Intraoperative Imaging of Metastatic Lymph Nodes Using a Fluorophore-conjugated Antibody in a HER2/neu-expressing Orthotopic Breast Cancer Mouse Model

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Abstract. We investigated in this study whether fluorescence imaging with a fluorophore-conjugated anti-human epidermal growth factor receptor 2 (HER2)/neu probe could be used to differentiate metastatic lymph nodes (LN) from normal LN to guide surgical resection. A fluorescent probe for detecting HER2/neu-expressing cells was generated by conjugation of the humanized anti-HER2/neu antibody trastuzumab with rhodamine. The green fluorescent protein-expressing breast cancer cell line 4T1-GFP was used for in vitro binding analysis and for the establishment of a model of HER2/neu expressing LN-metastatic breast cancer. All tumor-bearing mice were given a single intravenous injection of either rhodamine-conjugated anti-HER2/neu probe, or conjugated control IgG antibody, when LN metastasis developed. Each animal was imaged with both green and red fluorescence to assess in vivo binding of the rhodamine-conjugated anti-HER2/neu probe to the tumors and LN metastases. Hematoxylin and eosin (H&E) staining and immunohistochemistry was performed to confirm the presence of tumor and metastasis as well as HER2/neu expression. The imaging probe was able to bind to HER2/neu-expressing 4T1-GFP tumor cells in vitro. The primary tumors and LN metastases from the animals which were treated with rhodamine-conjugated anti-HER2/neu probe exhibited a visible red fluorescence signal. The fluorescent axillary LN metastasis was easily distinguishable from the surrounding normal tissue and normal LN. Sensitivity of 78% (14 out of 18) and specificity of 100% (20 out of 20) could be achieved with the rhodamine-conjugated anti-HER2/neu probe for the detection of LN metastasis. These data support further investigation of the fluorophore-conjugated anti-HER2/neu antibody to detect LN metastasis in the surgical setting.

The metastatic status of the sentinel lymph node (SLN) in patients with breast cancer is a predictor of recurrence and survival and is important for treatment strategy (1, 2). For some patients inadequate intraoperative identification of lymph node (LN) metastasis can lead to incomplete LN resection at the time of surgery. The current method for localizing SLN consists of an injection of a blue dye or a radio-labeled colloid (3). However, both procedures present some disadvantages, such as the lack of tumor specificity, the use of radioactivity, and risk of allergic reactions (4). Some studies have investigated the use of near-infrared (NIR)-emitting cadmium-based quantum dots (QDs) for fluorescence-guided SLN mapping in breast cancer (5, 6). However, the toxicity of the components of NIR-emitting QDs that have been observed thus far has remained a major obstacle to their clinical use (7, 8).

Tumor-associated antigens have been utilized in clinical tests as an aid for cancer diagnosis (9). Conjugating a monoclonal antibody targeted to a tumor-associated antigen with a fluorophore is an alternative to current non-specific methods of SLN detection (10). Some studies have demonstrated fluorophore-conjugated antibodies to be effective and specific probes for guiding excision of primary tumor, a procedure termed fluorescence-guided surgery (FGS) (11, 12). Fluorescence imaging with a conjugated antibody allows a simple optical readout, high resolution and specificity, as well as a wider dynamic range than dye absorption or scintigraphy (13).

In the present study, we investigated a fluorophore-conjugated antibody to human epidermal growth factor receptor 2 (HER2)/neu (trastuzumab) as a probe for...
intraoperative detection of LN metastasis in an orthotopic mouse model of breast cancer (14). HER2/neu is a tyrosine kinase receptor that is overexpressed in 20%-25% of invasive human breast carcinomas and which is associated with increased biological aggressiveness and a poor clinical prognosis (15, 16). Trastuzumab is a humanized monoclonal antibody targeting the HER2/neu extracellular domain and has been approved for treatment of patients with HER2/neu-overexpressing breast cancer (17).

