Lymph Node Metastasis in Breast Cancer Xenografts Is Associated with Increased Regions of Extravascular Drain, Lymphatic Vessel Area, and Invasive Phenotype

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Abstract

Interactions between the tumor stromal compartment and cancer cells play an important role in the spread of cancer. In this study, we have used noninvasive in vivo magnetic resonance imaging (MRI) of two human breast cancer models with significantly different invasiveness, to quantify and understand the role of interstitial fluid transport, lymphatic-convective drain, and vascularization in the regional spread of breast cancer to the axillary lymph nodes. Quantitative fluorescence microscopy was done to morphometrically characterize lymphatic vessels in these tumors. Significant differences in vascular and extravascular transport variables as well as in lymphatic vessel morphology were detected between the two breast cancer models, which also exhibited significant differences in lymph node and lung metastasis. These data are consistent with a role of lymphatic drain in lymph node metastasis and suggest that increased lymph node metastasis may occur due to a combination of increased invasiveness, and reduced extracellular matrix integrity allowing increased lymphatic development, as well as tumor cells. It is also possible that lymph node metastasis occurred via the cancer cell–bearing tumoral lymphatic vessels. The congestion of these tumoral lymphatics with cancer cells may have restricted the entry and transport of macromolecules.

Introduction

The presence of lymph node metastasis significantly influences breast cancer prognosis. The 5-year survival rate from breast cancer drops from 96% to 75% with regional spread, and to 20% with distant spread (1), with axillary lymph node–positive patients more likely to develop distant metastasis.

The extracellular matrix (ECM) presents one of the earliest lines of defense against the invading and disseminating cancer cell. Cancer cells invade surrounding tissues to grow and disseminate by secreting proteolytic enzymes, such as serine proteases and matrix-degrading metalloproteinases, or by inducing secretion of these enzymes by tumor stromal cells (2–4). It is possible that these proteolytic enzymes reduce the integrity of the tumor stroma and promote tumoral lymphatic development, as well as the movement of macromolecules through the ECM and supporting stroma of solid tumors is important to delimitate critical mechanisms in cancer invasion and metastasis.

Recently, we developed a noninvasive method for characterizing the extravascular transport of macromolecules through the ECM of solid tumors in vivo using magnetic resonance imaging (MRI), to better understand the role of the tumor-associated lymphatic system in the clearance of macromolecules and interstitial fluid (5). Here, for the first time, we have used this method to characterize differences in vascularization (vascular volume, permeability surface area product) and lymphatic-convective transport (macromolecular fluid transport rates/volumes, fraction of draining/pooling voxels) in vivo, in two human breast cancer xenografts preselected for differences in invasiveness. Immunofluorescent microscopy was used to quantify differences in lymphatic vessel morphology in these tumors.

Consistent with the highly invasive characteristics of the MDA-MB-231 cells, lymphatic vessels containing cancer cells were frequently detected in tumors derived from these cells. These tumors also exhibited higher vascular volume and permeability, as well as areas of drainage compared with the less invasive MCF-7 tumors. Although the tumoral lymphatic vessel area was higher in MDA-MB-231 tumors compared with MCF-7 tumors, macromolecular drain mostly occurred convectively through the ECM because the contrast agent was rarely observed within the tumoral lymphatic vessels. Therefore, macromolecular transport most likely followed paths of least resistance through the ECM; the increased drainage area observed in the more invasive tumors with a greater capacity for degrading ECM was consistent with this possibility.

Materials and Methods

Tumor model and inoculations. MCF-7 cells or MDA-MB-231 cells were inoculated in the upper left thoracic mammary fat pad of female severe combined immune deficient mice. 10⁶ cancer cells were inoculated in a volume of 0.05 mL HBSS (Sigma, St. Louis, MO). All experimental animal protocols were approved by the Institutional Animal Care and Use Committee.

