Molecular Imaging Reveals Trastuzumab-Induced Epidermal Growth Factor Receptor Downregulation In Vivo

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Previous in vitro studies demonstrated that treating tumors expressing both epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 with trastuzumab resulted in increased EGFR homodimerization and subsequent rapid down-regulation of EGFR. We investigated whether molecular imaging using near-infrared fluorescence (NIRF) imaging and PET probes could sensitively detect trastuzumab-induced EGFR downregulation in vivo. Methods: The F(ab)2 antibody fragment PaniF(ab)2 was generated by digesting the anti-EGFR monoclonal antibody panitumumab. PaniF(ab)2 was labeled with either a NIRF dye or 68Ga, and optical imaging and small-animal PET imaging of Dye-PaniF(ab)2 and 68Ga-PaniF(ab)2, respectively, were performed in HT-29 tumor-bearing nude mice treated with trastuzumab or untreated control. Results: Longitudinal NIRF imaging studies revealed significantly reduced tumor uptake of Dye-PaniF(ab)2 on days 5 and 7 in trastuzumab-treated HT-29 tumors, compared with control. Western blotting confirmed the downregulation of EGFR after treatment with trastuzumab. Small-animal PET on day 5 after trastuzumab treatment also demonstrated decreased 68Ga-PaniF(ab)2 uptake in trastuzumab-treated HT-29 tumors. The tumor uptake value of 68Ga-PaniF(ab)2 obtained from PET imaging had an excellent linear correlation with the uptake value measured using biodistribution. Conclusion: The downregulation of EGFR induced by trastuzumab treatment could be detected noninvasively using optical and PET imaging. This molecular imaging strategy could provide a dynamic readout of changes in the tumor signaling and may facilitate the noninvasive monitoring of the early tumor response to drug treatment.

Key Words: molecular imaging; EGFR; HER2; immunotherapy; tumor response


Epidermal growth factor (EGF) receptor (EGFR, also known as ErbB1) and human epidermal growth factor receptor 2 (HER2; ErbB2) are members of the EGFR family of transmembrane protein tyrosine kinase receptors that also includes HER3 (ErbB3) and HER4 (ErbB4) (1). EGFR is upregulated in a variety of human tumors, which is correlated with tumor proliferation and invasion (2). EGFR can be activated by specific ligands including EGF and transforming growth factor-α. On activation by ligands, EGFR activates downstream signaling pathways by either forming homodimers or forming heterodimers with other EGFR family members (1,2). Unlike EGFR, HER2 has no natural ligand, and the activation of HER2 is induced usually by heterodimerization with other EGFR family members. HER2 is overexpressed in up to 30% of breast cancers (1); its expression is usually associated with a more aggressive tumor phenotype, particularly when the tumor cells coexpress EGFR (3).

Trastuzumab (Herceptin; Genentech, Inc.) is a recombinant humanized monoclonal antibody against the extracellular domain of HER2 (4). Clinical studies using trastuzumab as an adjuvant therapy or in combination with chemotherapy have shown promising results (5). However, the therapeutic efficacy of trastuzumab has not met expectations so far. For example, less than 30% of patients with metastatic breast cancer respond to first-line trastuzumab therapy (6). Moreover, most patients who are initial responders eventually acquire resistance to trastuzumab therapy (7).

High levels of HER2 expression may be considered to be a pretreatment predictor of trastuzumab efficacy (8). In clinical practice, the HER2 status of tumor tissues can be assessed using immunohistochemistry for HER2 protein expression and fluorescence in situ hybridization for DNA amplification. However, both of these methods require tumor tissues that are obtained from biopsies, which is an invasive procedure that cannot be repeated frequently. Moreover, the results of immunohistochemistry and fluorescence in situ hybridization may be variable because of tumor heterogeneity (9). Another method to assess HER2 levels in cancer patients is to quantify serum HER2 expression using immunoassays (10). However, immunoassays do not provide whole-body information or report the HER2 levels in primary and metastatic lesions. In fact, it is not certain that all HER2-positive (HER2+) tumors will benefit from trastuzumab therapy. Therefore, further studies are needed to develop novel approaches that could more accurately predict the efficacy of trastuzumab before commencing therapy or more sensitively identify responsive and nonresponsive tumors during trastuzumab therapy.

