Enhanced Sonographic Imaging to Diagnose Lymph Node Metastasis: Importance of Blood Vessel Volume and Density

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Abstract

Lymph node size is an important variable in ultrasound diagnosis of lymph node metastasis. However, the size criterion often leads to oversight of tumor-positive lymph nodes within the range of "normal" size, such that more accurate diagnostic criteria for lymph node metastasis are required. In this study, we show how diagnosis of lymph node metastasis can be improved by evaluating changes in blood vessel volume and density using a novel contrast-enhanced high-frequency ultrasound (CE-HFUS) system with Sonazoid. An MRL/Mpj-lpr/lpr (MRL/lpr) mouse model of lymph node metastasis was used in which lymph nodes are similar in size to humans. Metastasis via lymphatic vessels to proper axillary lymph nodes (proper ALN) was induced by injection of tumor cells into the subiliac lymph nodes. Within 21 days of injection, significant increases in blood vessel volume and density, but no increases in the size of the proper ALNs, were observed. The increase in blood vessel density was confirmed with immunohistochemical analysis and was positively related to tumor cell proliferation as measured using bioluminescence imaging. Together, our results showed that alterations in blood vessel volume and density precede alterations in lymph node size in the early stages of lymph node metastasis. Detection of these changes by ultrasonography may offer new criteria for early diagnosis of lymph node metastasis. Cancer Res 73(7); 2082–92. ©2013 AACR.

Introduction

Metastasis, a characteristic of many tumor types, is estimated to be responsible for the death of 90% of all patients with cancer (1). Early detection of metastasis in tumor-draining lymph nodes, that is, sentinel lymph nodes, during the management of skin, breast, colon, head and neck, and other cancers should improve assessment of the stage of cancer and facilitate selection of the most appropriate treatment (2). Several noninvasive imaging modalities are currently used for the detection of metastasis in tumor-draining lymph nodes, including computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT), and ultrasound (3–7).

Among these imaging modalities, CT and MRI are used to determine nodal size, which is used to define the status of regional lymph nodes. A nodal size greater than 10 mm in the short-axis diameter is the most widely accepted criterion for the diagnosis of cancer involvement (8). However, this size-based characterization of lymph node metastasis frequently leads to misdiagnosis as metastatic lymph nodes may be of normal size, whereas nonmetastatic lymph nodes may become abnormally enlarged due to reactive swelling (defined pathologically as reactive lymphadenopathy; ref. 9). Recently, Zhang and colleagues (10) have reported that both diffusion-weighted and superparamagnetic iron oxide-enhanced MRI can distinguish tumor metastatic lymph nodes from reactive lymph nodes. However, both methods have limited value in detecting microscopic tumor metastasis to the draining lymph nodes at the early stages.

PET using 2-[18F]fluoro-2-deoxy-D-glucose (FDG; a glucose analog radiolabeled with fluorine-18) is a functional method for tumor detection, based on the increased glucose metabolism of malignant tumors. Investigators in a previous study (11) have evaluated the role of FDG-PET for the detection of lymph node metastasis in patients with melanoma by comparing its results with those of postoperative histopathology. FDG-PET was reported to be capable of detecting 100% of metastases present in lymph nodes that were 10 mm or greater in their short-axis diameter, 83% of metastases that were 6 to 10 mm, and 23% of metastases that were 5 mm or less. However, FDG-PET was found to be highly sensitive (93%) only for the detection of metastases with more than 50% lymph node involvement or with capsular infiltration.

Current clinical ultrasound systems use a frequency of 3 to 15 MHz (12). The criteria used for diagnosing lymph node metastasis with gray-scale sonography are lymph node size (short-axis diameter/long-axis diameter), cortical thickness, shape, nodal borders, central necrosis, absence of echo-rich hilar structures, and extracapsular spread (13–15). Among these criteria, nodal size and depiction of necrosis are the

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most important sonographic criteria (16). As technology has developed and with the introduction of power Doppler sonography, it has become possible to evaluate the pattern of intranodal vessels and measure blood flow velocity and vascular resistance with spectral Doppler gates (15, 17). Kagawa and colleagues (18) have reported that blood flow signals become scattered and that the scattering index increases as the metastatic lymph node size increases.