MRI paradigm. Mice were imaged 4 to 5 weeks postinoculation with tumor volumes in the range of 168.42 ± 79.6 mm³ (n = 5 per cell line). MRI was done on a Bruker Avance 4.7 T instrument equipped with shielded gradients using a custom-built RF volume coil placed around the body of the animal. The tail vein was catheterized before placing each animal in the magnet, for administration of the macromolecular contrast agent (MMCA) albumin-GdDTPA. Because mice were imaged up to 140 minutes inside the magnet, an additional catheter containing the anesthetic solution (a mixture of 0.5 mL ketamine and 0.5 mL acepromazine in 1.0 mL saline) was inserted s.c. for injecting additional anesthetic as required.

Multislice relaxation rate (T₁⁻¹) maps of the tumor were obtained using a saturation recovery method combined with fast-T₁ SNAPSHOT FLASH imaging [flip angle = 10° and echo time = 2 ms], as previously described (5). Six to eight (1 mm thick) coronal slices of the mouse cross-section, including the tumor, were acquired (128 × 128 matrix, 32 mm field of view, number of averages = 8) for 3 relaxation delays (100, 500, and 1,000 ms) for each slice. These MRI acquisition variables translate into an in-plane spatial...
resolution of $250 \times 250 \mu m^2$. Multislice maps of the fully relaxed magnetization ($M_0$) were also acquired at the beginning of each MRI experiment, using a recovery delay of 7 seconds. Images were acquired in two “phases” corresponding to the biphasic kinetics of the MMCA as described previously (5). The first or “early phase” is composed of images obtained before i.v. administration of 0.2 mL of 60 mg/mL albumin Gd-DTPA in saline (dose of 500 mg/kg) and repeated every 7 minutes, starting at 3 minutes postinjection, up to 31 minutes. Because macromolecular drain in and around tumors either by convection or by the lymphatics is a slow event (6–8), with rates of 2.3 to 3.7 $\mu L/g-min$ (7), a second block of MR data was continuously acquired for up to 140 minutes postcontrast. This second block of acquisitions was classified as the “late phase” of the MMCA, as it comprises of late drainage events within the tumor ECM.

At the end of the scans, mice were sacrificed and blood $T_1$ values determined from blood samples taken from the inferior vena cava. All tumors, lungs, and both ipsilateral and contralateral axillary lymph nodes were excised and fixed in buffered formalin for subsequent sectioning and staining.

**Detection of vascular and extravascular transport variables.** Variables describing the vascular and extravascular transport of the MMCA in MCF-7 and MDA-MB-231 tumors were calculated from the MMCA tissue concentration-time curve $\Delta R_{\text{tissue}}(t)$ normalized to the MMCA concentration in the blood $\Delta R_{\text{blood}}(t)$, by assuming three distinct tumor compartments: (a) the intravascular space, (b) the perivascular space that the MMCA first extravasates into, and (c) a more distant compartment consisting of regions of the ECM within which macromolecular transport events such as convective and lymphatic drain occur. For both early and late phases of the MR experiment, MMCA uptake was modeled as a linear function of time and $R_1$ values determined for every tumor in each group of animals. Additionally, because we had no *a priori* knowledge of the shape of the distributions for each of the assessed variables, the one-tailed nonparametric Mann-Whitney $U$ test was used to determine if the median vascular/extravascular MRI variables for MDA-MB-231–bearing animals were significantly greater than for MCF-7–bearing animals. Because we used a nonparametric test with five assessed variables, the one-tailed nonparametric Mann-Whitney $U$ test was used to determine if the median vascular/extravascular MRI variables for MDA-MB-231–bearing animals were significantly greater than for MCF-7–bearing animals. Because we used a nonparametric test with five assessed variables, the one-tailed nonparametric Mann-Whitney $U$ test was used to determine if the median vascular/extravascular MRI variables for MDA-MB-231–bearing animals were significantly greater than for MCF-7–bearing animals. Because we used a nonparametric test with five assessed variables, the one-tailed nonparametric Mann-Whitney $U$ test was used to determine if the median vascular/extravascular MRI variables for MDA-MB-231–bearing animals were significantly greater than for MCF-7–bearing animals.

