Ultrasound-guided photoacoustic imaging for the selective detection of EGFR-expressing breast cancer and lymph node metastases

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Abstract: We assessed the use of ultrasound (US)-guided photoacoustic imaging (PAI) and anti-EGFR antibody-conjugated gold nanorods (anti-EGFR-GNs) to non-invasively detect EGFR-expressing primary tumor masses and regional lymph node (LN) metastases in breast tumor mice generated by injecting MCF-7 (EGFR-negative) or MDA-MB-231 (EGFR-positive) human breast cells using a preclinical Vevo 2100 LAZR Imaging system. Anti-EGFR-GNs provided a significant enhancement in the PA signal in MDA-MB-231 tumor and the axillary LN metastases relative to MCF-7 tumor and non-LN metastases. We demonstrated that US-guided PAI using anti-EGFR-GNs is highly sensitive for the selective visualization of EGFR-expressing breast primary tumors as well as LN micrometastases.

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1. Introduction

Breast cancer is a heterogeneous disease comprising several biologically distinct subtypes, and the majority of breast cancer-related deaths are mainly due to incurable metastatic diseases [1]. Triple negative breast cancer (TNBC), lacking human epidermal growth factor receptor 2 (HER2), and estrogen receptor (ER) and progesterone receptor (PR) expression, are associated with an aggressive natural history compared with other disease subtypes. Targeting the epidermal growth factor receptor (EGFR) has been proposed as a promising imaging biomarker for selective detection and treatment of EGFR-positive TNBC including metastases, but it is still in the early stages [2–4].

Photoacoustic imaging (PAI), which uses the phenomena of optical absorption, rapid thermoelastic expansion, and acoustic pressure wave generation, has been introduced as a modality that can break through the fundamental limitation of the existing pure optical imaging techniques and can be performed using various endogenous and exogenous contrast agents [5]. PAI systems have been commercialized to meet the growing demand for studying models of human disease in small animals due to the availability of relatively inexpensive and convenient tools for clinical application. Recently, ultrasound (US)-guided functional PAI using exogenous contrast agents was introduced as a promising approach for the more sensitive and accurate detection of tumor and lymph node (LN) metastases in vivo [6–8]. Gold nanoparticles with strong optical absorption in the near-infrared spectral range were developed as PA contrast agents to obtain target-specific information on molecular or cellular processes with high sensitivity and specificity, offering considerable advances in preclinical
research and clinical applications, especially in oncology [5, 7]. Gold nanoparticles have been shown to be non-toxic to human cells but are still undergoing initial investigation in clinical cancer trials [9–11]. In recent years, gold nanorods (GNs) with distinctive rod shape-dependent optical properties have been proposed as attractive antibody-conjugated PA contrast agents to enable the selective detection of primary tumors and metastases \textit{in vivo} [11–13], yet the study of \textit{in vivo} US-guided PAI using molecular targeted GNs to more sensitively and specifically target and identify primary tumor as well as a small number of metastatic cancer cells in regional LN in breast cancer models is still its infancy.

The aim of this study is to assess the use of US-guided PAI and anti-EGFR antibody-conjugated GNs (anti-EGFR-GNs) to detect the EGFR-expressing primary tumor mass and regional LN metastases in a mouse model of human TNBC. We show that EGFR-positive primary breast tumor and early LN micrometastases can be selectively and sensitively visualized by contrast-enhanced PAI using anti-EGFR-GNs.

2. Methods

2.1 Preparation of gold nanorods (GNs) and anti-EGFR antibody-conjugated GNs (anti-EGFR-GNs)

GNs and anti-EGFR-GNs were purchased from Nanopartz Inc. (Loveland, CO, USA). In brief, highly stable 10 x 41 nm cetyltrimethylammonium bromide (CTAB)–coated GNs with longitudinal plasmon resonance at 808 nm were synthesized using a proprietary seed growth method. An SH-terminated branched amine polymer manufactured by Nanopartz was used to replace the CTAB. BS3 chemistry was used to attach the amine polymer to the primary amine of the anti-EGFR antibodies. It was calculated that there are over 100 amines/nm$^2$ which results in a loading of 16 antibodies per GN. Centrifugation and dialysis against sterile phosphate buffered saline were used in all steps of binding and purification. Loading of antibodies was determined using a proprietary method incorporating HPLC and UVVIS. Further sterilization and endotoxin testing was performed.