However, among the current ultrasound techniques available, even high-resolution ultrasound (6–11 MHz) combined with power Doppler sonography is unable to detect metastatic deposits smaller than 4.5 mm in diameter in positive sentinel lymph nodes (19, 20); in fact, it can detect only 22.2% of metastatic deposits larger than 4.5 mm (20). This is due to limitations in spatial resolution, operator dependence, the short time window available for imaging, and the limited field of view. Another limiting factor in the development of diagnostic ultrasound imaging has been a lack of appropriate animal models for studying lymph node metastasis (21).

Because the lymph node size of conventional mice is 1 to 2 mm, changes in the internal structure of murine lymph nodes from the onset of metastasis cannot be detected using current ultrasound systems. To overcome the limitations imposed by the use of traditional mouse models and sonography, the present study used 3-dimensional (3D) contrast-enhanced high-frequency ultrasound (CE-HFUS) in combination with the contrast medium, Sonazoid, to examine a mouse model of lymph node metastasis established by our previous research (22). Specifically, a CE-HFUS system set at a frequency of 35 MHz, was used to examine tumor development from the onset of metastasis in MRL/lpr mice, which have lymph nodes that are enlarged to approximately 8 mm in diameter (similar in size to human lymph nodes). The injection of tumor cells into the subiliac lymph nodes (SiLN) of these mice was used to induce metastasis through a single lymphatic vessel to the proper subiliac lymph nodes (SiLN) of these mice was used to induce to human lymph nodes). The injection of tumor cells into the subiliac lymph nodes (SiLN) of mice anesthetized with 2% iso-propanolol results in the proper ALN of the 12 mice, measured using a digital caliper, 45 g; 14–18 weeks old). The mean longitudinal diameters of the SiLN and proper ALN of the 12 mice, measured using a digital caliper, were 7.7 ± 1.7 mm (mean ± SD; n = 12) and 7.4 ± 1.5 mm (n = 12), respectively. Lymph node metastasis was induced in the proper ALN of mice in the metastasis group (n = 6) by injection of 1 × 10^6 KM-Luc/GFP cells, suspended in 30 μL of PBS, into the SiLN of mice anesthetized with 2% isoflurane (Abbott Japan Co., Ltd.) in oxygen. PBS (30 μL) was injected into the SiLN of mice in the negative control group (n = 5). The day of inoculation was defined as day 0.

To visualize the lymphatic vessel between the proper ALN and the SiLN, indocyanine green (ICG; excitation wavelength, 805 nm; emission wavelength, 840 nm; Daiichi Sankyo Co.) was used to approximate the size of the tumor by injection of 1 × 10^6 KM-Luc/GFP cells, suspended in 30 μL of PBS, into the SiLN of mice anesthetized with 2% isoflurane (Abbott Japan Co., Ltd.) in oxygen. PBS (30 μL) was injected into the SiLN of mice in the negative control group (n = 5). The day of inoculation was defined as day 0.

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Detection of metastasis in the proper ALN by in vivo bioluminescence imaging

Metastasis to the proper ALN was assessed using an in vivo imaging system (IVIS; Xenogen; ref. 27). Each mouse was anesthetized with 2.0% isoflurane in oxygen before intraperitoneal injection with 150 mg/kg of luciferin (Promega Co.). After 10 minutes, luciferase bioluminescence activity was measured for 30 seconds using the IVIS. This procedure was carried out on days 0, 4, 9, 14, and 21 postinoculation.

Evaluation of time-dependent changes in signal intensity in the proper ALN

Sonozoid (Daiichi Sankyo), a lipid-stabilized suspension of perfluorocarbon microbubbles, was used as an ultrasound contrast agent (28). A ζ potential and particle size analyzer (ELS-Z-2; Otsuka Electronics) revealed that Sonozoid had a median diameter of 2.46 ± 0.12 μm (n = 3; mean ± SD) and a mean ζ potential of −37.0 ± 1.1 mV (n = 3). To investigate the in vivo stability of Sonozoid, 2-dimensional (2D) dynamic contrast-enhanced imaging was conducted using a CE-HFUS system (VEVO770; VisualSonics Inc.) with a 35 MHz transducer (RMV-703; axial resolution 50 μm, focal length 10 mm; VisualSonics Inc.) set at 50% transmission power. For these
experiments, an additional group of mice was used that had received no tumor cells (n = 5). Mice were anesthetized with 2% isoflurane in oxygen and placed onto a stage maintained at 38°C (TM150, VisualSonics Inc.). While the transducer remained in a stable position, 2D contrast-enhanced images of the center of the proper ALN were taken serially, until 12 minutes after slow injection of 100 μL Sonazoid into the tail vein; respiration gating was used to suppress imaging artifacts due to respiration. A region of interest was subsequently drawn around the proper ALN and a time–intensity curve was created for determination of the optimal diagnostic window.