Materials and Methods

Cell culture. The green fluorescent protein (GFP)-transfected breast cancer cell line 4T1-GFP was obtained from AntiCancer, Inc. (San Diego, CA, USA). The cells were cultured in RPMI-1640 (GIBCO/BRL, Life Technologies, Grand Island, NY, USA) supplemented with 1% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT, USA) at 37°C in 5% CO2 saturated humidity. The medium was supplemented with penicillin/streptomycin (GIBCO/BRL).

Animal care. Forty BALB/C nu/nu nude female mice, aged 4-6 weeks and weighing 20-25 g, were purchased from the Beijing Kelihua Laboratory Animal Center (Beijing, P.R. China). The mice were maintained in a high efficiency particulate air (HEPA)-filtered environment at 24-25°C and 50-60% humidity. The animals were fed autoclaved laboratory rodent diet. Animal experiments were approved by the Animal Welfare Committee of Nanjing Origin Biosciences (Nanjing, P.R. China).

Surgical orthotopic implantation. Tumor stocks were made by subcutaneously injecting 4T1-GFP cells at a density of 5×10⁶ cells/100 μl into the flank of nude mice. The tumor tissues harvested from s.c. growth in nude mice were inspected and any grossly necrotic or suspected necrotic or non-GFP tumor tissues were removed. Tumor tissues were subsequently cut into small fragments of approximately 1 mm³. For surgical orthotopic implantation (SOI), the animals were anesthetized by injection of 0.03 ml of solution of 50% ketamine, 38% xylazine, and 12% acepromazine maleate, and the surgical area was sterilized using iodine and alcohol. The right second mammary gland was chosen for orthotopic implantation. A small incision was made along the medial side of the nipple. The mammary fat pad was exposed through blunt dissection. A small cut was made in the fat pad which was then bluntly expanded to form a small pocket. Two pieces of tumor tissue, previously prepared as described above, were sutured into the pocket using an 8-0 nylon suture. The skin was closed with a 6-0 silk suture (14, 18). All surgical procedures and animal manipulations were conducted under HEPA-filtered laminar-flow hoods with a ×8 surgical microscope model MZ650 (Nanjing Optic Instrument Inc., Nanjing, P.R. China) equipped with D510 long-pass and HQ600/50 band-pass emission filters (Chroma Technology, Surrey, BC, Canada) was used for imaging. Selective excitation of GFP was produced through an illuminator equipped with HQ470/40 and HQ540/40 excitation band-pass filters (Chroma Technology). Images were processed and analyzed with the use of Image Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Preparation of fluorescent probe. A fluorescent probe for detecting HER2-expressing cells was generated by conjugation of trastuzumab (Herceptin; Genentech, San Francisco, CA, USA) with rhodamine using the EasyLink Rhodamine Conjugation Kit (Abcam, Cambridge, MA, USA). Rhodamine is a red fluorescent dye that has an excitation wavelength of 570 nm and an emission wavelength of 590 nm. For rhodamine conjugation, the trastuzumab antibody and control IgG antibody were reconstituted in amine-free phosphate-buffered saline (PBS), pH 7.4. Five hundred milliliters of the 2 mg/mL antibody were added to the reactive dye for each conjugation. Before adding the antibody to the EasyLink mix, 50 μl of EL-Modifier reagent was added to the antibody. The antibody–dye mixtures were incubated in the dark at room temperature (20-25°C) for 3 h. After incubation for 3 h, 50 μl of EL-Quencher FD reagent was added and mixed gently. Antibody and dye concentrations in the final sample were determined using spectrophotometric absorbance. For each conjugation, the molar ratio of fluorophore to antibody was 4-5 to 1.