2.2 Cell culture and reagents

The following human breast cancer cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea): MCF-7, BT-474, HCC-1954, HCC-1937, MDA-MB-453 and MDA-MB-231 cells. The HCC-1954, HCC-1937, MDA-MB-453 and MDA-MB-231 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (WelGENE, Daegu, Korea) containing 10% FBS and supplemented with a 1% antibiotic solution containing penicillin and streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). The BT-474 and MCF-7 cells were grown in DMEM (WelGENE, Daegu, Korea) containing 10% FBS and supplemented with a 1% antibiotic solution containing penicillin and streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). The medium was replenished every 3-4 days. For bioluminescence imaging MDA-MB-231-Luc cells stably expressing firefly luciferase were established using lentivirus. All cells were passaged weekly and cultured in a 5% CO$_2$ incubator at 37°C.

2.3 Animals and the xenograft tumor model

All animal experiments were approved by the Seoul National University Hospital Biomedical Research Institute Animal Care and Use Committee (IACUC). A total of 32 female Balb/c nude mice (5–6 weeks old) were used for PA and US imaging and histological studies. Indocyanine green (ICG) (Sigma-Aldrich, St Louis, MI, USA), non-targeted GN, and anti-EGFR-GNs (10 nm × 40 nm, Nanopartz Inc., Lovel, CO, USA) were dissolved in physiological buffered solution. Six healthy mice were randomly assigned to one of 2 experimental groups, those that received an ICG injection (n = 3) and those that received a GN injection (n = 3). To identify and image the axillary LN of mice, 30μl of ICG (1 mM,
final 5pmol/g mouse) or GN (1 nM, final 0.005 pmol/g mouse) was injected into the right forepaw pad of each mouse, a 3-4 mm thick section of chicken breast tissue was overlaid on the axillary LN, and the mouse was imaged using PA and US imaging.

To develop the tumor model, $1 \times 10^6$ viable MDA-MB-231, MDA-MB-231-Luc and MCF-7 cells were suspended in 0.1 ml of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and injected into the right fat pad of the first mammary gland of female Balb/c nude mice. Tumor formation was monitored for 4-8 weeks after implantation. In order to monitor primary tumor growth and metastases non-invasively, the luciferase bioluminescence images were obtained and quantified using an in vivo bioluminescence imaging system. Tumor volume was measured with calipers and by US imaging using a modified ellipsoidal formula for volume (volume = $1/2[length \times width^2]$) [14]. When the average tumor volume of 20 tumor-bearing mice injected with either MDA-MB-231 cells (MDA-MB-231 tumor) or MCF-7 cells (MCF-7 tumor) reached 150-200 mm³, the mice were randomly assigned to one of 4 experimental groups and were injected intravenously via a tail vein with either 100μl of GN(100 nM, final 0.5 pmol/g mouse) or anti-EGFR-GNs (100 nM, final 0.5 pmol/g mouse): group 1, MDA-MB-231 tumor-GN(n = 5); group 2, MCF-7 tumor-GN(n = 5); group 3, MDA-MB-231 tumor-anti-EGFR-GN(n = 5); and group 4, MCF-7 tumor-anti-EGFR-GN(n = 5). The mice were then imaged using non-invasive PA and US imaging.

To identify and image the axillary LN metastases from MDA-MB-231/Luc-tumor-bearing mice (n = 6) at 8 weeks, 100μl of anti-EGFR-GNs (100 nM, final 0.5 pmol/g mouse) was injected into the primary tumor, and 150 μg/g of D-luciferin (Promega, San Luis Obispo, CA, USA) was injected into the peritoneum, followed by bioluminescence, PA and US imaging.

