A Mammaglobin-A Targeting Agent for Noninvasive Detection of Breast Cancer Metastasis in Lymph Nodes

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Abstract

Pathologic axial lymph node (ALN) status is an important prognostic factor for staging breast cancer. Currently, status is determined by histopathology following surgical excision of sentinel lymph node(s), which is an invasive, time consuming, and costly procedure with potential morbidity to the patient. Here, we describe an imaging platform for noninvasive assessment of ALN status, eliminating the need for surgical examination of patients to rule out nodal involvement. A targeted imaging probe (MamAb-680) was developed by conjugation of a mammaglobin-A–specific monoclonal antibody to a near-infrared fluorescent dye. Using DNA and tissue microarray, mammaglobin-A was validated as a cell-surface target that is expressed in ALN-positive patient samples but is not expressed in normal lymph nodes. In vivo selectivity was determined by i.v. injection of MamAb-680 into mice with mammaglobin-A–positive and -negative mammary fat pad (MFP) tumors; and by peritumoral MFP injection of the targeted imaging probe in mice with spontaneous ALN metastases. Fluorescence imaging showed that probe was only retained in positive tumors and metastases. As few as 1,000 cells that endogenously express mammaglobin-A were detected in ALN, indicating high sensitivity of this method. Translation of this approach offers considerable potential as a noninvasive clinical strategy to stage breast cancer. Cancer Res; 71(3); 1050–9. ©2010 AACR.
provide anatomic maps and do not detect tumor cells present in lymph nodes.

Development of imaging agents that selectively target cancer cells with no cross-reactivity to nontumor cells is a long-term goal for cancer imaging and could allow for the noninvasive staging of breast cancer by detection of tumor cells in ALNs. However, to date, there has been no definitive study for the development of a targeted imaging method for detection of tumor cells specifically in ALNs.

Breast cells are typically only present in the ALNs when breast cancer has spread from the primary tumor. Therefore, cell-surface marker(s) that discriminate breast epithelial cells from lymphoid cells can be used to detect metastasis (26–28). A comprehensive gene expression analysis of breast epithelial cells versus lymphoid cells determined that mRNA encoding mammaglobin-A protein can be used to distinguish epithelial cells from lymphoid cells (29). The mammaglobin-A gene (SCGB2A2) encodes a 10-kDa glycoprotein and is a member of the epithelial secretoglobin family 2A (30, 31). Several studies have demonstrated that mammaglobin-A is exclusively expressed in breast tissue and can be used as a marker for the detection of micrometastasis in SLN (for review see ref. 32). Mammaglobin-A has also been investigated as a molecular marker for targeted therapy of breast cancer (33, 34).

In the present study, we applied a high-resolution in vivo fluorescence imaging technique to noninvasively detect lymphatic metastasis of human breast cancer cells in a mouse model. For this purpose, a monoclonal antibody specific for binding to mammaglobin-A was conjugated to a near-infrared (NIR) fluorescent dye, (termed MamAb-680) and delivered to the lymphatic system by peritumoral injection into the mammary fat pad (MFP) of nude mice, allowing imaging of mammaglobin-A expressing cells that have spread to the ALN. Thus, we have combined the specificity of a mammaglobin-A–specific antibody, which binds to tumor cells, with the power of in vivo fluorescence imaging to demonstrate a noninvasive targeted method for detection of metastatic cells in lymph nodes. This novel approach offers a powerful targeted tool for in vivo studies of tumor cells within the lymphatic system, detection of tumor cells in lymph nodes, and for following the efficacy of antitumor therapy.

Materials and Methods

Cell culture

Human breast cancer cells, mammaglobin-A expressing ZR-75.1 (35–37) and nonexpressing MDA-mb-231 (37) were grown in RPMI 1640 (Life Technologies) containing 10% FBS (Life Technologies), 0.03% L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin in 5% CO2 at 37°C. Both cell lines were obtained from American Type Culture Collection (ATCC; 76x110). Cells were monitored by microscopy and confirmed the development of targeted imaging method for detection of tumor cells specifically in ALNs.

Human breast cancer cells, mammaglobin-A expressing ZR-75.1 (35–37) and nonexpressing MDA-mb-231 (37) were grown in RPMI 1640 (Life Technologies) containing 10% FBS (Life Technologies), 0.03% L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin in 5% CO2 at 37°C. Both cell lines were obtained from American Type Culture Collection (ATCC; 76x110). Cells were monitored by microscopy and confirmed the development of targeted imaging method for detection of tumor cells specifically in ALNs.