Three-dimensional reconstruction of the microvasculature in the proper ALN using CE-HFUS with Sonazoid

A 3D image of the microvasculature within the proper ALN was reconstructed and time-dependent blood vessel development in the proper ALN was evaluated on days 0, 4, 9, 14, and 21 postinoculation, using a CE-HFUS system with a 35 MHz transducer fixed to a 3D stage control system (Mark-204-MS; Sigma Koki Co., Ltd.). Before and 180 seconds after intravenous bolus injection of 100 μL Sonazoid into the tail vein, consecutive B-mode images with a slice thickness of 100 μm were captured throughout the entire proper ALN for 3D imaging during the diagnostic window. The difference in the video intensity between the preinjection and postinjection image frames was highlighted as a green overlay in the B-mode anatomic images using the accompanying software (VisualSonics). The highlighted green areas were considered to be the extracted blood vessel images. Blood vessel densities and volumes in the proper ALN (whose boundaries were manually traced using sequential, parallel ultrasound scans, according to the anatomic and acoustic characteristics) were calculated using analysis software (VisualSonics Inc.). Values on day 0 were set at 100%. Changes in each individual animal, measured on days 4, 9, 14, and 21, were calculated and expressed as percentage changes. Throughout the imaging session, mice remained anesthetized, while resting on a heated stage by administration of 1 L/min of 2% isoflurane in oxygen, in accordance with the manufacturer’s protocol. Respiratory gating was used to synchronize data acquisition with the mouse respiratory cycle to reduce motion artifacts during image analysis. A 2D image of the maximum cross-section of the proper ALN was extracted from the 3D image.

Immunohistochemical analysis

After IVIS and ultrasound imaging had been conducted on day 21 postinoculation, metastatic lymph nodes were excised in a manner that allowed the researcher to remain blind about whether they had been excised from mice in the control (4 of 5 mice) or metastasis (4 of 6 mice) group. Lymph nodes were fixed overnight in 18.5% formaldehyde in PBS at 4°C (Rapid Fixative; Kojima Chemical Industry, Inc.), dehydrated, and then embedded in paraffin. The embedded specimens were cut into 2-μm serial sections and either stained with hematoxylin and eosin or immunostained for detection of LYVE-1–positive and CD31-positive cells using a Discovery XT automated staining processor (Ventana Medical Systems, Inc.). Immunostaining of lymphatic endothelial cells was carried out using a polyclonal rabbit anti-mouse LYVE-1 antibody (4 μg/mL; 103-PA50AG; Cosmo Bio Co. Ltd.) for 2 hours at room temperature, an anti-rabbit immunoglobulin G (IgG) Histofine MAX-PO (R) Kit (Nichirei Biosciences Inc.) for 16 minutes at room temperature, and 3,3′-diaminobenzidine (DAB). Immunostaining of vascular endothelial cells was carried out using a precluded polyclonal rabbit anti-CD31 antibody (1/100 dilution; sc-1506-R; Santa Cruz Biotechnology, Inc.) for 2 hours at room temperature, in combination with an anti-rabbit IgG Histofine MAX-PO (R) kit for 16 minutes at room temperature.

To obtain the mean blood vessel density of the macrovessels, defined as vessels with a diameter greater than or equal to 30 μm, the total macrovessel area was measured and the value obtained was divided by the total area of the specimen. The specimen boundary was measured under low magnification (∼40 or ∼100) using a microscope (BX51; Olympus Co.) and digital camera (DP72; Olympus). The hot-spot method was used to calculate the mean blood vessel density of the microvessels, defined as vessels with a diameter less than 30 μm (29). Specifically, 5 hot-spot fields with the highest microvessel density were selected under low magnification (∼40 or ∼100) and the blood vessel density was calculated by dividing the total macrovessel area, consisting of vasculature with a minor axis of 5 to 30 μm (∼200) by the CD31-positive hot-spot area (523 μm × 695 μm). Measurements of blood vessel density were made by 2 researchers to reduce the measurement error.