2.4 Western blot analysis

The cells were lysed in RIPA buffer (Sigma, St. Louis, MO, USA). The proteins were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to nitrocellulose membranes. The membranes were blocked using 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 and were incubated overnight at 4°C with primary antibodies directed against EGFR (Santa Cruz Biotechnology, Dallas, Texas, USA) and β-actin (Sigma, St. Louis, MO USA) and then were incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, Texas, USA). The blotted membranes were visualized using enhanced chemiluminescence reagents (GE Healthcare, Danderyd, Sweden). Band density quantification of the western blot was carried out using ImageJ software.

2.5 Bioluminescent imaging

In vivo bioluminescent imaging was conducted on the IVIS luminal II system (Caliper, Hopkinton, MA, USA) with the use of Living Image acquisition and analysis software. The mice were anesthetized with isoflurane after which they were intraperitoneally injected with 150 μg/g of the firefly luciferase substrate, D-luciferin (Promega, San Luis Obispo, CA, USA). To capture peak intensity, imaging of the tumor and LN area was conducted 10 min after the injection of D-luciferin to capture the peak intensity, which could well represent the primary tumor volume and the metastatic nodule area in the LN. Bioluminescence imaging was performed 4, 5, 6, 7 and 8 weeks after injection with $1 \times 10^6$ MDA-MB-231/Luc cells stably expressing firefly luciferase. The sum of all detected photon counts within an oval shaped region of interest (ROI), either tumor or lung, was quantified in units of mean photons per second per centimeter squared per steradian (p/s/cm²/sr) using Living Image® software.

2.6 PA and US imaging

A Vevo2100 LAZR high frequency US and PA imaging system (FUJIFILM VisualSonics Inc., Toronto, Ontario, Canada) equipped with a linear array transducer (LZ-550, 32-55 MHz...
center frequency linear array with integrated light source) was used to acquire all PA and US images. In this system, integrated fiber-optic transducers are employed to deliver nanosecond laser pulses into deep anatomical targets. Tissues differentially and specifically absorb the light causing transient thermoelastic expansions, generating acoustic pressure waves, which are detected by 256 sensitive piezoelectric elements. Transmitted US pulses are similarly received generating high-resolution images of microscopic anatomical structures. The photoacoustic and spatial dimensions of the collected US images were <14 mm (width) by <15 mm (depth). Clear gel was centrifuged (to remove air bubbles) and used to provide PA coupling between the probe and tumor. The laser was tuned to optical wavelengths from 730 nm to 830 nm, with a PA signal gain of 40 dB. Data were collected in triplicate at a frame rate of 5 Hz for 20 s. Laser fluence, measured by a Nova II power meter with a PE50BB sensor (Ophir-Spiricon, LLC, North Logan, UT, USA), was between 10 to 20 mJ/cm²; all laser energies were below the American National Standards Institute (ANSI) safe exposure level for human skin. PA images were averaged eight times, thus suppressing uncorrelated noise.

Images were analyzed using post-processing software tools (FUJIFILM VisualSonics Inc., Toronto, Ontario, Canada). Tumor boundaries, as identified on the digitally stored US images, were outlined manually for quantification of the PA signal over the area. Each PA image was normalized by the measured fluence to correct for the pulse-to-pulse laser energy variations.

2.7 Histological analysis

Histological analyses of axillary LN and primary tumors were performed. The axillary LN was removed after injection of ICG or non-targeted GN. The tumors and organs were removed 48 h after the injection of non-targeted GN or anti-EGFR-GNs. The metastases detection of axillary LN was removed 48 h after the injection of anti-EGFR-GNs. The excised tissues were fixed with 4% buffered formalin and embedded in paraffin blocks. Tissues were sectioned into 4µm-thick sections. Hematoxylin and eosin (H&E) staining, immunostaining and immune gold-silver staining (Sigma, St. Louis, MO USA) were performed. EGFR expression in tumors and axillary LNs were investigated by immunostaining, using antibodies for EGFR (Santa Cruz Biotechnology, Dallas Texas) and an appropriate secondary antibody. Cytokeratin expression in isolated axillary LNs was investigated by immunostaining, using antibodies for Cytokeratin 8/18/19 (Abcam, Cambridge, MA, USA) and an appropriate secondary antibody. Tissue sections were subsequently stained with counter hematoxylin solution (Millipore Ltd., Darmstadt, Germany) for 1 min. Histological images of stained tissues were acquired using a microscope (Leica, Wetzlar, Germany) equipped with a CCD camera (Leica, Wetzlar, Germany).

