Early diagnosis of lymph node metastasis: Importance of intranodal pressures

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Key words
Early diagnosis, EPR effect, intranodal pressure, lymph node metastasis, lymphatic network, mouse model of metastasis

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Regional lymph node status is an important prognostic indicator of tumor aggressiveness. However, early diagnosis of metastasis using intranodal pressure, at a stage when lymph node size has not changed significantly, has not been investigated. Here, we use an MXH10/Mo-lpr/lpr mouse model of lymph node metastasis to show that intranodal pressure increases in both the subiliac lymph node and proper axillary lymph node, which are connected by lymphatic vessels, when tumor cells are injected into the subiliac lymph node to induce metastasis to the proper axillary lymph node. We found that intranodal pressure in the subiliac lymph node increased at the stage when metastasis was detected by in vivo bioluminescence, but when proper axillary lymph node volume (measured by high-frequency ultrasound imaging) had not increased significantly. Intravenously injected liposomes, encapsulating indocyanine green, were detected in solid tumors by in vivo bioluminescence, but not in the proper axillary lymph node. Basic blood vessel and lymphatic channel structures were maintained in the proper axillary lymph node, although sinus histiocytosis was detected. These results show that intranodal pressure in the proper axillary lymph node increases at early stages when metastatic tumor cells have not fully proliferated. Intranodal pressure may be a useful parameter for facilitating early diagnosis of lymph node metastasis.

The status of tumor-draining lymph nodes (LNs) is a prognostic factor for determining the cancer stage and thus treatment planning. The detection of metastasis in tumor-draining LNs has been carried out using non-invasive imaging methods, including computed tomography (CT), MRI, PET, and single photon emission CT ultrasound (US). Pooled estimates for sensitivity, on a per-neck basis, for CT, MRI, PET, and US are 52% (95% confidence interval, 39–65%), 65% (34–87%), 66% (47–80%), and 66% (45–77%), respectively, when used to detect cervical metastases in patients with clinically N0 head and neck cancer. Computed tomography, MRI, PET, and US do not differ in sensitivity and specificity, with the exception that CT shows superior specificity to US in patients with clinically N0 head and neck cancer. Recently developed hybrid imaging technologies (CT/MRI/US) and PET functional imaging have offered improvements, but additional advances are required to reduce costs and further increase sensitivity and specificity.

Intranodal pressure (INP) correlates positively with tumor volume in primary breast cancer, and has been used to predict metastasized LN size in the operating theater. Tumor-secreted lymphangiogenic factors and metastasis-promoting chemokines flow toward peritumoral lymphatic vessels, changing the microenvironment in draining LNs to one favoring deposition, survival, and growth of metastases. During these processes, alterations in blood vessel volume and density precede changes in downstream LN size. Therefore, INP elevations in draining LNs may indicate metastasis.

In the absence of more clinically relevant metastasis models, studies have used xenogeneic grafts in immune deficient nude or SCID mice. Here, we used MXH10/Mo-lpr/lpr (MXH10/Mo/lpr) inbred mice, which develop systemic swelling of LNs that reach up to 10 mm in diameter (similar in size to human LNs). The basic structures of the LNs (including the medulla, paracortex, and cortex) and lymphatic channels are preserved.

In the present study, tumor cells were injected into the subiliac LN (SiLN) to induce metastasis (through connecting lymphatic vessels) to the proper axillary LN (PALN). We show that INP increases in these LNs during the course of metastasis.

Materials and Methods
The Institutional Animal Care and Use Committee of Tohoku University (Sendai, Japan) approved all in vivo study protocols.

Cell culture. Malignant fibrous histiocytoma-like KM-Luc/GFP cells, expressing a fusion of the luciferase and enhanced green fluorescent protein genes, were cultured as previously described. C3H/He mouse mammary carcinoma (FMA-Luc) cells, expressing the luciferase gene, and B16F10 mouse melanoma cells (Cell Resource Center for Biomedical
Research, Institute of Development, Aging, and Cancer, Tohoku University) were maintained in RPMI-1640 medium supplemented with 10% FBS, 1% L-glutamine–penicillin–streptomycin, and 1 mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA). Cell lines were incubated at 37°C, 5% CO2/95% air until 80% confluence was achieved. Lack of Mycoplasma contamination was confirmed on the inoculation day (MycoAlert Mycoplasma Detection Kit; Lonza Rockland, Allendale, NJ, USA).