In vitro fluorescence imaging of probe binding to HER2/neu expressing cells. HER2/neu expressing 4T1-GFP cells at 30% confluence were incubated with rhodamine-conjugated anti-HER2/neu probe or control-conjugated IgG antibody at final concentrations ranging from 12.5 to 50 μg/ml and incubated at 37°C in 5% CO2 for 24 h. After incubation, the cells were washed three times with PBS (GIBCO/BRL) and then fixed in 4% formaldehyde for 20 minutes. The cells were analyzed using a confocal microscope equipped with fluorescence filters (Olympus, Tokyo, Japan). Confocal microscopy was performed at ×20 and ×40 original magnification. All images were recorded using identical settings.

Histology and immunohistochemistry (IHC). Tumors and LN were surgically removed following in vivo imaging. The tissue samples were fixed in 10% formalin and stained with H&E for standard light microscopy to examine tumor and metastasis histology. For HER2/neu expression in tumors and metastases, formalin-fixed paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated in graded ethanol solutions, and rehydrated in phosphate-buffered saline (PBS, pH 7.2). To enhance antigen retrieval, sections were microwaved for 5 min. Each slice was treated with two drops of 3% H₂O₂-...
methanol solution for 15 min at 37˚C and washed with PBS. Tissue sections were incubated overnight at 4˚C with diluted primary antibody to HER2/neu (1:500; Abcam). After washing in PBS, the slices were incubated with horseradish peroxidase-labeled secondary antibody (1:200; Maixin Bio-Tech Co., Ltd, Fuzhou, China) for 30 min at room temperature. After color development using diaminobenzidine (Maixin Bio-Tech Co., Ltd), the tumor sections were counterstained in hematoxylin and mounted in a neutral resin medium.

Results

Model of HER2/neu-expressing LN-metastatic breast cancer. The HER2/neu expressing LN metastatic breast cancer model was established by orthotopically implanting 4T1-GFP into nude mice. The breast primary tumor and LN metastasis were easily visualized by fluorescence imaging. It took 32 days for the 4T1 breast primary tumor to develop visible LN metastases observed by fluorescence imaging with a skip flap. Eighteen out of 40 (45%) tumor-bearing mice were found to develop axillary LN metastases on the same side of the breast primary tumor. At autopsy, primary and metastatic tumors were harvested and stained with antibody to HER2/neu to verify HER2/neu expression in vivo. All primary tumors and axillary LN metastases displayed strong HER2/neu expression (Figure 1). All LN metastasis detected by GFP imaging were confirmed by H&E histology, indicating complete correlation between fluorescence imaging and histology.

In vitro probe binding to HER2/neu-expressing breast cancer cells. In vitro probe binding to HER2/neu expressing cells was assessed by incubating 4T1-GFP breast cancer cells with the rhodamine-conjugated anti-HER2/neu probe or control IgG antibody. Confocal fluorescence microscope was used to visualize probe binding to cancer cells. As shown in Figure 2, the rhodamine-conjugated anti-HER2/neu probe was observed to bind to 4T1-GFP cancer cells. A positive binding signal was indicated by strong red fluorescence as compared to the lack of signal of the conjugated IgG antibody (Figure 2B). No significant difference in fluorescence intensity on 4T1-GFP cells was found for the probe concentrations tested (data not shown).

In vivo imaging of primary breast tumors and LN metastases. The mice bearing orthotopic breast tumors and LN metastases were given a single dose of rhodamine-conjugated anti-HER2/neu probe or IgG antibody by tail vein injection. Twenty-four hours after the injection, the primary tumor and local LNs were exposed and imaged under both green and red fluorescence illumination. Under white light, LN metastasis could not be differentiated from normal LN. However, under green fluorescence imaging, not only was the primary tumor easily visible, but very small axillary LN metastases were clearly detected. The primary tumors and LN metastases from the animals which had received rhodamine-conjugated anti-HER2/neu probe exhibited a visible red fluorescence signal above the background autofluorescence when animals were illuminated under red fluorescence (Figure 3). The fluorescent axillary LN metastases were easily distinguishable from the surrounding normal tissue and normal LN. The tumor-bearing mice which received rhodamine-conjugated control IgG did not display any red fluorescence signal. All LN metastasis, both HER2/neu+ and HER2/neu− were confirmed by H&E staining (Figure 3). This result indicates that the rhodamine-conjugated anti-HER2/neu probe specifically binds to HER2/neu-expressing tumors and metastases in vivo.