Statistical analysis

All measurements are presented as either mean ± SD or mean ± SEM values. Differences between groups were determined by two-way ANOVA followed by the Tukey–Kramer test (Figs. 1D, 4B, and Fig. 5A) or Student t test (Fig. 6B). Measurement of the correlation between luciferase activity (bioluminescence imaging) and blood vessel density (ultrasound imaging) in metastatic lymph nodes was determined using the Spearman rank correlation coefficient test (Fig. 5B). A P value of less than 0.05 was considered to represent a statistically significant result. Statistical analyses were conducted using Excel 2007 (Microsoft) with Statcel2 software.

Results

Metastasis in the proper ALN

Figure 1A shows an anatomic drawing of a 24-week-old MRL/lpr mouse that developed systemic lymphadenopathy to a remarkable extent. A single lymphatic vessel (which cannot be seen by the naked eye) and a single superficial epigastric vein extend in parallel from the SiLN to the proper ALN (Fig. 1A). When ICG solution was injected in the vicinity of the efferent lymphatic vessel in the SiLN, it reached the proper ALN through the lymphatic vessel (Fig. 1B).

Figure 1C and D illustrates measurement of the luciferase activity of cells growing in both the SiLN and the proper ALN on days 0, 4, 9, 14, and 21 postinjection of either KM-Luc/GFP cells into the SiLN of the metastasis group (n = 6) or PBS into the SiLN of the control group (n = 5). The results show that the
luciferase activities in both the SiLN and the proper ALN increased over time in the metastasis group. In the SiLN, there was a steep increase in luciferase activity on day 4, which was followed by a further slight increase up to day 21 ($P < 0.01$ on day 21, control-SiLN vs. treated-SiLN); in the proper ALN, a gradual increase up to day 21 was observed ($P < 0.05$ on day 21, control-proper-ALN vs. treated-proper-ALN). In the control group, the luciferase activities of both the SiLN and the proper ALN remained at baseline levels over the course of the experimental period.

**Determination of the diagnostic window**

To determine the optimal diagnostic window in which the blood vessel imaging in the presence of Sonazoid was conducted, we investigated the time–intensity relation for B-mode imaging of the proper ALN without metastasis (Fig. 2). As an injected solution is generally not evenly distributed throughout the circulation immediately after injection, a concentration difference is produced in the bloodstream, which varies depending on the duration of the administration. In the present study, dense Sonazoid acoustic signals were detected near the hilum of the proper ALN 1 second after injection and throughout the proper ALN 10 seconds after injection, whereas scattered acoustic signals were detected throughout the proper ALN after 60 seconds. We found that the concentration of Sonazoid in the blood became uniform over time. Figure 2B shows the contrast intensity throughout the proper ALN over time. Immediately
after tail-vein injection of Sonazoid, the mean gray-scale value initially increased dramatically but then decreased gradually until 168.0 ± 13.7 seconds postinoculation (n = 5; mean ± SD); subsequently, it remained at this decreased level. On the basis of this determination of the optimal diagnostic window, all ultrasound imaging was conducted between 180 and 420 seconds.

Evaluation of the changes in blood vessel volume and lymph node size in the proper ALN

As changes in vascularization and tumor size are important parameters that determine tumor progression (30, 31), changes in blood vessel volume and nodal size of the proper ALN with (n = 6) and without (n = 5) metastasis were measured using 3D CE-HFUS. Figure 3 shows 2D images of the maximum cross-section of the proper ALNs extracted from the 3D images. It may be seen that the acoustic signals in the lymph nodes did not change over time in the control group (Fig. 3A) but increased over time throughout the lymph nodes in 5 of 6 mice in the metastasis group (Fig. 3B), with localized dense acoustic signals found in 1 of 6 mice in the metastasis group (Fig. 3C).