Polymerase chain reaction analysis of vascular endothelial growth factor family expression. Expression of vascular endothelial growth factor (VEGF)-A, VEGF-B, VEGF-C, and VEGF-D in KM-Luc/GFP and FM3A-Luc cells was examined. B16F10 cells were used as the positive control for VEGF-C expression. RNA was extracted (RNeasy Mini Plus kit; Qiagen, Düsseldorf, Germany), and isolated RNA (1 μg) was reverse-transcribed with ReverTra Ace (Toyobo, Osaka, Japan). The negative control used sterile distilled water. Table S1 shows the PCR primers used.

Mice. MXH10/Mo/lpr mice (weight, 27–37 g; 14–16 weeks old) were maintained at the Institute for Animal Experimentation, Graduate School of Medicine, Tohoku University. Induction of PALN. Cells, 2.0 × 10^7 KM-Luc/GFP or 2.0 × 10^4 FM3A-Luc cells in 60 μL vehicle (20 μL PBS plus 40 μL of 400 mg/mL Matrigel; Collaborative Biomedical Products, Bedford, MA, USA), were injected into the SI-LNs of mice anesthetized with 2% isoflurane in oxygen (Abbott, Lake Forest, IL, USA). In controls, 60 μL vehicle was injected (Figs.1A and 1B). Inoculation (24-gauge needle) was guided by high-frequency US imaging (VEVO770; VisualSonics, Toronto, ON, Canada) using a 25-MHz transducer (RMV-710B; VisualSonics) (Fig. 1C).(17) The inoculation day was defined as day 0. Three groups were used for evaluation of tumor growth/metastasis, PALN size, and INP: control, KM-Luc/GFP, and FM3A-Luc (Fig. 1A). The KM-Luc/GFP group was divided into two subgroups (day 3 and day 6), and the FM3A-Luc group into three subgroups (day 6, day 10, and day 14).

Induction of solid tumor. Solid tumor was produced by s.c. injection of 2.0 × 10^7 KM-Luc/GFP or 2.0 × 10^4 FM3A-Luc cells (suspected in 60 μL PBS/Matrigel vehicle) into the right or left flank of the mouse.

Detection of tumor growth and metastasis. Metastasis to the PALN was assessed using in vivo bioluminescence imaging (IVIS; Xenogen, Waltham, MA, USA) on days 0, 3, and 6 for PALN was assessed using injection of 2.0 × 10^4 GFP or 2.0 × 10^4 M2e GFP cells, and days 0, 3, 6, 10, and 14 for FM3A-Luc cells.(18) Metastasis was considered successful when the ICG was detected by IVIS at the same time points.

Measurement of INP in SI-LN and PALN. The mouse was anesthetized and an arc-shaped incision made in the abdominal skin from the SI-LN to the PALN (Fig. 1B). As indocyanine green (ICG) injected into the SI-LN seldom flowed across the midline to the contralateral side and always flowed from the SI-LN to the PALN, it is unlikely that surgery damaged the lymphatic vessels connecting the SI-LN and PALN or influenced INP measurements. A 21-gauge hypodermic needle was connected to a pressure transducer (BLPR2; World Precision Instruments, Sarasota, FL, USA) through a three-way stopcock (Terumo, Tokyo, Japan) filled with physiological saline. The pressure transducer was connected to a directly coupled amplifier system (Bridge8; World Precision Instruments) linked to a computer running analysis software (LabScribe2; iWorx Systems, Dover, NH, USA). A zero reading was obtained with the needle open to the air at the level of the node to be measured.(19) Intranalional pressure was measured (0.02 s sampling rate) with the needle inserted for 5 min into the central region of the LN.

Measurement of PALN and solid tumor volume. Proper axillary LN volume was measured using high-frequency US imaging (VEVO770) with a 25-MHz transducer (RMV-710B), on days −1 (before inoculation), 2, and 5 for KM-Luc/GFP cells, and days −1, 2, 5, 9, and 13 for FM3A-Luc cells.(4) Solid tumor volume was calculated from digital caliper measurements as: π/6 × width^2 × length.