Molecular imaging is a robust tool for the noninvasive detection of biologic processes and monitoring the pharmacodynamics of biomarkers in vivo. HER2 was proposed as a specific biomarker for assessing the response of tumors to trastuzumab therapy. Although trastuzumab labeled with 111In (11), 89Zr (12), and 64Cu (13) has already been tested in humans for specific HER2 targeting,
these radiotracers may not be optimal for monitoring the efficacy of trastuzumab treatment because of potential self-blocking and competitive inhibition after several rounds of trastuzumab therapy. To overcome this, McLarty et al. (14) developed 111In-labeled pertuzumab, an antibody that recognizes the distinct epitope of HER2 that is targeted by trastuzumab. This study demonstrated that the radiotracer could sensitively monitor trastuzumab-induced HER2 downregulation in vivo. In addition to pertuzumab, several other HER2-targeting agents that recognize distinct epitopes from trastuzumab, such as an 124I-labeled diabody (15) and an 18F-labeled Affibody (16), were also developed. All of these radiotracers have reported high HER2 specificity, and imaging results demonstrated that the molecular imaging of HER2 expression is a sensitive biomarker for assessing the early tumor response to trastuzumab treatment.

EGFR is commonly expressed in HER2+ tumors, and the synergistic interaction between these 2 EGFR family members could lead to a more malignant phenotype and poor prognosis (3, 17, 18). Using an engineered β-gal system, Wehrman et al. (19) reported that treating EGFR- and HER2-coexpressing cells in vitro with trastuzumab inhibited the formation of HER2–EGFR heterodimers and subsequently increased EGFR homodimer formation in the presence of EGF. The increased number of EGFR homo- dimers resulted in increased EGFR internalization and downregulation on the basis of these findings, we hypothesized that molecular imaging of EGFR expression in vivo could be used for characterizing the process of trastuzumab-induced EGFR downregulation noninvasively and thereby monitoring tumor responses to trastuzumab. We used the F(ab′)2 fragment of panitumumab (Vectibix; Amgen, Inc.), a humanized anti-EGFR monoclonal antibody, as the EGFR-targeting agent, which was then labeled with either an optical imaging dye or 68Ga. The downregulation of EGFR induced by trastuzumab treatment in a preclinical animal model was assessed using optical and PET imaging.

MATERIALS AND METHODS

Cell Culture and Animal Model

HT-29 human colon cancer, A549 human lung carcinoma, and A431 human epidermoid carcinoma cell lines were obtained from American Type Culture Collection. Cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO2. All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee at Peking University. To establish the HT-29 tumor model, 5 x 10^6 tumor cells were inoculated subcutaneously into the right front flanks or right thighs of female BALB/c nude mice. Tumor growth was measured using calipers, and tumor volume was calculated using the formula volume = length x width^2/2.

Expression of EGFR and HER2 in Cell Lines

Panitumumab and trastuzumab were radiolabeled with Na125I (Perkin-Elmer) as described previously (20). 125I-panitumumab and 125I-trastuzumab were then used as the EGFR- and HER2-specific radioligands, respectively. The expression levels of EGFR and HER2 in HT-29, A549, and A431 cells were determined by radioligand saturation binding assays, as described previously (20). The results were expressed as the number of receptors per tumor cell.

Effects of Trastuzumab on EGFR Expression In Vitro

Serum-starved HT-29, A549, and A431 tumor cells were treated with or without trastuzumab (25 μg/mL) at 37°C for 2 h, and human EGF (hEGF) (20 ng/mL) was added for the last 10 min before cells were harvested. After being washed with ice-cold phosphate-buffered saline (PBS), cells were lysed and analyzed by Western blotting to determine the EGFR levels.

In Vivo Trastuzumab Therapy

HT-29 tumor–bearing nude mice were separated into 2 groups and injected intraperitoneally with trastuzumab (200 μg per mouse) or PBS (n = 9 per group) for 5 d (from days 1 to 5). Tumor size and body weight were measured every other day. On day 5, 4 animals from each group were sacrificed, and tumor samples were harvested. Half of each tumor was frozen and then cut into 5-μm-thick sections for immunofluorescence staining. The remaining half of each tumor was lysed, and the EGFR levels were determined by Western blotting.