2.8 Statistical analyses

All experiments were expressed as the means ± standard deviations calculated from at least three independent experiments. Comparison of PA signal between pre- and post-contrast PA images was performed with paired Student t-test analysis. A p value less than 0.05 was considered statistically significant. SPSSV 17.0 software (Cary, NC, USA) was used for all statistical analyses.

3. Results

3.1 In vivo PAI and histological analysis of axillary LNs of mice

Figs. 1(a) and 1(c) show representative fusion images of US B-mode and PA-mode before and after the injection of ICG and GN. The dynamics of ICG and GN uptake by the axillary LN were monitored using PAI, and the contour of the axillary LN region can be clearly seen in the fusion images of PA and US B-mode images. The mean PA signal amplitude of the
Axillary LNs of ICG-injected mice before injection was 1.60 ± 0.30 AU, and the PA signal amplitudes at 0.5 h, 1 h, 2 h, 4 h and 24 h post-injection were 4.68 ± 0.48 AU, 4.89 ± 0.32 AU, 3.83 ± 0.06 AU, 7.50 ± 0.54 AU, 1.95 ± 0.05 AU, respectively [Fig. 1(b)]. A significant change in the PA signal in the axillary LN was observed at 0.5 h post-injection, indicating significant accumulation of ICG in the axillary LN regions, and was strongest 4 h after the injection of ICG (an approximately 4-fold increase relative to the pre-injection signal, \( p<0.05 \)). The PA signal measured from the axillary LNs just before the injection was similar to that 24 h after the injection of ICG. The PA signal amplitudes in the axillary LNs of GN-injected mice before injection and 4 h, 12 h, 24 h, 48 h and 72 h post-injection were 2.06 ± 0.07 AU, 3.29 ± 0.17 AU, 6.42 ± 0.45 AU, 9.48 ± 0.73 AU, 11.96 ± 0.59 AU, 9.38 ± 0.59 AU, respectively [Fig. 1(d)]. The PA signal amplitude in the LNs of GN-injected mice was significantly increased 4 h post-injection and reached a steady state at 48 h post-injection, indicating a maximal accumulation of GNS in the axillary LN regions (an approximately 6-fold increase relative to the pre-injection signal, \( p<0.05 \)).

Fig. 1. Analysis of US-guided photoacoustic (PA) images and histology of the axillary LN of mice injected with ICG or gold nanorods (GN). (a) Non-invasive and dynamic fusion of US and PA images of the axillary LN region before and 0.5 h, 1 h, 2 h, 4 h and 24 h after ICG injection (5 pmol/g mouse). (b) Plot of PA signal amplitude in axillary LNs versus pre- and post-injection time of ICG. (c) Non-invasive and dynamic fusion of US and PA images of axillary LN region before and 4, 12 h, 24 h, 48 h and 72 h after GN injection (0.005 pmol/g mouse). (d) Plot of PA signal amplitude in axillary LNs versus pre- and post-injection time of GN. The results represent the mean ± standard deviation in the ICG-mice group (n = 5) and the GN-mice group (n = 5), \( *p<0.05 \), \( **p<0.01 \). (Arbitrary units (AU)). (e, f) Photographs of isolated axillary LN before and after injection of ICG or GN. (g) H&E staining of microsectioned axillary LN isolated from mice 4 h post-injection of ICG. (h) H&E staining and silver staining of microsectioned axillary LN isolated from mice at 48 h post-injection of GN. The control (Cont) is a microsectioned axillary LN without injection of ICG or GN. Scale bar: 100 μm.
ICG-LNs were observed as dark green in color, and an obvious color change was not detected in isolated GN-LNs [Figs. 1(e) and 1(f)]. H&E staining showed that no histological change was observed in ICG-LNs isolated from mice 4 h post ICG injection compared with LNs that were not injected [Fig. 1(g)]. A large amount of accumulated GNs in the cortex and medulla area of GN-LNs isolated from mice 48 h post-injection was detected by silver staining, but there were no histological changes in the tissue as detected by H&E staining [Fig. 1(h)].