**Assay for blood and lymphatic vessel functionality by immunostaining.** In separate bench-top experiments, two MCF-7–bearing and four MDA-MB-231–bearing animals were anesthetized and 0.2 mL of 60 mg/mL biotinylated albumin-GdDTPA (8) was administered i.v. Animals were sacrificed 3 hours later and tumors, lungs, and axillary lymph nodes were excised and fixed in buffered formalin for subsequent immunostaining for

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**Figure 1.** Representative single-slice functional MRI maps showing (A) draining and (C) pooling voxels for an MDA-MB-231 tumor (arrows)–bearing animal. MMCA concentration-time curve typical of a (B) draining voxel (within cross-hairs in A) exhibiting slow uptake of the MMCA during the early phase followed by elimination of the MMCA during the late phase and (D) pooling voxel (within cross-hairs in C) exhibiting some uptake of the MMCA during the early phase followed by enhancement during the late phase.
blood vessels, lymphatic vessels, and the presence of the biotinylated contrast agent. Colocalization of contrast agent within the lumen of blood or lymphatic vessels as assessed by fluorescent microscopy was used to assess functionality.

Adjacent 5-μm-thick formalin-fixed, paraffin-embedded tumor sections obtained at 500 μm intervals were cut onto silanized glass slides, cleared of paraffin in Histoclear II (National Diagnostics, Atlanta, GA), and rehydrated by means of graded alcohol baths. After rinsing in double-distilled water, slides were transferred to a preheated (95-100°C) citrate buffer [10 mmol/L sodium (pH 6.0), 0.5 mL Tween] and heated in a microwave for 15 minutes at 95°C to 100°C, and then cooled for 30 minutes. Slides were then blocked in PBS-5% FCS for at least 30 minutes.

Double staining for lymphatic and blood vessels. Lymphatic vessels were detected using an antibody to the lymphatic endothelial hyaluronan receptor LYVE-1 (10). Briefly, slides were incubated overnight at 4°C with 4.7 μg/mL of rabbit anti-mouse LYVE-1 antibody (Research Diagnostics, Inc., Flanders, NJ), rinsed (×2) in PBS, and incubated with goat anti-rabbit Alexa-Fluor 488 (Molecular Probes, Inc., Eugene, OR) secondary antibody for at least 90 minutes at room temperature. For detection of blood vessel endothelia, slides were incubated overnight at 4°C with 20 μg/mL rat anti-mouse CD34 (clone MEC14.7) antibody (Cell Sciences, Canton, MA) after another PBS rinse. Slides were then washed again in PBS before being incubated with goat anti-rat Cy3-conjugated secondary antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for at least 90 minutes at room temperature. Slides were counterstained with Hematoxylin-2 (Richard Allan Scientific, Kalamazoo, MI) and mounted in an aqueous mounting medium, Faramount (DAKO Corp., Carpenteria, CA). After LYVE-1 and CD34 staining, sections obtained from mice injected with biotinylated albumin-GdDTPA were incubated with streptavidin Marina Blue (Molecular Probes) secondary antibody for at least 90 minutes at room temperature, followed by hematoxylin-2 counterstaining. Green fluorescence in the tissue sections identified lymphatic endothelial cells of lymphatic vessels, red fluorescence identified vascular endothelial cells and blood vessels, and where applicable, blue fluorescence identified regions containing the biotinylated MR contrast agent.

Digitized immunofluorescence microscopy. All slides were viewed (at ×20) using a Nikon ECLIPSE-TS100 microscope (Nikon Instruments, Inc., Melville, NY) equipped with Plan-fluor lenses and filters for detecting GFP/FITC, RFP/TRITC and HOECHST/4′,6-diamidino-2-phenylindole fluorescence. Regions of interest (ROI) with lymphatic vessels (i.e., LYVE-1-positive structures) were identified on each slide at high magnification (×40) for further analysis. Each field of view with lymphatic vessel ROIs or hotspots was digitized using a Nikon Coolpix 5000 digital camera (Nikon Corporation, Tokyo, Japan) and morphometric image processing was conducted off-line using the ImageJ program.3

Figure 2. A, representative multislice functional MRI maps of pooling and draining voxels for an MDA-MB-231-bearing animal (top) and an MCF-7-bearing animal (bottom). B, box-and-whisker plot comparing the fraction of pooling and draining voxels between MDA-MB-231 and MCF-7 xenografts. The length of the box is the interquartile range (i.e., the middle 50% of the data). Line through the middle of each box, median. The upper adjacent value is the largest observation ≥25th percentile + 1.5 × (interquartile range), whereas the lower adjacent value is the smallest observation ≤25th percentile − 1.5 × (interquartile range), and are displayed as T-shaped lines extending from each end of the box. *, P < 0.1, one-sided Mann-Whitney U test. C, box-and-whisker plot comparing the volume of extravasated fluid pooled/drained between MDA-MB-231 and MCF-7 xenografts. *, P < 0.1, one-sided Mann-Whitney U test.