Western Blotting

Detailed procedures are described in the supplemental materials and methods (supplemental materials are available at http://jnm.snjmjournals.org). After Western blotting, the images were opened in ImageJ (http://rsb.info.nih.gov/ij). A region of interest (ROI) was drawn on the first lane, and the same size and shape ROI was applied to all other lanes and the background to quantify the intensity of each band.

Longitudinal Near-Infrared Fluorescence (NIRF) Imaging of EGFR Expression

The EGFR-targeting NIRF agent was generated by conjugating the F(ab′)2 fragment of panitumumab, named PaniF(ab′)2, with DyLight680-NHS ester (Pierce Biotechnology, Inc.). PaniF(ab′)2 was generated by digesting panitumumab using a F(ab′)2 preparation kit (Pierce Biotechnology, Inc.). The identification of PaniF(ab′)2 was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Supplemental Fig. 1). The immunoreactive fraction of 125I-labeled PaniF(ab′)2 was measured to be 77.57% ± 3.73% using HT-29 cells by the method previously described (21). A cell competition binding assay also confirmed that the EGFR immunoreactivity of PaniF(ab′)2 was similar to that of intact panitumumab (Supplemental Fig. 2). PaniF(ab′)2 was conjugated with DyLight680-NHS using a previously described method (22). Detailed procedures are described in the supplemental materials and methods. The DyLight680-NHS-PaniF(ab′)2 conjugate, named Dye-PaniF(ab′)2, was stored at 4°C for further in vivo applications.

Serial NIRF imaging of the tumor response to trastuzumab therapy was performed on days 0, 3, 5, and 7 in the HT-29 tumor model. Mice were injected intravenously with 0.5 nmol of Dye-PaniF(ab′)2, and in vivo NIRF imaging was then performed 4 h after injection. After a baseline scan was obtained on day 0, 5 daily treatments of trastuzumab (200 μg per mouse intraperitoneally) or PBS were administered on days 1, 2, 3, 4, and 5 in the treatment and control groups, respectively (n = 5 per group). NIRF imaging was repeated on days 3, 5, and 7 under the same conditions. For each scan, mice were anesthetized by the inhalation of 2% isoflurane in oxygen; NIRF imaging was then performed using an IVIS small-animal imaging system (Xenogen) (excitation, 675 nm, emission, 720 nm) as previously described (22, 23). Identical illumination settings were used to acquire all scans. To quantify the tumor uptake of Dye-PaniF(ab′)2, the ROI was drawn for each tumor using Living Image software (Xenogen). The fluorescence intensity was presented as the average radiant efficiency in the unit of [p/s/cm2/sr]/[μW/cm2]. Tumor uptake was then calculated by normalizing the fluorescence intensity of the tumor with the total injection dose.

68Ga Radiolabeling and Small-Animal PET Imaging

PaniF(ab′)2 was conjugated to 1,4,7-triazacyclononane-1,4,7-triactic acid (NOTA) under standard isothiocyanate-amine reaction conditions. NOTA-PaniF(ab′)2 was then radiolabeled with 68Ga. Detailed procedures are described in the supplemental materials and methods.
HT-29 tumor–bearing nude mice were treated with trastuzumab (200 μg per mouse daily) or PBS by intraperitoneal injection for 5 d (from days 1 to 5). On day 5, mice were injected via the tail vein with 3.7 MBq of 68Ga-PaniF(ab′)(2) under isoflurane anesthesia. At 3 h after injection, 5-min static small-animal PET images were acquired (n = 4 per group). The ROI-derived percentage injected dose per gram of tissue (%ID/g) was determined using a previously described method (24). For a blocking study, a group of 4 HT-29 tumor–bearing mice were injected intravenously with 1 mg of panitumumab 4 h before injection of 3.7 MBq of 68Ga-PaniF(ab′)(2). At 3 h after injection, the mice underwent PET scanning. Biodistribution of 68Ga-PaniF(ab′)(2)

Immediately after PET imaging, HT-29 tumor–bearing nude mice were sacrificed and dissected. Blood, tumors, major organs, and tissues were harvested and weighed; the radioactivity in the tissues was then measured using a γ-counter (Packard). The results are presented as %ID/g. The tumor and muscle uptake values of 68Ga-PaniF(ab′)(2) determined by biodistribution were correlated with those determined by PET quantification using GraphPad Prism 4.0 (GraphPad Software). The correlation coefficient (R²) was then calculated applying a linear fit to measure the strength of the association.