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Mouse anti-mammaglobin-A mAb, 1:50, (Clone 304-1 A5, Thermo Scientific) was used for staining. The slides were scanned in the Moffitt Analytical Microscopy Core Facility (AMC) using an Aperio ScanScope XT digital slide scanner (Aperio). The digital image of each sample was evaluated by two reviewers (M.M.B. and M.C.L.). Positive staining was arbitrarily set as membranous (partial or complete) and cytoplasmic immunoreactivity in greater than or equal to 5% of tumor cells. Results were recorded as positive or negative.

Conjugation of antibody to dye

Fifteen micrograms of human mammaglobin-A–specific mouse mAb (Zeta Corp.) was incubated with 10 μg VivoTag-S 680 (VisEn Medical) and purified with a Sephadex G25 column (Roche). Protein (A280) and dye (A680) absorbance was determined using an ND-1000 spectrophotometer (Nanodrop) and used to confirm the number of fluorophore molecules conjugated to each antibody molecule.

Microscopic studies

Cells and tissues from positive and negative xenograft tumors were fixed with cold methanolacetone and incubated with 1 μg/μL MamAb-680 and 5.0 μg/mL of wheat germ agglutinin (WGA; Invitrogen) for 30 minutes. After 3 washes with PBS, coverslips were mounted using mounting medium with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories, Inc.). Micrographs were acquired at 200 Hz in the Moffitt Analytical Microscopy Core using a Leica DMI6000 inverted microscope and TCS SP5 tandem confocal scanner, through a 63X/1.40NA Plan Apochromat oil immersion objective lens with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories, Inc.) using an Aperio ScanScope XT digital slide scanner (Aperio). The digital image of each sample was evaluated by two reviewers (M.M.B. and M.C.L.). Positive staining was arbitrarily set as membranous (partial or complete) and cytoplasmic immunoreactivity in greater than or equal to 5% of tumor cells. Results were recorded as positive or negative.

Orthotopic implantation of cells into ALN

Female nu/nu mice 6 to 8 weeks old were implanted with an estrogen-release pellet (see above). Two days after implantation, luciferase expressing ZR-75.1 cells were injected into the ALN using ultrasound image guidance. Mice were anesthetized with 3% to 4% isoflurane using a nose-cone manifold and restrained on the stage of a VEVO 770 high-resolution small-animal ultrasound imaging system (VisualSonics) using tape; ultrasound gel was applied to the area over the axillary node; the 40-MHz ultrasound probe was placed in the probe guide and the node located by mechanically adjusting the probe guide to resolve the nodes; a 1-cc syringe with a 29-gauge needle was loaded with 100 to 100,000 cells in a 20-μL volume of 1:1 Matrigel and sterile PBS and positioned in the needle guide so that the end of the needle could be moved into the node and cells injected. Ultrasound images were acquired at the time of each injection. Four hours after injection of cells, animals were anesthetized and 300 μL of 15 mg/mL β-luciferin potassium salt (GoldBio) was introduced via intraperitoneal (i.p.) injection. Five minutes after the injection, a bioluminescence image was acquired using standard bioluminescence settings on the IVIS-200. Twenty-four hours after injection of cells, MamAb-680 was injected into the MFP proximal to axillary nodes, and fluorescence imaging was performed using the IVIS-200 as described above for pharmacodynamic and biodistribution studies.

Statistics

Data are represented as mean ± SD and the t test was used to determine significance.

Results

Mammaglobin-A expression in patient tissue samples

Several studies have demonstrated high expression of mammaglobin-A in breast cancer (for review see ref. 32).
For further confirmation and to characterize mRNA expression in patient tissue samples, including lymph node metastases and normal tissues, we analyzed publicly available DNA microarray data sets. Mammaglobin-A mRNA was highly and generally expressed in breast tumors, breast cancer lymph node metastases, and in normal breast (Fig. 1). A high percentage (83%) of lymph node metastases expressed mammaglobin-A. In contrast, mammaglobin-A was not expressed in normal lymph nodes. Also, other organs involved in toxicity or drug clearance, that is, liver, kidney, heart, lung, and spleen did not express mammaglobin-A mRNA.

To determine mammaglobin-A protein expression in patient samples, immunohistochemistry (IHC) was performed on a breast cancer tissue microarray containing 250 samples. Positive staining was observed in the ductal epithelium of 63% of normal breast tissues (Supplementary Fig. S1A and B), 80% DCIS, 53% invasive ductal carcinoma without metastasis, 43% invasive ductal carcinoma with metastasis, and 45% lymph node with macrometastasis of breast cancer (Supplementary Fig. S1C and D).