3.2 In vivo EGFR-targeted PAI and histological analysis of a xenograft tumor mouse model

The TNBC cell lines (HCC-1937 and MDA-MB-231) had high EGFR protein expression (as detected by western blot) relative to the HER2-expressing cell lines (BT474, HCC1954 and MDA-MB-453), whereas the ER-positive MCF-7 cells did not express EGFR [Fig. 2(a)]. Silver staining showed that MDA-MB-231 cells exposed for 24 h to anti-EGFR-GNs (120 pmol/L) had a specifically and selectively higher internalization of the GNs than MCF-7 cells [Fig. 2(b)].

The mean PA signal amplitudes in MDA-MB-231 tumors of anti-EGFR-GNs-injected mice pre-injection and 2 h, 4 h, 8 h, 24 h, 48 h and 72 h post-injection were 1.63 ± 0.09 AU, 3.77 ± 0.26 AU, 4.13 ± 0.29 AU, 5.98 ± 0.59 AU, 9.91 ± 0.91 AU, 8.60 ± 0.39 AU, 5.95 ± 0.50 AU, respectively. The mean PA signal amplitudes in MCF-7 tumors of anti-EGFR-GNs-injected mice pre-injection and 2 h, 4 h, 8 h, 24 h, 48 h and 72 h post-injection were 1.66 ± 0.06 AU, 2.82 ± 0.25 AU, 3.06 ± 0.29 AU, 4.93 ± 0.34 AU, 6.82 ± 0.31 AU, 4.05 ± 0.54 AU, 2.75 ± 0.28 AU, respectively [Figs. 2(c) and 2(d)]. The maximal value of the PA signals was reached 24 h post-injection in both groups and was significantly higher in MDA-MB-231 tumors (approximately 2-fold higher) compared with MCF-7 tumors at 48 h and 72 h post-injection ($p<0.05$). H&E staining did not show any necrotic areas in the tumors of either the MCF-7 or MDA-MB-231 group [Fig. 2(e) upper]. MDA-MB-231 tumor tissue showed higher EGFR expression than MCF-7 tumor tissue [Fig. 2(e) middle]. Silver staining showed a large amount of anti-EGFR-GNs accumulated in the MDA-MB-231 tumors, whereas no anti-EGFR-GNs were seen in the MCF-7 tumors [Fig. 2(e) lower].
Fig. 2. Analysis of EGFR expression in human breast cancer cell lines and US-guided PAI and histology acquired in MCF-7 and MDA-MB-231 primary tumors of mice intravenously injected with anti-EGFR-GNs. (a) EGFR expression in MCF-7, BT-474, HCC-1954, HCC-1937, MDA-MB-453 and MDA-MB-231 human breast cancer cell lines (by western blot). (b) Silver staining of MCF-7 and MDA-MB-231 cells incubated with or without 120 pmol/L of anti-EGFR-GNs for 24 h. Control (Cont) was incubated without 120 pmol/L of anti-EGFR-GNs. (c) Non-invasive and dynamical fusion of US and PA images of MCF-7 and MDA-MB-231 tumors before and 2 h, 4 h, 8 h, 24 h, 48 h and 72 h after intravenous injection with anti-EGFR-GNs (0.5 pmol/g mouse). (d) Plot of PA signal amplitude in MCF-7 and MDA-MB-231 tumors versus pre- and post-injection of anti-EGFR-GNs. The results represent the mean ± standard deviation in the MCF-7-mice group (n = 5) and MDA-MB-231-mice group (n = 5). **p<0.01, Arbitrary units (AU). (e) H&E staining, EGFR immunostaining and silver staining of microsectioned MDA-MB-231 and MCF-7 primary tumors isolated from mice 48 h post-injection of anti-EGFR GN. Scale bar: 100 μm.