Statistical Analysis

Quantitative data were expressed as mean ± SD. Means were compared using the Student’s t test. P values of less than 0.05 were considered to be statistically significant.

RESULTS

Trastuzumab-Induced EGFR Downregulation in EGFR- and HER2-Coexpressing Cell Lines

The radioligand binding assay demonstrated that HT-29 and A549 cells were EGFR-positive (EGFR1 and HER21), whereas A431 cells were EGFR-negative (HER22) (Figs. 1A and 1B). HT-29 (EGFR1, HER21), A549 (EGFR1, HER21), and A431 (EGFR- and HER2-negative) cells were then used to investigate the effects of trastuzumab on the downregulation of EGFR. As shown in Figure 1C, EGFR expression was unaffected by intraperitoneal injection of trastuzumab (lanes 1 and 2). However, in the presence of HER2-targeting antibody (lanes 3 and 4), trastuzumab treatment caused a 30%–50% reduction in EGFR expression. A blocking study in HT-29 tumor–bearing nude mice was performed to confirm the in vivo EGFR-targeting efficacy of Dye-PaniF(ab′)(2). As shown in Supplemental Figure 3, serial NIRF imaging studies (on days 0, 3, and 7) demonstrated a blocking effect. The in vivo EGFR-targeting efficacy of Dye-PaniF(ab′)(2) was confirmed in a blocking study in HT-29 tumor–bearing nude mice treated with or without trastuzumab, followed by presence or absence of HERG (Figs. IC, I J, lane 3 vs. lane 4).

In Vivo Therapeutic Efficacy of Trastuzumab

As shown in Figure 2A, trastuzumab treatment suppressed HT-29 tumor growth. A difference in the average fractional tumor volumes (V/V0) between the trastuzumab and control groups was observed beginning on day 4. On day 10, mice were injected via the tail vein with 3.7 MBq of 68Ga-PaniF(ab′)(2), and tumor and muscle uptake values of 68Ga-PaniF(ab′)(2) were determined. The ROI-derived percentage injected dose per gram of tissue (%ID/g) was determined using a previously described method (24). The results are presented as %ID/g. The tumor and muscle uptake values of 68Ga-PaniF(ab′)(2) determined by biodistribution were correlated with those determined by PET quantification using GraphPad Prism 4.0 (GraphPad Software). The correlation coefficient (R²) was then calculated applying a linear fit to measure the strength of the association. Statistical Analysis

Quantitative data were expressed as mean ± SD. Means were compared using the Student’s t test. P values of less than 0.05 were considered to be statistically significant.
the downregulation of EGFR after trastuzumab treatment.  

In control tumors on days 5 (3.52% ± 0.07, 6P = 0.01) and 7 (3.17% ± 0.07, 6P < 0.01). Taken together, these data confirm the accuracy of noninvasive PET imaging to quantify the downregulation of EGFR after trastuzumab treatment.

DISCUSSION

Although molecular imaging techniques have been extensively investigated in the field of oncology, relatively few approaches were applied to provide a deeper understanding of the biologic dynamics of tumor progression that extends beyond merely mapping the density of a target and localizing the lesions (25). Recently, pioneering works have been conducted to measure the androgen receptor pathway (26,27) and the aberrant MYC signaling (28) using PET. These studies demonstrated that molecular imaging of a biomarker that is overexpressed in tumors enables the systematic measurement of oncogenic signaling pathways associated with therapeutic interventions (25,29).