**ZR-75.1 breast cancer cells express mammaglobin-A**

ZR-75.1 breast cancer cells endogenously express mammaglobin-A (35–37) and MDA-mb-231 cells do not (37). To confirm this, mammaglobin-A mRNA expression was quantified by qRT-PCR in ZR-75.1 cells, but MDA-mb-231 cells did not express. Western blot and immunocytochemistry (ICC) also confirmed protein expression (Supplementary Fig. S2A and B).

**Antibody and MamAb-680 characterization**

Three different mammaglobin-A monoclonal antibodies were evaluated for sensitivity and specificity by Western blot and ICC (Supplementary Fig. S2C and D). From these studies, a specific mAb (Zeta Corp.) was selected for conjugation to NIR dye (VivoTag-S 680). To evaluate the antibody–dye conjugation and to verify that MamAb-680 retained binding specificity, ICC using the dye-labeled primary antibody was performed on the endogenously expressing ZR-75.1 cells, MDA-mb-231 cells engineered to express mammaglobin-A (Supplementary Fig. S2E) and the nonexpressing MDA-mb-231 cells. MamAb-680 bound only to expressing cells (Fig. 2A). Hence, the conjugated agent retained specificity for mammaglobin-A protein.

**Mammaglobin-A is expressed on the cell surface**

Zuo and colleagues recently reported that mammaglobin-A is directly associated with the surface of breast cancer cells (34). As described above, permeabilized fixed cells were used for ICC. To verify cell-surface expression, ZR-75.1 cells were incubated with MamAb-680 at 4 °C, and agent was observed at the cell-surface colocalized with agglutinin dye (Fig. 2B). Western blots of membrane protein extracts of mammaglobin-A-positive cells stained positive for mammaglobin-A protein (Supplementary Fig. S2F).

**MamAb-680 selectively accumulates in positive tumors**

To determine the specificity of MamAb-680 targeting in vivo, ZR-75.1 and MDA-mb-231 cells were implanted in the right and left MFP of female nude mice. After tumor growth to approximately 500 to 800 mm³ in volume, MamAb-680 was i.v. injected. As shown in Figure 3A, ZR-75.1 tumors retained higher levels of the agent signal compared with MDA-mb-231. Signal in the positive tumor was quantified as an 8.6 ± 0.8 SD (n = 4, P < 0.001) fold enhancement relative to the negative tumor, 24 hours postinjection. As a second test of agent specificity, a blocking experiment was performed where ZR-75.1 tumor-bearing mice were preinjected with an excess (250 μg) of unlabeled mammaglobin-A antibody, followed by injection of 50 μg of MamAb-680. The unlabeled mammaglobin antibody effectively reduced the fluorescence signal 6.6-fold in the tumor relative to the previous experiment without addition of blocking antibody (Fig. 3B). These results demonstrate the in vivo targeting specificity of MamAb-680.

To confirm the presence of mammaglobin-A protein in vivo, sections from flash-frozen tumors were stained with MamAb-680, a nuclear stain, (DAPI), and a cell-surface/cytoplasmic stain, WGA, and analyzed using confocal microscopy (Fig. 2C). Mammaglobin-A staining was only observed in tumors from the positively expressing cell line but not in the negative line. Ex vivo images of the corresponding center sections of the tumors confirmed MamAb-680 localization to the positive tumor relative to the negative tumor (Fig 2D).

**Pharmacodynamics and biodistribution of MamAb-680**

To assess the pharmacodynamics of tumor uptake and clearance, MamAb-680 was i.v. injected and images acquired at intervals from 5 minutes to 12 days postinjection. Fluorescence signal increased for 24 hours and then slowly decreased at later time points (Fig. 4A). Elevation of

![Figure 1. DNA microarray expression profile of mammaglobin-A in breast cancer and normal tissues. Data are represented as mean ± SD. Note the log₁₀ scale.](image-url)
fluorescence in the positive tumor relative to the negative tumor was detected from 4 hours to 10 days after injection.

For biodistribution studies, mammaglobin-positive and -negative tumor-bearing mice were i.v. injected with MamAb-680 and 24 hours later, tumors and organs were removed and imaged _ex vivo_. Figure 4B demonstrates that fluorescence was present in the positive tumor but has largely cleared from the negative tumor and other organs.

**MamAb-680 detects tumor cells in ALNs**

Previously, it was not reported that ZR-75.1 cells form ALN metastases. To determine this, $5 \times 10^6$ ZR-75.1/Luc cells were implanted into the right MFP of estrogen-pelleted mice. Three weeks after implantation, tumors were surgically removed. Two weeks later, the tumor had regrown and bioluminescence imaging showed metastasis to the ALN (Fig. 3C). Hence, ZR-75.1 cells are a suitable model for ER$^+$ breast cancer lymph node metastasis.