However, in MCF-7 and MDA-MB-231 tumors injected with non-targeted GNs, the no significant difference in PA signals between the 2 groups at 2 h, 4 h, 24 h, 48 h and 72 h of
the time points was observed [Figs. 3(a) and 3(b)]. Non-targeted GNs, though being less accumulated in MDA-MB-231 tumors compared to anti-EGFR-GNs, can be used in in vivo PAI application for generally detecting tumor due to the enhanced permeability and retention (EPR) effect that is property by which non-targeted GNs tend to accumulate in tumor tissue much more that they do in normal tissues. Silver staining showed that accumulation of non-targeted GNs were not seen in the MCF-7 and MDA-MB-231 tumors at 72 h [Fig. 3(c) lower].

![Fig. 3. Analysis of US-guided PAI and histology acquired in MCF-7 and MDA-MB-231 primary tumors of mice intravenously injected with non-targeted GNs. (a) Non-invasive and dynamical fusion of US and PA images of MCF-7 and MDA-MB-231 tumors before and 2 h, 4 h, 8 h, 24 h, 48 h and 72 h after intravenous injection with non-targeted GNs (0.5pmol/g mouse). (b) Plot of PA signal amplitude in MCF-7 and MDA-MB-231 tumors versus pre-and post-injection of non-targeted GNs. The results represented the mean ± standard deviation in the MCF-7-mice group (n = 5) and MDA-MB-231-mice group (n = 5). **p<0.01, Arbitrary units (AU). (c) H&E staining and silver staining of microsectioned MDA-MB-231 and MCF-7 primary tumor isolated from mice 48 h post-injection of non-targeted GNs. Scale bar: 100 μm.

3.3 In vivo EGFR-targeted PAI and histological analysis of LN metastases

An obviously enhanced PA signal (3.67 ± 0.18 AU) was detected in axillary LNs with metastases of MDA-MB-231-Luc tumors (n = 2) at 24 h after intratumoral injection with anti-EGFR-GNs (0.5 pmol/g mouse) compared with that of the pre-injection (1.53 ± 0.12 AU) Figs. 4(a) and 4(c)]. No significant PA signal change was observed in non-metastatic axillary LNs of the MDA-MB-231-Luc tumor-bearing mice (n = 4) after intratumoral
injection with anti-EGFR GNs (pre vs post, 1.57 ± 0.15 AU vs 1.61 ± 0.13 AU) [Figs. 4(b) and 4(c)]. In vivo and ex vivo strong bioluminescence signals were obtained in primary tumors, but no signal was detected in the axillary LNs showing the enhanced PA signals using an IVIS imaging system [Fig. 4(d)]. As expected, H&E staining as well as EGFR and Cytokeratin 8/18/19 immunostaining of axillary LN isolated from a mouse with a strong PA signal revealed the presence of MDA-MB-231-Luc cells in the cortex and medulla area of the LN [Fig. 4(e) upper and middle]. Silver staining and showed that the anti-EGFR-GNs injected into the tumor drained to the axillary LN harboring metastases, confirming the correlation with the increased PA signals [Fig. 4(e) lower]. In contrast, no cancer cells and anti-EGFR-GNs were detected in those axillary LNs that showed no changes in PA signals [Fig. 4(f)].

4. Discussion and conclusion

PAI using various exogenous contrast agents (methylene blue, ICG and GNs) is a rapidly emerging non-ionizing and noninvasive imaging technology that integrates the merits of strong optical contrast with high spatial resolution to image the course and flow of lymphatic vessels as well as to selectively detect breast tumors, including metastases [5–8]. Our results
showed that non-invasive EGFR-targeted US-guided PAI is sensitive enough to detect the primary tumor and metastatic LNs in a mouse model of human TNBC. PAI easily integrates the clinical US imaging system and has shown tremendous potential in accurately detecting primary tumor and LN metastases while simultaneously providing structural, functional and molecular information at clinically relevant penetration depths [5]. Because breast cancer cells often invade regional lymph nodes (LN) receiving the drainage from a tumor, accurate detection of regional LN metastases can help to determine the stage of the breast cancer and develop an appropriate treatment plan for the breast cancer patient [13, 15–17]. Moreover, a sentinel node biopsy guided by ICG or PAI can avoid postoperative complications, such as seroma formation, lymphedema, and limited motion, of an axillary LN dissection [18, 19]. In an effort to improve on accurate identification and safe dissection of metastatic LNs, non-invasive imaging modalities including bioluminescence imaging, positron emission tomography (PET), US and magnetic resonance imaging (MRI) have been tested in animal models and patients but currently lack the specificity and sensitivity to discriminate between metastatic and normal LNs.