Morphometric image analysis of tissue sections. Automated morphometric analysis of each digitized fluorescent image was done using an in-house program written by us for ImageJ. Briefly, images were converted to grayscale, normalized, filtered, binarized, and morphometrically closed. All binarized objects (excluding objects at the image edges) in the final image >90 pixels in size were counted as features [i.e., either as being positive for LYVE-1 (i.e., lymphatic) or for CD34 (blood vessel)]. The program was run using over 200 images corresponding to nonoverlapping lymphatic and blood vessel ROIs for each tumor. Two morphometric variables were computed for each digitized image: (a) the fractional area occupied by LYVE-1-positive structures (A%) and (b) the circularity (C = 4π × area/perim²) of each structure, with a value of 1.0 indicating a perfect circle and values approaching 0.0 indicating increasingly elongated vessels. The two-tailed nonparametric Mann-Whitney U test for independence of medians was used to determine if there was a significant (a = 0.05) difference between these variables for MCF-7 and MDA-MB-231 bearing animals, respectively.

Assay for lymph node and lung metastasis. Tumor-positive lymph nodes and lungs from all MCF-7- and MDA-MB-231-bearing animals were identified by optical microscopy examination of H&E-stained tissue sections, and significant differences (a = 0.05) were evaluated using a Fisher’s exact test.

Results

Figure 1 illustrates the two distinct contrast signatures used to identify tumor ROIs. Voxels were classified from the late phase of the protocol as pooling if the MMCA concentration within the extravasated fluid increased with time, or as draining if the MMCA concentration decreased over time relative to the early phase of the MRI protocol. Based on this classification, Fig. 2A illustrates differences in MMCA draining and pooling between the two tumor types. Overall, a significantly (P = 0.0159) greater percentage of draining voxels was identified in MDA-MB-231 tumors compared with MCF-7 tumors (Fig. 2B), whereas the number of pooling voxels was not significantly different. The median volume of extravasated fluid drained in MDA-MB-231 tumors was significantly greater (P = 0.0754); that is, more negative than for MCF-7 tumors (Fig. 2C), whereas the median volume of extravasated fluid that pooled was not significantly different between the two tumor types.

For the two types of tumors studied, the median PS and Vp for MDA-MB-231 tumors was elevated (P = 0.0754 for each) compared with that of MCF-7 tumors (Fig. 3).

Results from the morphometric immunohistochemical studies obtained from these tumors are shown in Fig. 4. Tumoral lymphatic vessels exhibiting green fluorescence were often observed in the tumor margins of both tumor models (Fig. 4A and B) with virtually no overlap with CD34-stained blood vessels. Overall, these lymphatic vessels were primarily peripheral and tended to be elongated, or ellipsoidal in MDA-MB-231 tumors in contrast to smaller, more circular lymphatics often found in MCF-7 tumors (Fig. 4C). These morphologic differences were reflected by (a) the significantly (P = 0.011) greater median circularity of lymphatic vessels in MCF-7 tumors compared with MDA-MB-231 tumors (Fig. 4C) and (b) the significantly (P < 0.001) greater median fractional area of LYVE-1-positive structures in MDA-MB-231 tumors than in MCF-7 tumors (Fig. 4D). Lymphatic vessel lumina