To investigate whether MamAb-680 can be delivered through the lymphatics and are selectively retained in ALN bearing mammaglobin-A expressing metastases, agent was injected peritumorally into the MFP. At 24 hours postinjection, a strong fluorescence signal was obtained from the area of the ALN (Fig. 3D), which colocalized with the bioluminescence image of the luciferase expressing metastases (3C). After imaging, the metastases were removed and determined to be composed of cancer cells by hematoxylin and eosin (H&E) staining and pathologic examination (3E).

Agent selectivity for positive lymph nodes was also determined using an orthotopic model of lymph node metastasis. ZR-75.1/Luc cells were directly injected into the ALN using ultrasound image guidance (Fig. 5A) and were detected by bioluminescence (Fig. 5B). MamAb-680 was delivered by MFP injection and was observed to have traversed to the lymph node within 4 hours (Fig. 5C). Fluorescence signal was retained in lymph nodes implanted with mammaglobin-A–positive cells (Fig. 5D) long after clearance from the MFP and negative lymph nodes. Positive lymph nodes were resolved as early as 4 hours and were detected up to at least 7 days postinjection. Our results show that _in vivo_ lymphatic imaging using MamAb-680 provided a specific and durable signal in mammaglobin-A expressing lymph node metastases.

One million MDA-mb-231 cells were injected into the ALN as a mammaglobin-A–negative control using the same method and amounts described above, then MamAb-680 was injected into the MFP and imaged (Fig. 5E). A corresponding postinjection bioluminescence image confirmed the presence of cells in the ALN (Supplementary Fig. S3A). Minimal signal was detected in the draining lymphatics at 4 hours postinjection and no signal was observed at 24 hours (Fig. 5F). At 48 hours, agent was cleared from the animal. Hence, the fluorescence signal is specific for mammaglobin-A–expressing cells in the ALN. As an additional control, ALN were sham injected with PBS and Matrigel (no cells). In this case, the same result was observed as seen for the mammaglobin-A–negative cells above (Supplementary Fig. S3D and E).
To determine the sensitivity of the agent, a range in number of ZR-75.1/Luc (1,000 to 1 million) were injected into ALN via ultrasound guidance. For confirmation of successful cell implantation, bioluminescence images were acquired and as few as 1,000 cells were detected (Fig. 6A). Four hours after cell injection, MamAb-680 was injected into MFP proximal to the ALN and fluorescence images were acquired 24 hours after injection (Fig. 6B). Bioluminescent and fluorescent signals were quantified by drawing a ROI encompassing the tumor cells in the ALN. Signal intensity decreased with cell number and at least 1,000 cells were detectable above background (Fig. 6B and Supplementary Fig. S4A and B).

**Discussion**

To the best of our knowledge, this is the first report of the development of a targeted method for noninvasive detection of breast cancer cells in lymph nodes. Using a novel molecular imaging agent (MamAb-680) and a mouse model, we have demonstrated the noninvasive detection of breast cancer lymph node metastases bearing a specific marker, mammaglobin-A. The agent was generated by conjugating a NIR fluorescent dye to a monoclonal antibody that is determined to be highly specific for binding to mammaglobin-A.

Because breast cells are found in ALNs primarily in the case of regional metastasis, markers generally specific for breast cells can be used for detection of nodal disease. Here, we have used mammaglobin-A that is expressed in a percentage of all types of breast cancer, including lymph node positive samples, and its expression in normal tissues is restricted to the intraductal epithelia of the mammary gland. Mammaglobin-A mRNA was not detected in any other tissue including normal lymph nodes. Gene expression profiling of breast epithelial cells versus lymphoid cells has shown that mammaglobin-A is a robust mRNA for distinguishing epithelial cells from lymphoid cells (29), which is in agreement with the results reported herein.

IHC staining for mammaglobin-A in a breast tissue microarray revealed that 45.2% of lymph nodes with macro-metastases were positive for mammaglobin-A. This was lower than frequencies (48%–84%) reported previously by several groups using different antibodies (30, 40–43). Although
mammaglobin-A is a member of the secretoglobin family and is generally considered to be secreted, our results showed that mammaglobin protein is associated with the surface of breast cancer cells, which is in agreement with Zuo and colleagues (34). Hence, the membrane-associated mammaglobin-A can be utilized as a molecular marker for targeting imaging agents to breast cancer metastases.

We have reported for the first time that ZR-75.1 breast cancer cells form spontaneous metastases to ALNs in nude mice. MDA-mb-231, an invasive breast cancer cell line, is widely used in breast cancer lymph node metastasis studies, and these cells require at least 6 to 8 weeks after implantation to form detectable metastases (44). However, ZR-75.1 cells were detected in the ALN as early as 5 weeks after xenografting the cells into the MFP. Hence, ZR-75.1 cells are a robust model for breast cancer lymph node metastasis studies.