Table 1 shows the sensitivity and the specificity of rhodamine-conjugated anti-HER2/neu probe for the detection of LN metastases in the HER2/neu-expressing LN-metastatic model of breast cancer. A total of 14 out of 18 tumor-bearing mice with LN metastases detected by green fluorescence displayed positive LN metastases with the rhodamine-conjugated anti-HER2/neu probe, indicating a sensitivity of 78%. All 22 tumor-bearing mice with normal LN were found to be negative with the rhodamine-conjugated anti-HER2/neu probe, demonstrating a specificity of 100%.

Discussion

In this study, we investigated whether fluorescence imaging could be used to accurately differentiate LN metastases from normal LNs in an orthotopic model of breast cancer using a fluorophore-conjugated, HER2/neu-targeted antibody. The
4T1-GFP orthotopic breast cancer mouse model has a high LN metastasis rate. Real-time imaging of tumor and metastasis progression, as well as good correlation with histological assessment of malignancy, was possible with this model (21-22).

The imaging probe used in our study consisted of trastuzumab conjugated with the fluorescent dye rhodamine which was specific for the HER2/neu receptor. The imaging probe demonstrated good binding to HER2/neu-overexpressing tumor cells in vitro. In vivo probe-binding experiments demonstrated that the binding of the rhodamine-conjugated anti-HER2/neu probe was specific for LN metastasis, since no fluorescence was visible in normal LNs. The distinction between LN metastasis and normal LNs during surgery of mammary carcinomas is clinically important both for successful surgery and as an indication of the prognosis of the patient after surgery. This observation could be advantageous in clinical settings since one of the pitfalls of blue dye for detection of the SLN consists of the false positives related to the fast migration of the dye through the lymphatic chain (3, 23).

Our study demonstrated that the rhodamine-conjugated anti-HER2/neu probe has a sensitivity of 78% and a specificity of 100% for the detection of HER2/neu expressing LN metastases compared to histological detection of LN metastases, the standard in clinical diagnosis. Therefore, this fluorescence-labeled probe has exceptional potential as an aid for intraoperative detection of LN metastases for HER2/neu-expressing breast cancer because the probe is highly specific, fluorescent, non-radioactive, and easily visible, potentially enabling FGS (24).

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Figure 2. In vitro probe binding to human epidermal growth factor receptor 2 (HER2)/neu-expressing breast cancer cells was assessed by incubating 4T1-GFP cancer cells with the red fluorescent rhodamine-conjugated anti-HER2/neu probe or control-conjugated IgG antibody as described in the Materials and Methods. A: 4T1-GFP cancer cells illuminated by green fluorescence imaging. B: Binding of rhodamine-conjugated antibody to HER2/neu to 4T1-GFP cancer cells illuminated by red fluorescence imaging. C: Merged image of A and B.

Figure 3. In vivo imaging of primary breast tumor and lymph node (LN) metastasis. In vivo imaging was performed 24 hours after intravenous delivery of the rhodamine-conjugated anti-HER2/neu probe as described in the Materials and Methods. A representative mouse shown with orthotopic 4T1-GFP breast tumor and LN metastasis was illuminated with green (A) and red fluorescence (B), and histology was confirmed by H&E staining of the LN (C) (x400 magnification). A representative mouse shown with orthotopic breast tumor and normal LN was illuminated with green (D) and red fluorescence (E), and histology was confirmed by H&E staining of the LN (F) (x400 magnification).
Conflicts of Interest

None of the Authors have a conflict of interest in regard to this study.

References