Production of ICG liposomes. 1,2-Distearoyl-sn-glycero-3-phosphatidylcholine (MC8080; NOF, Tokyo, Japan) and 2, distearoyl-sn-glycerol-3-phosphatidylethanolamine-methoxy-PEG (DSPE-PEG2000-OMe) (DSPE-020CN; NOF; 94:6 mol/mol) were dissolved in chloroform. Following solvent removal (thaw rotation, 60 mPa, 65°C, 2 h), the liposome film was dissolved in 80 μM ICG (in PBS). Following several freeze–thaw cycles to form large unilamellar vesicles, ICG liposome diameter was adjusted to <200 nm using extruding equipment (NorthEast Lipids, Burnaby, Canada) and sizing filters (100, 200, and 600 nm porous columns). Unencapsulated ICG was removed by PD-10 columns (GE Healthcare, Little Chalfont, UK). After sterilization (0.45-μm pore filter; Millipore, Billerica, MA, USA), the lipid concentration (Phospholipid C-test; Wako Pure Chemical Industries, Osaka, Japan) was adjusted to 0.5 mg/mL. The ICG liposome zeta potential and size were 2.2 mV and 134.7 ± 9.39 nm (mean ± SD, n = 2), respectively (ELSZ-2, Otsuka Electronics, Osaka, Japan). Light absorption characteristics were measured with a spectrum analyzer (UV2600; Shimadzu, Kyoto, Japan). To confirm ICG liposome stability at mammalian body temperature, 1 mL aliquots was incubated at 37°C. Any ICG released was removed by PD-10 columns, and absorbance at 800 nm (to detect any remaining ICG) measured after 0 min, 5 min, 1 h, 6 h, and 24 h (SpectraMax M2/M2e multidetection microplate reader; Molecular Devices, Sunnyvale, CA, USA). Approximately 20% of the ICG liposomes had collapsed after 48 h at 37°C.

Leakage of ICG liposomes from vasculature. Indocyanine green leakage from SI-LN and PALN vasculature was measured by IVIS, with solid tumors used as a positive control. Indocyanine green liposomes were injected i.v. on day 6 (KM-Luc/GFP) or 14 (FM3A-Luc) after cell inoculation, and leakage measured 24 h after injection. The mouse was then killed, and the harvested SI-LN, PALN, and solid tumor tissues weighed and homogenized in 1 mL PBS (T25 Basic Ultra-Turrax S1; IKA Works, Staufen, Germany). The fluorescence intensity of supernatant in 48-well plates (500 μL/well) was measured by IVIS.

Histological analysis. The LNs were excised from the mice after ICP measurements had been made. Some LNs were fixed overnight in 10% formalin at 4°C, dehydrated, embedded in paraffin, serially sectioned (3–5 μm), and either stained with H&E or immunostained for LYVE-1-positive, CD31-positive cells (Discovery XT Automated Staining Processor; Ventana Medical Systems, Tucson, AZ, USA). Immunostaining of lymphatic endothelial cells was carried out using polyclonal rabbit anti-mouse LYVE-1 antibody (4 μg/mL, 2 h at room temperature) (103-PA50AG; Cosmo Bio, Tokyo, Japan) in combination with biotinylated anti-rabbit IgG (20 min at room temperature; Vector Laboratories, Burlingame, CA, USA) and
A (i) Control

Control Group
(n = 12)

PBS & matrigel injection

Time (days)

Pressure measurement & sacrifice

(ii) KM-Luc/GFP

Day 3 Group
(n = 12)

Tumor implantation

Pressure measurement & sacrifice

Time (days)

Day 6 Group
(n = 12)

Tumor implantation

Pressure measurement & sacrifice

(b) DC amplifier system

PC

Pressure transducer

Anesthesia machine

T-shape stopcock

Needle

Syringe

Saline

SiLN

B (iii) FM3A-Luc

Day 6 Group
(n = 12)

Tumor implantation

Pressure measurement & sacrifice

Day 10 Group
(n = 12)