In general, 2D images provide data about only 1 cross-section, unless there is 3D symmetry and uniformity that allows for extrapolation. Thus, a 2D image cannot be used to evaluate internal structural changes when vessels are localized to a particular area, as shown in Fig. 3C. Therefore, the changes in blood vessel volume and nodal size in the proper ALN were measured using 3D CE-HFUS. Figure 4 illustrates the 3D vessel structures of the proper ALN with metastasis on days 0, 4, 9, 14, and 21; the increase in blood vessel volume over time is shown in Fig. 4A and the changes in blood vessel volume and nodal size are presented in Fig. 4B. The day of cell injection into the SiLN was set at day 0 and blood vessel volume and lymph node size on day 0 were each set at 100%. The lymph node size of the control group (n = 5) was observed to remain constant over the experimental period (96% ± 2% at day 21; P > 0.05, day 0 vs. 21), whereas that of the metastasis group (n = 6) seemed to increase slightly although statistical significance was not reached (116% ± 10% at day 21; P > 0.05, day 0 vs. 21). The blood vessel volume of the control group (n = 5) also remained constant up to day 21 (87% ± 2% at day 21; P > 0.05, day 0 vs. 21), whereas that of the metastasis group (n = 6) increased over time. Blood vessel volume in the metastasis group at day 21 was significantly different from that at day 0 (190% ± 28% at day 21; P < 0.01, day 0 vs. 21). Importantly,
the magnitude of the change in blood vessel volume in the metastasis group at day 21 was found to be significantly different from the magnitude of the change in lymph node size ($P < 0.05$). This finding suggests that angiogenesis is a more important parameter than nodal size for evaluating the progression of lymph node metastasis in the early stages.

**Quantitative analysis of blood vessel density in the proper ALN with metastasis**

Blood vessel density in the proper ALN was investigated using 3D CE-HFUS, with vessel density normalized to the density measured on day 0. Figure 5A shows that there was no significant change in normalized blood vessel density over time in the control group ($n = 5$) but a 1.62-fold increase on day 21 compared with day 0 in the metastasis group ($n = 6$; $P < 0.05$, day 0 vs. 21). In addition, the normalized blood vessel density of the metastasis group was found to be 1.72-fold greater than that of the control group on day 21 ($P < 0.05$, control vs. metastasis). Figure 5B shows the correlation between the normalized blood vessel density measured using 3D CE-HFUS and the normalized luciferase activity reflecting the proliferation of tumor cells as measured using IVIS. There was a positive correlation between the 2 factors [Spearman rank correlation coefficient ($r_s$) = 0.763, $n = 28$].

**Evaluation of blood vessel density by immunohistochemical analysis**

Analysis of structural changes in the proper ALNs, with and without metastasis, revealed that the basic structures of the lymph nodes, such as the medulla, paracortex, and cortex, and the lymphatic channels had been preserved in the control group. Figure 6 shows that blood vessels could be discretely observed in the control group. In contrast, continuous invasion of tumor cells from the marginal sinus was detected in the cortex of the metastasis group, as well as a reduction in the size of LYVE-1–positive regions by the expansion of metastasis and an increase in the number and size of the blood vessels of the lymph node, even in non-metastatic regions. Quantification of blood vessel density (measured as blood vessels per unit area) in the proper ALNs, with and without metastasis, on day 21 revealed that the densities of both the microvessels (vessels with a width < 30 µm) and macrovessels (vessels with a width ≥ 30 µm) of the metastasis group were greater than those of the control group. Specifically, the total vessel density of both...
microvessels and macrovessels in the metastatic proper ALNs was found to be 1.5-fold greater than that of the control proper ALNs ($P < 0.01$). These changes correspond well with the 1.72-fold increase in the metastasis group compared with the control group, as shown in Fig. 5A.

**Discussion**

The present study is the first to show, using a 3D CE-HFUS system in combination with Sonazoid that in lymph nodes undergoing metastasis, increases in blood vessel volume and density precede changes in nodal size. Despite the fact that microscopic lymph node metastases may be present that cannot be observed macroscopically and that there may be small lesions that cannot be detected even by current ultrasonography techniques, it is nonetheless very important to use ultrasonography before treatment to assess lymph nodes less than 10 mm in diameter that cannot be detected by CT or MRI, as a diagnostic procedure for lymph node metastasis (32). This study, using a 3D CE-HFUS system with Sonazoid, demonstrated that the increases in blood vessel volume and density were 1.9- and 1.6-fold greater on day 21, respectively, than on day 0, and the increase in blood vessel density was positively related to tumor cell proliferation. In contrast, no significant difference was found between the nodal size of the proper ALN on day 0 and that on day 21, most likely because the normal lymph node tissue had been partially replaced by infiltrating tumor cells, causing only a slight increase in nodal size. In addition, neither the emergence of an echogenic region due to central necrosis (33) nor the absence of hilum (16) was detected in metastatic lymph nodes until the last day of the experiment (day 21). These results indicate that blood vessel volume and density may be used as ultrasound parameters for diagnosis of early lymph node metastasis.