Figure 3. A, corresponding multislice functional MRI maps of the vascular volume for the MDA-MB-231-bearing (top) and MCF-7-bearing animals (bottom) shown in Fig. 2. B, box-and-whisker plot comparing Vv between MDA-MB-231 and MCF-7 xenografts, for all animals. *, P < 0.1 with the one-sided Mann-Whitney U test. C, corresponding multislice functional MRI maps of the permeability surface area product for the MDA-MB-231-bearing (top) and the MCF-7-bearing animals (bottom) shown in Fig. 2. D, box-and-whisker plot comparing PS between MDA-MB-231 and MCF-7 xenografts, for all animals. *, P < 0.1, one-sided Mann-Whitney U test.
in MDA-MB-231 tumors were frequently packed with cancer cells (Fig. 5A-C). In contrast, the lumen of even the most dilated lymphatic vessels in MCF-7 tumors (Fig. 5D-F) rarely contained cancer cells. Whereas none of the MDA-MB-231 tumors imaged exhibited intratumoral lymphatics (i.e., lymphatic vessels at distances ≥ 500 μm from the tumor margin), such vessels were detected in two MCF-7 tumors. However, neither of these two animals exhibited lung or lymph node metastases. Almost none of the tumor sections, irrespective of the type of tumor, exhibited colocalization of the LYVE-1 stain and the MMCA albumin GdDTPA, indicating that the most of the lymphatic vessels were not channeling or eliminating any appreciable amount of MMCA from the tumor interstitial space at the time point studied. Thus, as shown in Fig. 6A and B, although MMCA tracks were observed within the ECM, MMCA was rarely detected within the lumen of the tumoral lymphatics.

A significantly \( (P < 0.001) \) greater proportion of MDA-MB-231-bearing animals exhibited cancer-positive proximal axillary lymph nodes (11 of 12 versus 0 of 7 animals; Fig. 7A-C) and a significantly \( (P = 0.027) \) greater proportion of cancer-positive lungs (7 of 13 versus 1 of 11 animals; Fig. 7D-F) compared with MCF-7-bearing animals. These data are summarized in Table 1.

Discussion
As summarized in Table 2, several variables were found to be significantly different between the two breast cancer models studied here. The MRI data revealed that the more invasive MDA-MB-231 tumors exhibited higher vascular permeability \( (PS) \) and vascular volume \( (V_V) \), and a larger number of draining voxels that in turn drained a larger volume of fluid exudate compared with the less invasive MCF-7 tumors. From the morphometric analyses of the microscopy data, a greater fractional area of tumoral lymphatic vessels and a larger number of tumoral lymphatic vessels containing tumor cells were detected in MDA-MB-231 tumors. A higher incidence of metastatic deposits was detected in the lymph nodes and lungs of mice with MDA-MB-231 tumors compared with MCF-7 tumors.

The values of \( V_V \) and \( PS \) derived from the early phase of the MR experiment were consistent with previously published data for these tumor models (5, 11). Prior studies have also detected higher endogenous levels of vascular endothelial growth factor (VEGF), a potent angiogenic and permeability factor, in MDA-MD-231 xenografts compared with MCF-7 tumors, which explains the higher \( PS \) and \( V_V \) measured in the former (11). Also consistent with earlier results, the increased permeability combined with the high invasiveness associated with MDA-MB-231 cells (12) resulted in significantly higher metastatic lung deposits. Tumor dissemination is thought to occur concomitantly via lymphatics and blood capillaries, and not initially via lymphatics and subsequently through the bloodstream once the regional lymph nodes are overwhelmed (13). In this study, 6 of the 11 mice with extensive lymph node deposits also exhibited extensive metastases to the lungs, suggesting a pattern of preferential nodal metastasis for invasive breast cancer. This pattern supports previous observations that the destruction of tumor cells in the lymph node environment does not seem to be as successful as the destruction of tumor cells in blood vessels (13). The potential protective effect on tumor cells of the larger volume of interstitial fluid exudate in the MDA-MB-231 tumors is also consistent with this scenario (14, 15).

![Figure 4](https://www.aacrjournals.org/)