Small-Animal PET and Biodistribution of 68Ga-PaniF(ab’)2

Trastuzumab-induced EGFR downregulation was investigated further by small-animal PET and biodistribution using 68Ga-PaniF(ab’)2. On day 5, 68Ga-PaniF(ab’)2 PET imaging and biodistribution analysis were performed. As shown in Figure 4A, 68Ga-PaniF(ab’)2 was visualized clearly in the tumors of the control group. In contrast, decreased 68Ga-PaniF(ab’)2 uptake was observed in trastuzumab-treated tumors. Importantly, no apparent tumor accumulation of 68Ga-PaniF(ab’)2 was observed in the tumors blocked by competitive inhibition. The uptake values in tumors were measured from ROI analyses and are shown in Figure 4B. The uptake of 68Ga-PaniF(ab’)2 in trastuzumab-treated tumors was significantly lower than in the control tumors (1.15 ± 0.42 vs. 2.70 ± 0.51 %ID/g, 6P < 0.01). After blocking with cold panitumumab, the tumor uptake of 68Ga-PaniF(ab’)2 was inhibited significantly (from 2.70 ± 0.51 to 0.77 ± 0.15 %ID/g, 6P < 0.001).

To validate the accuracy of the PET quantification, a necropsy-based biodistribution experiment was performed. As shown in Figure 4C, predominant uptake of 68Ga-PaniF(ab’)2 was observed in the blood and liver in both control and trastuzumab groups. However, the uptake of 68Ga-PaniF(ab’)2 in trastuzumab-treated HT-29 tumors was significantly lower than in control tumors (1.58 ± 0.16 vs. 2.44 ± 0.30 %ID/g, 6P < 0.01). The values obtained from quantified PET images were then compared with the values derived from the biodistribution study. As shown in Figure 4D, a linear relationship was found between the 68Ga-PaniF(ab’)2 uptake quantified using PET ROI analysis and the necropsy-based biodistribution study, with a correlation coefficient (R2) of 0.80. These data demonstrated the accuracy of noninvasive PET imaging to quantify the downregulation of EGFR after trastuzumab treatment.
In this study, we documented that the use of molecular imaging could quantitatively reflect trastuzumab treatment-induced changes in EGFR expression in vivo. Molecular imaging of the feedback loop interaction between EGFR and HER2, the 2 well-established tumor biomarkers of the EGFR family, could facilitate better understanding of the EGFR signaling pathway and cross-talks between EGFR family members. The dynamic readout of changes in tumor EGFR signaling on anti-HER2 treatment would also open the possibility for early assessment of tumor response to therapy.

HER2 has been used as a biomarker for monitoring the treatment efficacy of trastuzumab in preclinical studies. Several PET radio-tracers targeting HER2 have been investigated to determine the expression levels of HER2 noninvasively, thereby assessing the early tumor response to trastuzumab (14–16). However, data are controversial, because several studies questioned whether trastuzumab treatment downregulated HER2 (14,15,30,31). Possible explanations for these controversial results include the variability of different cell lines and tumor tissues and the insensitivity of immunohistochemistry staining methods for measuring HER2 levels (14). Because HER2 is the direct target of trastuzumab, the actions of trastuzumab on HER2 are likely to be dynamic, depending on the rapid internalization and reexpression of HER2.

A previous study demonstrated that trastuzumab could modulate EGFR. Molecular imaging of EGFR to predict tumor responses to trastuzumab therapy may facilitate the determination of trastuzumab-induced EGFR downregulation, investigation of the underlying mechanisms by which antitumor drugs modulate EGFR. Molecular imaging of EGFR to predict tumor responses to trastuzumab is limited to tumors that express both EGFR and HER2. In the clinical setting, the coexpression of EGFR and HER2 usually stands for a more malignant cancer phenotype (3,17,18). Therefore, using molecular imaging of the pharmacodynamics of EGFR to predict the tumor response to trastuzumab might have possible clinical applications for aggressive tumor phenotypes.

**CONCLUSION**

Using optical and small-animal PET imaging, we were able to noninvasively assess the EGFR downregulation induced by trastuzumab treatment. This noninvasive molecular imaging strategy may facilitate the determination of trastuzumab-induced EGFR downregulation, investigation of the underlying mechanism, and detection of biologic behaviors in vivo.

**DISCLOSURE**

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