In general, normal tissue is invaded and replaced by tumor cells at an early stage of metastasis, resulting in changes in the microenvironment. These changes include release of metalloproteinases that degrade the extracellular matrix, secretion of growth factors to promote neovascularization (34), and development of sinus hyperplasia (35). In the diagnosis of lymph node metastasis by B-scan sonography, the clinical criteria currently used are increases in nodal size and cortical thickness, changes in nodal shape, infiltration of surrounding structures, presence of inhomogeneous internal echo patterns (including necrosis), absence of echogenic hilar structures, and extracapsular spread (14, 36). However, these changes only become pronounced once the tumor has already grown to some extent. In fact, other studies have found that the size and shape of lymph nodes with small metastatic tumors are within the reference range for their anatomic positions (37) or only slightly larger than those of tumor-free lymph nodes (38). Anticipatory changes in blood vessel volume and density at earlier stages may be

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**Figure 4.** Changes in blood vessel volume and nodal size of proper ALNs determined using 3D CE-HFUS. **A,** 3D images of the blood vessel structure of the proper ALN with metastasis obtained on days 0, 14, and 21. The vessels are highlighted in green. **B,** changes in blood vessel volume and lymph node size. Metastasis refers to a proper ALN with metastasis ($1 \times 10^7$ cells/min; $n = 6$) and Control to a proper ALN without metastasis ($n = 5$). Open and closed circles represent the blood vessel volumes within the proper ALNs of the control and metastasis groups, respectively. Open and closed triangles represent the sizes of the proper ALNs of the control and metastasis groups, respectively. Values at different time points were normalized by comparison with those on day 0. On day 21, the magnitude of the change in blood vessel volume was larger than the magnitude of the change in lymph node size. $^*$, temporal change within each group; $^\#$, comparison between groups. $^*$ or $^\#$, $P < 0.05$; $^{**}$ or $^{**\#}$, $P < 0.01$; calculated using two-way ANOVA followed by Tukey-Kramer testing. Error bars indicate the SEM.
between groups became significant on day 21. A, quantitative assessment of the change in blood vessel density of control (n = 5) and metastatic (n = 6) proper ALNs using 3D CE-HFUS. The blood vessel density of the metastatic proper ALNs increased with time and the difference between groups became significant on day 21. *, P < 0.05 versus day 0 in the metastasis group; #, P < 0.05 versus control on day 21; determined by two-way ANOVA followed by Tukey-Kramer testing. Mean ± SEM values are shown. B, relationship between normalized blood vessel density determined using 3D CE-HFUS and normalized luciferase activity determined with IVIS. Using analysis of Spearman rank correlation coefficient, a linear correlation (r_s = 0.763) was found between normalized luciferase activity and normalized blood vessel density during tumor progression (P < 0.01). This relationship indicates that both these noninvasive imaging methods may be used to follow the progression of metastasis.

Figure 5. Quantitative assessment of metastasis in the proper ALN using 3D CE-HFUS and an IVIS. Values were normalized by comparison with those obtained on day 0. A, quantitative assessment of the change in blood vessel density of control (n = 5) and metastatic (n = 6) proper ALNs using 3D CE-HFUS. The blood vessel density of the metastatic proper ALNs increased with time and the difference between groups became significant on day 21. *, P < 0.05 versus day 0 in the metastasis group; #, P < 0.05 versus control on day 21; determined by two-way ANOVA followed by Tukey-Kramer testing. Mean ± SEM values are shown. B, relationship between normalized blood vessel density determined using 3D CE-HFUS and normalized luciferase activity determined with IVIS. Using analysis of Spearman rank correlation coefficient, a linear correlation (r_s = 0.763) was found between normalized luciferase activity and normalized blood vessel density during tumor progression (P < 0.01). This relationship indicates that both these noninvasive imaging methods may be used to follow the progression of metastasis.

overlooked when using conventional ultrasonography techniques for diagnosis.

Our research group has previously reported that use of 2D CE-HFUS with acoustic liposomes was capable of evaluating antitumor angiogenic effects in a subcutaneous tumor model (39). Combined with Sonazoid, the 3D CE-HFUS system used in the present study allows for in vivo quantitative monitoring of tumor vascularity during the progression of lymph node metastasis. This approach provides several advantages. Sonazoid is a microbubble ultrasound agent with a stable shell, resulting in a relatively long time window being available for imaging. The use of a 3D stage control system avoids the operator-dependence of selection that occurs for a 2D scan. In addition, compared with conventional vascular visualization methods (40), the combination of this approach with a respiratory gating technique allows for visualization of vascular regions with reduced effects of noise and tissue movement during ultrasound measurements. Finally, while conventional functional ultrasound (power Doppler imaging) is more sensitive for large mature vessels (41), the imaging technique presented in this study enables the assessment of both large and small vessels, through which microbubbles normally pass freely.

