnosed with invasive breast cancer between 2001 and 2008. Patients with a previous history of breast cancer, documented BRCA1 or BRCA2 mutations, or who underwent neoadjuvant therapy were not eligible for this study. Patients with isolated tumor cells or micrometastases as well as those diagnosed with negative lymph node status who later died of disease were excluded from analysis. To ensure consistency, diagnosis of every specimen was made by a single breast pathologist from hematoxylin and eosin (H&E) stained slides; grade was assigned using the Nottingham Histologic Score [11, 12]. ER and PR status were determined by immunohistochemistry by a commercial clinical laboratory (MDR Global, LLC, Windber, PA, USA); HER2 status was determined by fluorescence in situ hybridization using the PathVysion HER2 kit according to manufacturer's protocol (Abbott Laboratories, Abbott Park, IL, USA).

2.2. RNA Isolation, Amplification, aRNA Labeling and Hybridization. For each case, hematoxylin- and eosin-stained slides were examined by a dedicated breast pathologist and tumor areas marked for laser microdissection. One to six serial sections (8 μm thick) were cut, mounted on glass PEN foil slides (W. Nuhsbaum, Inc., McHenry, IL, USA), stained using the LCM staining kit (Applied Biosystems, Foster City, CA, USA) and microdissected on an AS*LMD* laser microdissection system (Leica Microsystems, Wetzlar, Germany). Slide preparation, staining and cutting were performed within 15 minutes to preserve RNA integrity.

RNA was isolated from laser microdissected tumor cells using the RNAqueous-Micro kit (Applied Biosystems, Foster City, CA, USA) and treated with DNase I to remove any contaminating genomic DNA. RNA integrity was assessed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA was converted to biotin-labeled aRNA using two rounds of amplification with the MessageAmpII aRNA Amplification kit (Applied Biosystems, Foster City, CA, USA), and the concentration and quality of the aRNA samples measured with the NanoDrop ND-1000 (NanoDrop Products, Wilmington, DE, USA) and the 2100 Bioanalyzer, respectively. Hybridization, washing, staining and scanning were performed using the HG U133A 2.0 arrays (Affymetrix, Santa Clara, CA, USA) according to manufacturer's protocol.

2.3. Analysis and Statistics. Affymetrix gene expression data was imported into Partek Genomics Suite 6.5 (Partek, Inc, St Louis, MO, USA) as CEL files using default Partek parameters. Raw data was preprocessed, including background correction, normalization and summarization using robust multiarray average (RMA) analysis and expression data log2 transformed. Differential gene expression analysis was performed using one-way ANOVA using lymph node status as the variable. Gene lists were created using a cut-off of $P < .001$, >1.5-fold change. Hierarchical clustering was performed using the Gene Expression module.

3. Results

3.1. Clinicopathological Characteristics. The average number of metastatic lymph nodes in the node-positive group was 4.69 (range 1–19). The average age at diagnosis did not differ significantly between those with (55.0 years) and those without (57.7 years) lymph node metastasis. None of the pathological features evaluated differed significantly between groups (Table 1). To date, 24/39 (62%) of the node-negative patients have been disease-free for ≥5 years and none have died of disease. In contrast, 6/35 (17%) of node-positive patients died of disease with an average survival of 34 months, one (3%) has progressed from stage IIIa to stage IV, and 47% have remained disease-free for at least five years.

3.2. Gene Expression. Statistical analysis revealed significant differences in expression levels for 15 probes between tumors from patients with lymph node metastases and those without (Table 2). These genes correspond to 11 genes (KIAA1609 and SLC27A2 are each represented by two independent probes) with known function, one uncharacterized gene and one probe that represents a UniGene EST cluster only. These results suggest that primary breast tumors with different metastatic capacities are more similar than different in gene expression as the small number of genes differentially expressed does not differ significantly ($P = .25$) from what would be expected by chance. Hierarchical clustering analysis was able to correctly classify 4/41 (90%) of the lymph node-negative tumors but only 23/35 (66%) of the node-positive tumors (Figure 1).