**Figure 4.** A, photomicrograph obtained at ×20 from a 5-μm-thick MDA-MB-231 tumor section showing elongated peripheral lymphatic vessels stained positive for LYVE-1. B, photomicrograph obtained at ×20 from a 5-μm-thick MCF-7 tumor section showing more circular peripheral lymphatic vessels stained positive for LYVE-1. C, box-and-whisker plot comparing the histologically assessed median circularity of LYVE-1-positive structures obtained using hotspot analysis from MDA-MB-231 and MCF-7 tissue sections, respectively. *, \( P < 0.05 \) with the two-sided Mann-Whitney \( U \) test. D, box-and-whisker plot comparing the histologically assessed median fractional area of LYVE-1-positive structures obtained using hotspot analysis from MDA-MB-231 and MCF-7 tissue sections, respectively. *, \( P < 0.05 \), one-sided Mann-Whitney \( U \) test.
The average values of the median rate of interstitial fluid drain from MDA-MB-231 and MCF-7 tumors of 0.06 ± 0.01 and 0.04 ± 0.01 mL/g-h, respectively, were comparable with the ranges of elimination rates for tumor interstitial fluid of 0.06 to 0.31 and 0.14 to 0.22 mL/g-h reported by other investigators (7, 16). Consistent with observations in our earlier study (5), the majority of MMCA drainage in these tumors occurred primarily via convective flow through the ECM. The infrequent detection of albumin-GdDTPA within the tumoral lymphatic vessels of either tumor type further confirm these earlier observations. It is possible that MMCA transport through the ECM occurs along the pathways of least resistance (15). In healthy tissue, elastin fibers within the ECM are arranged to direct fluid exudate into the lymphatics (17). In tumors, however, this arrangement is compromised due to ECM remodeling that often accompanies tumor growth (18). In addition, factors such as elevated interstitial fluid pressure, blood vessel permeability, hydraulic conductivity, blood pressure (19), and the pressure exerted by proliferating cancer cells (20) have each been known to affect the intratumoral transport of macromolecules.

Figure 5. A, photomicrograph obtained at ×40 from a 5-μm-thick MDA-MB-231 tumor section showing LYVE-1-stained lymphatic vessels. B, phase contrast image of the same tumor section stained with hematoxylin. C, image in (B) overlaid with the image in (A), with the transparency of the latter adjusted to enable visualization of the lymphatics (L) packed with tumor cells. D, photomicrograph obtained at ×40 from a 5-μm-thick MCF-7 tumor section showing LYVE-1-stained lymphatic vessels. E, phase-contrast image of the same tumor section stained with hematoxylin. F, image in (E) overlaid with the image in (D), with the transparency of the latter adjusted to visualize lymphatics (L), which have few, if any, tumor cells.

Figure 6. A, photomicrograph obtained at ×40 from a 5-μm-thick MDA-MB-231 tumor section showing LYVE-1 stained lymphatic vessels (green) and the MMCA albumin-GdDTPA (blue). The MMCA can be detected between the lymphatic vessels without colocalization of the two. B, a similarly double-stained field of view obtained at ×40 from a 5-μm-thick MDA-MB-231 tumor section illustrating the flow of MMCA along “pathways of least resistance” through the ECM, in between LYVE-1-positive structures. Hardly any colocalization of the two stains is evident, illustrating that the bulk of the transport in this tumor model was probably convective.
The more invasive tumor model, MDA-MB-231, exhibited a significantly higher number of draining voxels as well as larger extravascular fluid clearance, compared with MCF-7 tumors, which may reflect the integrity of its ECM. The MDA-MB-231 cell line is significantly more invasive than the MCF-7 cell line (12), and imaging studies have shown the ability of MDA-MB-231 cells to degrade Matrigel at a faster rate than MCF-7 cells (11). In addition, the more malignant MDA-MB-231 cells have also been shown to be associated with the production of more matrix metalloproteinases than the less-invasive MCF-7 cells (21). The resultant ECM remodeling by MDA-MB-231 cells may have resulted in increased pathways of least resistance for the transport of extravasated fluid, as well as tumor cells, which could explain the greater number of drainage regions and greater volume of extravasated fluid cleared from MDA-MB-231 tumors compared with MCF-7 tumors, as well as the increased lymph node metastases. The elevated permeability detected in the vasculature of the MDA-MB-231 tumors may have contributed to this movement of the MMCA through the ECM rather than through lymphatic channels.