Tumor implantation

Pressure measurement & sacrifice

Day 14 Group
(n = 12)

Tumor implantation

Pressure measurement & sacrifice

C

Lymph node

Needle

Cancer cells

D

KM-Luc/GFP

FM3A-Luc

Negative control (H2O)

Positive control (B16F10)

VEGF-A

VEGF-B

VEGF-C

VEGF-D

β-actin
diaminobenzidine. Immunostaining of vascular endothelial cells was achieved using pre-diluted polyclonal goat anti-CD31 antibody (1:100 dilution; 2 h at room temperature) (sc-1506; Santa Cruz Biotechnology, Dallas, TX, USA) in combination with biotinylated anti-goat IgG (20 min at room temperature; Vector Laboratories) and diaminobenzidine. The remaining LN specimens were prepared as frozen sections (10-µm slices), to detect the area of CD31-positive cells using immunofluorescence. Sections were fixed in 4% paraformaldehyde (15 min at room temperature), washed (PBS), incubated overnight at 4°C with purified rat anti-mouse CD31 primary antibody (1:100 in PBS with 3% BSA and 0.1% Triton-X; 553370, BD Pharmingen, San Diego, CA, USA), washed (PBS), incubated (40 min, 4°C) with Alexa 555-conjugated goat anti-rat secondary antibody (1:500; Life Technologies, Carlsbad, CA, USA), washed (PBS), and mounted with Vectorshield (Vector Laboratories). Histological images were captured using a fluorescence microscope (BX51; Olympus, Tokyo, Japan) and digital camera (DP72; Olympus). The red channel from each RGB image was exported into Photoshop CS3 (Adobe Systems, San Jose, CA, USA). To measure vessel density in each section, the five most vascularized fields (hot-spot areas) were selected under low magnification (×40 or ×100), and the percentage of each field stained for CD31 calculated (×200) using ImageJ software (NIH).^{(4)}

**Fig. 2.** Induction of metastasis in a mouse model. (A–D) Changes in luciferase activity. (A,C) Luciferase activities induced by KM-Luc/GFP cell injection into the subiliac lymph node (SiLN) (n = 24). Metastasis to the proper axillary lymph node (PALN) was detected on day 6. Luciferase activity increased in both the SiLN and PALN, but was higher in the SiLN. (B,D) Luciferase activities induced by FM3A-Luc cell injection into the SiLN (n = 36). Metastasis to the PALN was detected on day 14. Tumor growth was slower than for KM-Luc/GFP cells, but the trends in luciferase activity were similar. *P < 0.05, **P < 0.01 versus day 0 (one-way ANOVA and Tukey’s test). Mean ± SD values are shown. (E–H) Changes in lymph node size. (E,G) Change in PALN volume, metastasized by KM-Luc/GFP cells (n = 12). (F,H) Change in PALN volume, metastasized by FM3A-Luc cells (n = 12). Lymph node volume was measured using high-frequency ultrasound imaging. Mean ± SEM values are shown, normalized to those on day 1.
Results

Induction of metastasis to PALN. KM-Luc/GFP or FM3A-Luc cells were injected into the SiLN to provoke metastasis to the PALN. Both cell types expressed VEGF-A and VEGF-B but not VEGF-C; KM-Luc/GFP but not FM3A-Luc cells showed slight VEGF-D expression (Fig. 1D). Figure 2 shows the luciferase activity of KM-Luc/GFP cells in the SiLN and PALN (days 0, 3, and 6; Fig. 2A,C), and that of FM3A-Luc cells (days 0, 3, 6, 10, and 14; Fig. 2B,D). Luciferase activity in both LNs increased over time, with larger increases in the SiLN. Tumor cells were detected in the PALN on day 6 for KM-Luc/GFP cells and day 14 for FM3A-Luc cells. In subsequent experiments, INP measurements were made from the early stages of metastasis to day 6 (KM-Luc/GFP) or day 14 (FM3A-Luc).