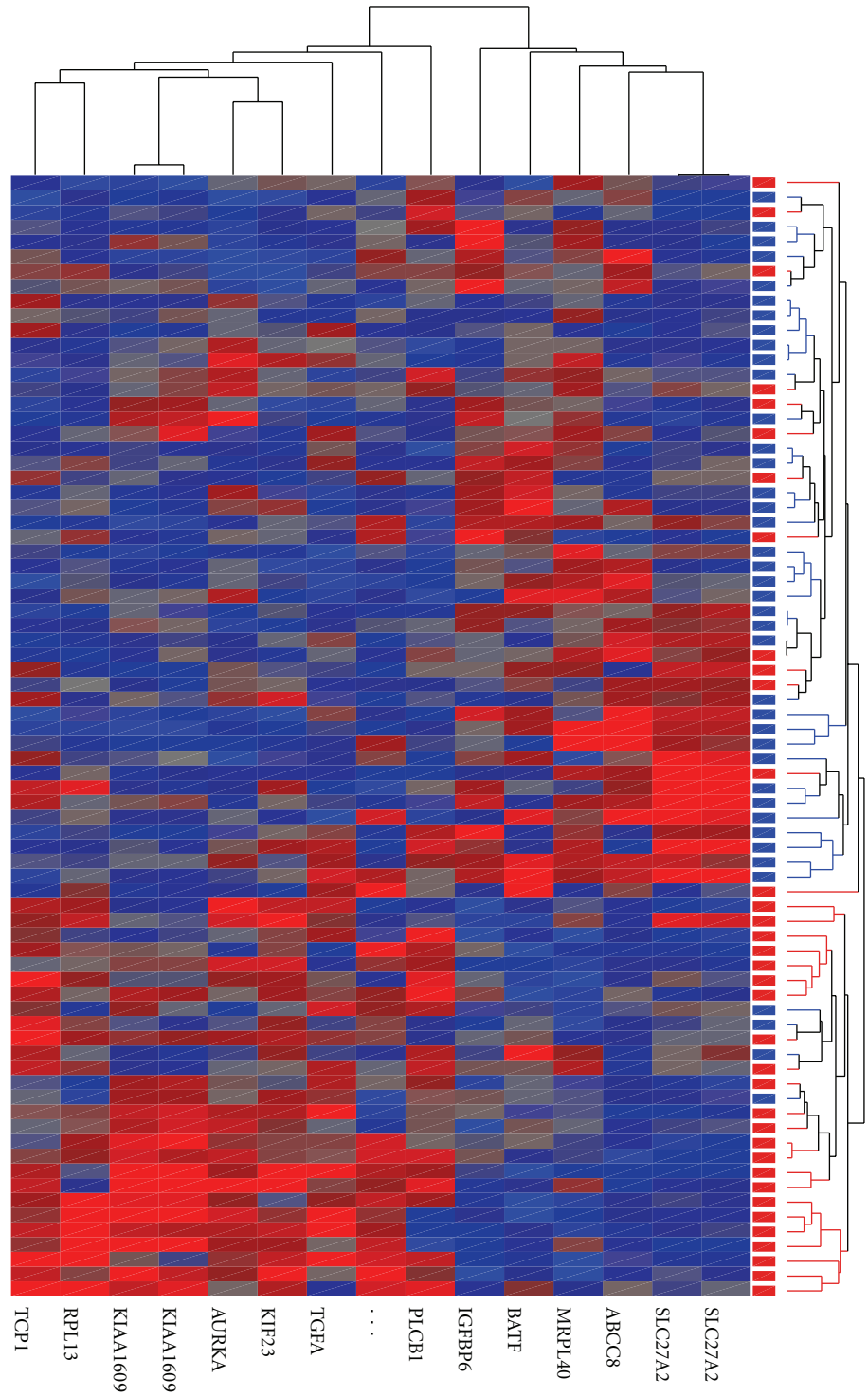

Figure 1: Heat map and hierarchical clustering of 76 primary tumor samples based on 15 differentially expressed probes. Tumors from patients with negative lymph node status are represented in the dendrogram by blue bars and tumors from patients with positive lymph node status are represented by red bars. 4/41 tumors with negative lymph nodes and 12/35 tumors with positive lymph nodes were classified incorrectly. Red squares: high expression, blue: low expression.