Although it has been shown that interstitial fluid flow can guide lymphangiogenesis and lymphatic modeling (22), the effects of interstitial fluid flow on lymphatic endothelial cell morphogenesis were only recently observed when Ng et al. (23) showed that interstitial fluid flow, and not planar shear alone, induced drastic lymphatic endothelial cell remodeling. The greater volume of interstitial fluid eliminated in the MDA-MB-231 tumors, compared with MCF-7 tumors, may therefore have also contributed to differential morphogenetic stimulation of lymphatic endothelial cells and the higher lymphatic vessel area measured with immunohistochemistry in MDA-MB-231 compared with MCF-7 tumors. The observed differences in the histologically assessed median circularity of LYVE-1-positive structures between the MDA-MB-231 tumors and MCF-7 tumors may be due to differences in

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**Table 1.** Summary of the cancer-positive and cancer-negative lymph nodes and lungs detected by microscopic evaluation of histologic slides stained with H&E, for invasive MDA-MB-231 and noninvasive MCF-7 tumors

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. animals CA+ve</th>
<th>No. animals CA−ve</th>
<th>Total</th>
<th>Fisher’s exact test</th>
</tr>
</thead>
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<td>Lymph node</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>11</td>
<td>1</td>
<td>12</td>
<td>0.0001</td>
</tr>
<tr>
<td>MCF-7</td>
<td>0</td>
<td>7</td>
<td>7</td>
<td></td>
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<tr>
<td>Lung</td>
<td></td>
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<tr>
<td>MDA-MB-231</td>
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<td>6</td>
<td>13</td>
<td>0.027</td>
</tr>
<tr>
<td>MCF-7</td>
<td>1</td>
<td>10</td>
<td>11</td>
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Abbreviations: CA+ve, cancer positive; CA−ve, cancer negative.
the compressive force arising from differences in cell proliferation. These observations are consistent with a study by Padera et al. (20), in which the authors showed that regions exposed to greater compressive forces contained nonfunctional, collapsed lymphatic vessels exhibiting low circularity.

Although MDA-MB-231 tumors exhibited greater fractional lymphatic vessel area, as mentioned earlier, these tumoral lymphatic vessels were nonfunctional in terms of our MMCA assay. Unlike MCF-7 tumors, lymphatic vessels in MDA-MB-231 tumors were frequently congested with cancer cells, which may partly explain their compromised ability to channel MMCA. Mice with MDA-MB-231 tumors exhibited a significantly higher proportion of cancer-positive lymph nodes than MCF-7 tumors. The lack of MMCA transport by itself does not preclude lymph node metastasis from these vessels. Transport mechanisms that govern the clearance of fluid exudate from the ECM may not necessarily govern the transport of tumor cells. Cell migration is a finely controlled process involving delicate cell-cell and cell-matrix interactions (14) and irrespective of their ability to transport fluid, tumoral lymphatics may promote metastasis by providing tumor cells with additional avenues for leaving the primary tumor site (24). Another possible route for lymph node metastases may be via the peritumoral lymphatics. Such a mechanism is consistent with studies by Dafni et al. (8) in which the same MMCA was detected within the peritumoral draining lymphatics. Cancer cells may travel along with the MMCA through the ECM into draining peritumoral lymphatics located in adjacent normal tissue. There are interesting parallels between the implications of our data and observations made using intravital multiphoton microscopy of the interaction between tumor cells and the ECM during cell migration and invasion (25, 26). Specifically, Condeelis et al. (25) have shown that the absence of a dense network of ECM promotes the movement of cancer cells along long ECM fibers. It is possible that macromolecules move through ECM tracks along the same pathways taken by cancer cells and accelerate their transport. A combined high-resolution MRI and intravital microscopy study examining this possibility, by characterizing MMCA transport and relating it to ECM fiber organization and cancer cell movement, is both feasible and likely to provide further insights.

In summary, our results suggest that the extravascular clearance of macromolecules for both tumors was primarily via convection or conduits of least resistance within the ECM. Collectively, the in vivo and ex vivo data suggest that the higher incidence of lymph node metastases observed in MDA-MB-231 tumors compared with MCF-7 tumors was most likely a consequence of their differential invasiveness, reduced ECM integrity resulting in increased drainage areas, and increased lymphatic vessel area. The data obtained here support the exploration of strategies to alter the integrity of the ECM as potential treatments to reduce lymph node metastasis. In addition, the clinical extension of the MRI approach described here will further advance our understanding of the mechanisms underlying lymph node metastasis.

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References