In vivo fluorescence imaging has emerged as an excellent method for the noninvasive imaging of tissues with high spatial resolution. Optical imaging allows for the real-time visualization of specific molecular markers, in disease, prognosis, staging, and therapy monitoring (45). Our in vitro and in vivo studies demonstrated a superb high selectivity of the agent, where 8.6 ± 0.8-fold enhancement of positive tumor was observed relative to the negative tumor. Although mammaglobin-A is expressed in normal breast, its distribution is limited to the epithelia, which is not accessible to the agent, which is injected into the parenchyma.

To determine the sensitivity of our method, we developed a novel, orthotopic xenograft model for lymph node metastasis, where precise numbers of cells were injected into the ALNs using ultrasound image guidance. As few as 1,000 cells were quantitatively detected in the ALN. Hence, our method may overcome the problem of interobserver variation in classification of metastatic disease as macrometastasis (>2 mm), micrometastasis (between 0.2 and 2 mm), or individual tumor cells or groups of cells (ITC, <0.2 mm; refs. 46, 47). One thousand cells represent a volume of approximately 1 nL, which is well below the size of a micrometastasis. In addition, detection of ITCs in prenodal lymph before colonization in the lymph node was shown to be possible.

In the future, the level of detection may be improved by using a fluorescent dye with longer excitation and emission wavelengths, for example, 800 to 900 nm; or through the use of another imaging modality, such as detection of positron or single photon emission, MRI, nanoparticle, or NIR dye-based PAT; or by using a more sophisticated fluorescence imaging system, for example, with spectral unmixing or fluorescence lifetime capability, compared with the surface radiance (planar) imaging system used in this study. In addition, our fluorescent targeted agents could be used intraoperatively for image-guided surgery to remove involved nodes and leave uninvolved nodes (48). To add therapeutic capability, targeted gold nanoparticles could be used for both noninvasive imaging via PAT as well as delivering therapy through
photothermal ablation (49). The targeted nanoparticles could thus be heated using laser light to destroy cells and tissue adhered to the agent (i.e., "molecular surgery.") Regional control in the lymph nodes after targeting metastatic cancer could eliminate the need for surgery to remove the diseased nodes and therefore reduce significant complications (e.g., lymphedema). Furthermore, cancer imaging of metastatic lymph nodes could provide response assessment to traditional therapeutic options such as cytotoxic or molecular targeted chemotherapy, and axillary radiotherapy.

According to the pharmacokinetics study, the optimal time for imaging was 24 hours after administration of the agent, at which time the agent had reached peak levels in the involved node and was cleared from surrounding tissues. However, positive lymph nodes were detected as early as 4 hours. Because this antibody-based agent is relatively large compared with synthetic peptide-based ligands, the time required for SLN uptake and clearance could be improved by the development of a smaller mammaglobin-A–specific ligands or peptibodies.

According to our IHC data, mammaglobin is expressed in 45.2% of lymph node positive samples. Therefore, agents specific for additional markers expressed in lymph node metastases will be required, in combination with the mammaglobin-A targeting probe, to detect all breast cancer lymph node metastases. For example, it has been reported that the combination of mammaglobin-A and cytokeratin-19 (CK19) is an optimal molecular marker set for the detection of clinically
actionable metastases in breast lymph nodes with 90% sensitivity and 94% specificity (29). Unfortunately, CK19 is not a cell-surface marker. Through gene expression profiling and IHC validation of protein expression in patient tissue samples, we are in the process of identifying additional cell-surface markers that in combination with mammaglobin-A will detect all breast cancer lymph node metastases.

In summary, our results demonstrate that the breast cancer targeted agent, MamAb-680 can be used for the noninvasive, in vivo detection of cancerous cells in mouse ALNs with high resolution and specificity. This targeted imaging strategy has potential for future translation into the clinic for SLN assessment and intraoperative surgical guidance. In the future, this agent may be improved by development of small peptide-based ligands and through the possible attachment to nanoparticles for delivery of imaging contrast and therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Figure 6. Sensitivity of detection of cells injected into the ALN. A, bioluminescence activity quantified for a range of injected cell numbers. Inset shows signal for a mouse injected with 1,000 cells. B, agent associated fluorescence for a range of cell numbers, 24 hours postinjection with MamAb-680. Inset shows fluorescence for a mouse injected with 1,000 cells. All data represent mean ± SD of pixel values within the ROIs.
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