Table 1: Clinical and pathological features of 76 invasive breast tumor specimens used in microarray analysis.

	Node negative ($n = 41$)	Node positive ($n = 35$)	P node− versus node+
Age			
<50 years	37%	34%	NS
≥50 years	63%	66%	
Histology			
IDCA	95%	86%	
ILCA	5%	9%	NS
Mixed	0%	5%	
Grade			
Well-differentiated	27%	9%	
Moderately-differentiated	29%	43%	NS
Poorly-differentiated	44%	48%	
Hormone receptor status[a]			
ER+/PR+	54%	51%	
ER+/PR−	22%	12%	NS
ER−/PR−	24%	37%	
HER2 Status			
Positive	20%	26%	NS
Negative	80%	74%	
Tumor Size			
T1	63%	42%	
T2	34%	54%	NS
T3	3%	4%	

[a] No cases of ER−/PR+ were identified in this group of tumors.

4. Discussion

Gene expression-based molecular signatures have been developed that can be used to predict intrinsic subtype, tumor grade, and risk of recurrence [13–15], each of which can be used as a prognostic tool. Although a signature specific to the development of local metastases may not predict overall outcome, such a signature would have both biological and clinical utility. Identification of genes involved in the successful establishment of metastatic tumors in the lymph nodes would improve our understanding of the metastatic process. Differentially expressed genes may represent those involved in the initiation of metastasis, altering cell motility, angiogenesis and invasion thus allowing primary tumor cells with metastatic potential to disseminate [16]. These genes would then serve as molecular targets against which novel therapeutics could be developed to prevent the early stages of metastasis. In addition, identification of a signature of metastasis would allow women at low risk of lymph node metastasis to be spared unnecessary surgical procedures and the ensuring complications of lymph node disruption as well as to identify the 8–10% of node-positive women diagnosed as node-negative by SLNB [17].

To this end, efforts have been made to develop a breast tumor molecular signature that differs between patients with and without lymph node metastasis. For example, evaluation of gene expression patterns of 176 candidate genes between primary tumors without lymph node metastasis and those with 10 or more positive lymph nodes revealed differences in gene expression, with significantly higher expression of ERBB2 ($P < .0001$) in tumors from node-positive compared to node-negative tumors [18]. From a pool of 89 primary tumors, data from 19 primary tumors without lymph node metastasis and 18 with ≥10 positive lymph nodes were compared to generate a metagene profile, enriched for genes involved in cellular immunity, capable of predicting lymph node status with 90% accuracy [19]. Finally, using Serial Analysis of Gene Expression in 27 invasive ductal carcinomas with either positive or negative lymph node status, 245 differentially expressed ($P < .05$) genes were detected; these results were validated in an independent set of tumors for seven of the genes [20].

In contrast, a number of research groups have failed to develop molecular signatures predictive of lymph node metastasis. Gene expression data from 129 primary breast tumors was used to successfully develop signatures correlating expression patterns with grade and ER and HER2 status but a signature for lymph node status could not be identified; the authors thus concluded that while there may be a biological propensity to metastasize, the influence of time and stochastic processes on tumor metastasis may preclude the identification of a signature of lymph node metastasis [21]. In a second study evaluating microarray data from 151 lymph node-negative and 144 lymph node-positive primary tumors, significant gene expression differences were not detected between tumor types. The authors then applied the lymph node metastasis signature described previously by Huang et al. to their own external data set and achieved a classification accuracy of only 50%, implying that the signature developed by Huang, using a small sample set and limiting analysis to patients with ≥10 positive lymph nodes, is not an effective predictor of nodal metastasis [22]. In addition, while the 70-gene poor prognosis signature that is the basis for the MammaPrint assay is effective at predicting risk of recurrence, it was ineffective in predicting lymph node status, leading the authors to conclude that hematogenous and lymphogenic metastases are driven by independent molecular mechanisms [23]. Similar to these studies, the fifteen probes found in our study to be differentially expressed were not effective in correctly classifying primary tumors, especially those with positive lymph nodes, by lymph node status.