Changes in PALN volume. Lymph node size is an important variable used for US diagnosis of LN metastasis. Next, we measured changes in PALN volume using 3D high-frequency US imaging; data are shown for KM-Luc/GFP cells (Fig. 2E,G) and FM3A-Luc cells (Fig. 2F,H). Proper axillary LN volume was normalized to that on day −1. There were no significant changes in PALN volume over time for either cell type, supporting a previous finding that evaluation of LN size is not sufficient for early diagnosis of LN metastasis.

Intranodal pressure in SiLN and PALN. Next, we investigated temporal changes in the INP in the SiLN and PALN (Fig. 3). In control (i.e., non-treated) mice, INP was higher in the SiLN than in the PALN (0.07 ± 0.34 mmHg vs. 0.02 ± 1.24 mmHg, n = 36), indicating a pressure gradient between these LNs. For KM-Luc/GFP cells, INP increased over time in both the SiLN (Fig. 3A; n = 24; P < 0.05, SiLN versus control on day 3; P < 0.01, SiLN versus control on day 6) and PALN (Fig. 3A; n = 24; P < 0.05, PALN versus control on day 6). Intranodal pressure in the SiLN and PALN appeared to increase over time for FM3A-Luc cells (Fig. 3A), but the changes were not significant.

Indocyanine green liposome leakage from vasculature. Angiogenesis, a hallmark of cancer, leads to immature, hyper-permeable tumor vessels and an elevation of interstitial fluid pressure in a solid tumor. To examine the enhanced permeability and retention (EPR) effect during the early stages of LN metastasis, we injected ICG liposomes i.v. and compared their leakages from the SiLN, PALN, and solid tumor. For the KM-Luc/GFP group at day 6, the average sizes of the solid tumor, SiLN, and PALN were 182.81 ± 49.15 mm³ (n = 5), 358.76 ± 73.69 mm³ (n = 5), and 195.49 ± 34.78 mm³ (n = 5), respectively. For the FM3A-Luc group at day 14, the average sizes of the solid tumor, SiLN, and PALN were 165.18 ± 87.74 mm³ (n = 5), 411.41 ± 33.00 mm³ (n = 5), and 157.90 ± 27.45 mm³ (n = 5), respectively. There were no significant differences in LN size between the KM-Luc/GFP and FM3A-Luc groups (one-way ANOVA). First, we used in vivo bioluminescence imaging to confirm solid tumor growth (Fig. 4A(i),B(i)) and metastasis progression (Fig. 4A(ii),B(ii)). Twenty four hours after injection, ICG liposomes were detected around the solid tumor (Fig. 4A(iii),B(iii)), but did not accumulate in the SiLN and PALN (Fig. 4A(iv),B(iv)).

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Figure 4(C,D) shows the luciferase activities of solid tumor (Fig. 4A(i),B(i)), SiLN, and PALN (Fig. 4A(iii),B(iii)). There were no significant differences in luciferase activities between solid tumor and SiLN (a site of tumor injection), whereas the luciferase activity of the solid tumor was significantly higher than that of the PALN (a site of metastasis) \((P < 0.01, \text{solid tumor versus PALN})\). Measurement of fluorescence / weight ratios in homogenized tissues revealed significant differences between solid tumor, SiLN, and PALN (Fig. 4E,F; \(P < 0.01\), versus solid tumor). Thus, under the present conditions, the EPR effect that accompanies angiogenesis in solid tumors was not detected in the SiLN or PALN.

**Histological findings.** Figure 5 shows representative images of the SiLN and PALN stained with H&E, anti-LYVE-1 antibody, or anti-CD31 antibody, under control conditions (day 3), or after inoculation with KM-Luc/GFP (day 6) or FM3A-Luc (day 14) cells. The basic internal structures (medulla, paracortex, cortex, and lymphatic channels) were preserved in tumor-containing SiLN and metastasized PALN. Discrete blood vessels were observed in all LNs (Fig. 5A), and lymphatic sinus histiocytosis in the PALN and SiLN (Fig. 5B). Next, we measured LN vessel density (the CD31-positive area) using immunofluorescence techniques (Fig. 6A). Blood vessel density did not differ between the control and metastasis groups (Fig. 6B,C). It has been noted that as mice age, their lymph node structures change over a period of several weeks. After 12 weeks, follicles tend to atrophy due to the accumulation of abnormal lymphocytes. We observed that the degree of atrophy was similar for control and metastatic lymph nodes, except for the portion of the lymph node comprising metastatic tumor.