We used the 3D CE-HFUS system, set at a frequency of 35 MHz, to examine the proper ALNs of MRL/lpr mice with lymph nodes enlarged to approximately the size of human lymph nodes (7.6 ± 1.3 mm, mean ± SD; n = 15). This technique allows for evaluation of tumor development from the onset of metastasis. Furthermore, this method was able to overcome the limitations imposed by the use of conventional clinical ultrasound systems, set at frequencies of 3 to 15 MHz and by mouse models with normal-sized (1–2 mm) lymph nodes (42). MRL/lpr mice show lymphadenopathy due to an accumulation of lpr T cells (B220+/Thy1.2+/CD4+CD8–) in the paracortical regions of the lymph nodes (43, 44). However, as these lpr T cells do not infiltrate the lymphoid follicle and the follicular dendritic cell network (45), the basic lymph node structures, including the medulla, paracortex, and cortex, and the lymphatic channels are maintained, allowing MRL/lpr mice to serve as an animal model of lymph node metastasis.

In general, multiple steps are required for tumor cells to metastasize from their primary site to regional lymph nodes, including detachment from the primary tumor mass, invasion into lymphatic vessels, transport through draining lymphatic vessels, arrest in lymph nodes, and survival and growth in lymph nodes (46). In an experiment using BALB/c mice and athymic BALB/c-nu/nu mice (47), organization of the lymphatic channels and the vasculature was observed before the establishment of metastasis in the sentinel lymph node with enlargement of lymph node sinuses and emergence of functional blood vessels that developed from high endothelial venules. This may be the reason why the number and size of the blood vessels increased not only in the metastatic foci but also in nonmetastatic regions. The total number of lymphatic vessels in metastatic lymph nodes decreases, because these vessels may collapse or find it difficult to penetrate into expanding primary tumor (Fig. 6A; refs. 48, 49). In the mouse model used in the present study, direct injection of cells into the SiLN via a lymphatic vessel resulted in the delivery of tumor cells to the proper ALN. This was followed by continuous invasion of cells that had metastasized into the marginal sinus, into the cortex and paracortex, and then into the medulla, possibly through the lymphatic and medullary sinuses; this invasion pattern in the proper ALN was similar to that of spontaneous lymph node metastasis. However, our previous experiments found that the incidence of metastasis to the proper ALN from the SiLN depended on the number of cells injected into the SiLN, the duration of the injection, and the SiLN volume (22). The spread of metastasis and subsequent blood vessel development may depend on the uniformity of the cells in the injection solution (Fig. 3B and C). The molecular biologic aspects of metastasis could not be investigated directly in the present study, whose methodology and findings were limited to the use of imaging techniques. In addition, potential differences between tumor metastatic lymph nodes and inflammatory lymph nodes were not investigated.
In conclusion, the results of the present study indicate that blood vessel volume and density are more important parameters than nodal size for sonographic evaluation of the progression of lymph node metastasis in the early stages. As a correlation was identified between vessel density and luciferase activity, a molecular marker of tumors, the use of 3D CE-HFUS in combination with PET (50) or MRI (10), together with specific molecular probes should be investigated as a means of further improving diagnostic accuracy. In future, our findings may allow for the early diagnosis of metastasis of some malignant tumors to superficial lymph nodes (such as melanoma, breast cancer, and head and neck cancers), or intraoperative diagnosis of metastatic lymph nodes.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

Conception and design: L. Li, S. Mori, M. Sakamoto, T. Kodama Development of methodology: L. Li, S. Mori, T. Kodama Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Li, S. Mori, M. Sakamoto, T. Kodama Writing, review, and/or revision of the manuscript: L. Li, S. Mori, S. Takahashi, T. Kodama

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Li, S. Mori, M. Sakamoto, T. Kodama

Study supervision: S. Mori, M. Sakamoto, S. Takahashi, T. Kodama

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Li, S. Mori, M. Kodama, T. Kodama Writing, review, and/or revision of the manuscript: L. Li, S. Mori, S. Takahashi, T. Kodama

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