A number of reasons may explain the discrepancy between those groups that have reported molecular signatures of lymph node metastasis and those that have failed to find gene expression differences. Study design may affect the ability to detect critical molecular alterations. Most studies identifying a signature of lymph node metastasis relied on small (<40 samples) sample sizes. Breast cancer is not a single disease but rather a complex mix of different architectures, grades and underlying subtypes which may necessitate the use of large number of samples to generate robust signatures [24]. In addition, multiple models were developed using patients with extremely discordant (negative lymph node status compared to ≥10 positive lymph nodes) phenotypes

Table 2: Fifteen probes demonstrating significant differences in expression level between tumors with and without lymph node metastases. KIAA1609 and SLC27A2 were represented by multiple probes.

Gene symbol	Accession number	Gene name	Probe ID	*P* value	Fold-change
Genes downregulated in node-positive primary tumors					
ABCC8	NM_000352	ATP-binding cassette, subfamily C (CFTR/MRP), member 8	210246_s_at	.000889	1.67
BATF	NM_006399	Basic leucine zipper transcription factor, ATF-like	205965_at	.000874	1.51
IGFBP6	NM_002178	Insulin-like growth factor binding protein 6	203851_at	.000679	1.55
MRPL40	NM_003776	Mitochondrial ribosomal protein L40	203152_at	2.14E-05	1.57
SLC27A2	NM_003645	Solute carrier family 27 (fatty acid transporter), member 2	205768_s_at	.000413	2.34
			205769_at	.000921	2.12
Genes upregulated in node-positive primary tumors					
—[a]	AL050145		215526_at	.000477	1.55
AURKA	NM_198433	Aurora kinase A	208079_s_at	.000512	1.80
KIAA1609	NM_020947	KIAA1609	221843_s_at	1.25E-05	1.66
			65438_at	1.53E-05	1.73
KIF23	NM_138555	Kinesin family member 23	204709_s_at	.000675	1.82
PLCB1	NM_015192	Phospholipase C, beta 1 (phosphoinositide-specific)	213222_at	.000612	2.05
RPL13	NM_033251	Ribosomal protein L13	214976_at	.000129	1.64
TCP1	NM_030752	T-complex 1	208778_s_at	.000183	1.51
TGFA	NM_003236// NM_001099691[b]	Transforming growth factor, alpha	205016_at	.000233	1.77

[a] This probe corresponds to UniGene cluster HS.225986 but not to a known gene.
[b] This probe represents both isoforms 1 and 2 of the TGFA gene.

[18, 19]; these models, therefore, may not apply to the majority of patients who have an intermediate number of positive lymph nodes [21]. Finally, validation of these signatures on independent sample sets has, to our knowledge, not been reported, and to date, while molecular portraits are used to determine tumor grade, subtype and prognosis, no clinical assay is available to determine lymph node status.

In addition to methodological concerns, lack of a signature of lymph node metastasis may be attributable to biological properties of primary breast tumors, such as the nature and number of cells within a primary tumor with metastatic potential. Injection of melanoma cells into mice demonstrated that tumor cells vary widely in their ability to produce metastases, and cells with metastatic potential are rare within the primary tumor [25, 26]. This view was challenged by the development of gene expression signatures such as the 70-gene poor prognosis signature and a molecular signature of metastasis developed from solid tumors [15, 27]; because these signatures were derived from bulk tumors, the authors concluded that the majority of cells in the primary tumor have the ability to metastasize. In fact, the ability to predict which tumors will metastasize based on gene signatures derived from primary tumors does not preclude the presence of small subpopulations of cells with full metastatic potential found in localized regions throughout the primary tumor [28, 29]. For example, comparison of gene expression patterns between cell line populations that have high compared to low metastatic potential to bone revealed that only a small fraction of cells demonstrated the full bone metastasis signature [30]. More recently, the sequencing of a basal-like primary breast tumor and corresponding brain metastasis revealed a significant enrichment of 20 mutations in the metastasis compared to the primary tumor, suggesting that metastases arise from a minority population of cells within the primary tumor [31]. If these models in which few cells within the primary tumor have full metastatic capacity are correct, genetic signatures from these rare cells will be masked by the majority of tumor cells which do not have full metastatic capacity.