Multifunctional contrast agents loaded with specific antibodies and therapeutic drugs have been suggested to improve the delivery limitations, such as enzymatic degradation, inadequate pharmacokinetics and tissue accessibility, of therapeutic antibodies and drugs, thereby reducing non-specific side effects and enabling higher dose delivery to target tissues over expressing the cell surface receptor [5, 10]. Rod-shape gold nanoparticles characterized by enhanced optical absorption and photostability are attractive PA contrast agents because of their production of strong PA signals as well as their ability to conjugate biomolecules to them for PAI. In the present study, we highlighted the potential of using PAI combined with intravenous anti-EGFR-GNs (0.5 pmol/g mouse) injections to selectively visualize EGFR-expressing breast cancer cells. In our study, the intravenously injected gold nanorod concentration is lower than previously reported doses [11, 20]. Moreover, the amount of anti-EGFR antibody (0.92 μg/g animal) loaded in the GNs was lower compared with a clinical trial using cetuximab [2] but was a sufficient amount for specific and selective targeting of EGFR-expressing cancer cells. The maximum accumulation of all tested nanoparticles after intravenous injection occurred in the liver and spleen. In agreement with most published reports [11], the very small amount of GNs accumulated in the liver and spleen was detected by silver staining.

Luke GP et. al. showed the in vivo application of PAI to non-invasively detect the LN micrometastases (<50μm) using intratumoral injection with 1.6 pmol of spherical gold nanoparticles targeted to the EGFR oral cancer animal models, but the LN micrometastatic foci (<50μm) was able to detected by using bioluminescence imaging method [13]. Very recently, although the functional spectroscopic PAI to measure the blood oxygen saturation in the LN led to less sensitivity and specificity to discriminate metastatic LN as compared to PAI using exogenous PA contrast agents, this approach might open up the suitable possibility for clinical applications [21]. In our study, PAI by intratumoral injection of 0.5 pmol/g mouse anti-EGFR-GNs, which is a significantly lower amount compared with that of previous reports [13, 21] clearly identified small colonies of EGFR-expressing cancer cells that spread to the axillary LNs, even though bioluminescence imaging technology did not detect the cancer cell in LNs. Our result showed that anti-EGFR-GNs-enhanced PAI is more sensitive than bioluminescence imaging for the early detection of breast cancer cells that have migrated to regional LNs. Our studies combining US-guided PAI with anti-EGFR-GNs may be able to achieve simultaneous sensitivity and specificity values approaching 100% to discriminate LN micrometastases from normal LN.

The anticancer therapy of multifunctional GNs loaded with cetuximab and a low dose of gemcitabine in an orthotopic pancreatic cancer model was superior to that of the antibody alone, drug alone or combination of the two [22], suggesting that anti-EGFR-GNs can be used for treating EGFR-expressing breast cancer cell LN metastases. Although the
concentration of anti-EGFR-GNs in this study is below the previously reported amount exhibiting toxicity, GNs are not yet accepted for widespread clinical use. Further studies regarding the pharmacokinetics, biodistribution and in vivo toxicity of anti-EGFR-GNs used as a clinical therapy need to be conducted because particles larger than 5 to 6 nm are not easily cleared from the body [11]. Our results suggest that non-invasive EGFR-targeted PAI is a relevant approach for use in the clinic to improve the selective and effective targeted delivery of chemotherapeutics into EGFR-expressing tumors and to guide real-time surgery of LN micrometastases and residual tumors with EGFR-expressing cancer cells, following some alterations in the system to make it functional and safe for humans.

In conclusion, US-guided PAI technology using anti-EGFR-GNs is feasible and highly sensitive. It allows for the selective visualization of EGFR-targeted primary tumors as well as LN micrometastases in a murine model of human TNBC. US-guided PAI combined with therapeutic drugs would be a novel theranostic system to simultaneously investigate non-invasive and longitudinal diagnosis and therapy of EGFR-positive TNBC in experimental studies.

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