**Discussion**

The present study demonstrates that INP measurement can detect tumor growth in a metastatic LN earlier than a volume-based evaluation using US imaging. The detection sensitivity of INP was similar to that of \textit{in vivo} bioluminescence imaging (Figs 2,3).

In our experimental system, tumor cells were injected into the SiLN to induce metastasis to the PALN through lymphatic vessels. However, some of the tumor cells may have reached the lung by way of the thoracoepigastric vein.(14,15) Intranodal pressure increased in both LNs over time, but was greater in the SiLN. Accumulation of ICG liposomes due to the EPR effect(25) was observed only in solid tumor after...
Representative immunofluorescence images of the SiLN and PALN under control conditions (n = 4) on day 3 or after inoculation with KM-Luc/GFP cells (n = 4) on day 6 or FM3A-Luc cells (n = 4) on day 14, showing staining for CD31 as a marker of blood vessels. Bar = 200 μm. (B,C) Mean values for the percentage CD31-positive area in the SiLN (B) and PALN (C). There were no significant differences in the entire lymph node blood vessel density between the control and metastasis groups (one-way ANOVA). Mean ± SEM values are shown. NS, not significant.

In controls, INP was higher in the SiLN than in the PALN. In the anatomical chart of a mouse, the efferent lymphatic vessel of the PALN is connected with the subclavian vein, and that of the SiLN with the PALN. The thoracoepigastric vein, which connects the subclavian vein and inferior vena cava, runs adjacent to the SiLN and PALN, and receives venous blood from these LNs through small branches. Thus, it is not surprising that INP is lower in the PALN than in the SiLN.

Bouta et al. measured INP in the popliteal LN of TNF-Tg mice and their wild-type littersmates (aged 4–10 months), and determined INP in wild-type mice to be 5.01 ± 0.41 mmHg. The difference between their value and ours may reflect the different strains of mice used and/or the anatomical location of the LN measured. Nathanson et al. reported that metastasis to an axillary sentinel LN in breast cancer was associated with higher INP than that in tumor-free LNs, and that clinical suspicion of metastasis correlated with INP in predicting macrometastases. We expect that INP may be an important parameter for detecting the early stages of LN metastasis, when LN size has not changed significantly.

In conclusion, the present study describes for the first time temporal changes in the INP of LNs connected by a lymphatic network, after the initiation of metastasis. Downstream LN INP increases at stages when metastatic tumor cells have not fully proliferated. Thus, INP may be a useful parameter for the early diagnosis of LN metastasis.

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Disclosure Statement

The authors have no conflict of interest.

References

1 Huang X, Zhang F, Lee S et al. Long-term multimodal imaging of tumor draining sentinel lymph nodes using mesoporous silica-based nanoprobes. Biomaterials 2012; 33: 4370–8.

2 Mumpecht V, Honer M, Vigi B et al. In vivo imaging of inflammation- and tumor-induced lymph node lymphangiogenesis by immuno-positive emission tomography. Cancer Res 2010; 70: 8842–51.

3 Seo Y, Aparici CM, Chen CP et al. Mapping of lymphatic drainage from the prostate using filtered 99mTc-sulfur nanocolloid and SPECT/CT. J Nucl Med 2011; 52: 1068–72.

4 Li L, Mori S, Kodama M, Sakamoto M, Takahashi S, Kodama T. Enhanced sonographic imaging to diagnose lymph node metastasis: importance of blood vessel volume and density. Cancer Res 2013; 73: 2082–92.

5 Liao LJ, Lo WC, Hsu WL, Wang CT, Lai MS. Detection of cervical lymph node metastasis in head and neck cancer patients with clinically N0 neck-a meta-analysis comparing different imaging modalities. BMC Cancer 2012; 12: 236.

6 Kuhn FP, Hullem M, Mader CE et al. Contrast-enhanced PET/MR imaging versus contrast-enhanced PET/CT in head and neck cancer: how much MR information is needed? J Nucl Med 2014; 55: 551–8.
Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Primer sequences used in this study.