Molecular heterogeneity within tumor subtypes may also preclude the identification of a single signature of metastasis. Breast tumors can be classified by their intrinsic subtypes, including luminal A, luminal B, HER2-positive and basal-like, based on different patterns of gene expression [13]. These subtypes have been associated with differences in relapse-free and overall survival with the basal-like and HER2-positive subtypes having the shortest survival times [32]. Not only do intrinsic subtypes have different prognoses, but recent studies have shown that each subtype has preferential sites of metastasis: bone was the predominant site of relapse in luminal and HER2-positive tumors but was infrequent in basal-like tumors. In contrast, basal-like tumors had frequent relapse in brain, lung and distant lymph nodes [33, 34]. Data supporting the idea that tumors with different phenotypes may metastasize differently was provided by a recent study which found nonoverlapping

signatures for the development of distant metastasis in lymph-node-negative ER-positive and ER-negative tumors, suggesting that there are different molecular mechanisms associated with metastasis depending on tumor biology [35]. Whether lymph node metastasis is similarly affected by tumor phenotypes such as ER status or intrinsic subtype remains to be determined.

The ability to metastasize may be influenced by not only the tumor cells but also the microenvironment, both local and distant. Dissemination of tumor cells from the primary site is one of the earliest steps of metastasis; successful invasion and migration of tumor cells requires a number of changes in the breast microenvironment including degradation of the extracellular matrix and angiogenesis. Distant tissue may be subjected to premetastatic niche conditioning, undergoing changes such as recruitment of bone-marrow derived cells that form a favorable environment for tumor cells to grow. Finally, the last stages of metastasis require tumors cells to successfully reach the secondary site, escape senescence and survive and proliferate within a foreign environment [16, 36]. Given the importance of the microenvironment, molecular characterization of the tumor component alone may not be sufficient in predicting metastatic behavior as a tumor with an aggressive profile may be growing within a nonpermissive microenvironment and vice versa. In fact, many signatures of poor prognosis or metastasis include the expression of stromal genes. Thus, consideration of only the tumor epithelial component may fail to capture the full metastatic potential of a primary tumor.

Finally, the ability to metastasize may depend not on biologic features of the primary tumor but on inherent host susceptibility. Outcrossing of a highly metastatic transgenic mouse to a variety of inbred mouse strains resulted in significant variability in the propensity to metastasize; since each animal received the metastatic transgene, the differences in metastatic capacity have been attributed to genetic background [37]. Linkage studies identified candidate metastasis modifier genes in mouse, including SipaI [38]. Follow up studies in humans confirmed that SIPA1 is a metastasis susceptibility gene [39, 40]. Thus, the ability to successfully metastasize may, at least in part, reflect a systemic, rather than tumor-driven, proclivity.

5. Conclusions

New molecular tools are needed that can effectively discriminate patients with and without the propensity to develop lymph node metastasis so that women at low risk may be spared potentially significant morbidities associated with surgical evaluation, and the false negative rate associated with SLNB can be reduced. In this study, 15 probes, representing 11 well-characterized and two hypothetical genes, were differentially expressed between tumor types; however, hierarchical clustering based on this gene signature was ineffective, especially for the lymph node-positive tumors, suggesting that a single molecular classifier for lymph node metastasis may not exist. The inability to derive molecular profiles of metastasis in primary tumors may reflect tumor heterogeneity, paucity of cells within the primary tumor with metastatic potential, influence of the microenvironment, or inherited host susceptibility to metastasis.

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