Optical imaging of metabolism in HER2 overexpressing breast cancer cells

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Abstract: The optical redox ratio (fluorescence intensity of NADH divided by that of FAD), was acquired for a panel of breast cancer cell lines to investigate how overexpression of human epidermal growth factor receptor 2 (HER2) affects tumor cell metabolism, and how tumor metabolism may be altered in response to clinically used HER2-targeted therapies. Confocal fluorescence microscopy was used to acquire NADH and FAD autofluorescent images. The optical redox ratio was highest in cells overexpressing HER2 and lowest in triple negative breast cancer (TNBC) cells, which lack HER2, progesterone receptor, and estrogen receptor (ER). The redox ratio in ER-positive/HER2-negative cells was higher than what was seen in TNBC cells, but lower than that in HER2 overexpressing cells. Importantly, inhibition of HER2 using trastuzumab significantly reduced the redox ratio in HER2 overexpressing cells. Furthermore, the combinatorial inhibition of HER2 and ER decreased the redox ratio in ER+/HER2+ breast cancer cells to a greater extent than inhibition of either receptor alone. Interestingly, trastuzumab had little impact upon the redox ratio in a cell line selected for acquired resistance to trastuzumab. Taken together, these data indicate that the optical redox ratio measures changes in tumor metabolism that reflect the oncogenic effects of HER2 activity within the cell, as well as the response of the cell to therapeutic inhibition of HER2. Therefore, optical redox imaging holds the promise of measuring response and resistance to receptor-targeted breast cancer therapies in real time, which could potentially impact clinical decisions and improve patient outcome.

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OCIS codes: (170.1790) Confocal microscopy; (170.2520) Fluorescence microscopy; (170.1530) Cell analysis; (170.1610) Clinical applications

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1. Introduction

The course of breast cancer treatment increasingly relies on the molecular phenotype of the tumor. For example, breast cancers that overexpress the estrogen receptor (ER) are often treated with ER-antagonists (e.g., tamoxifen, fulvestrant), while those that overexpress human epidermal growth factor receptor 2 (HER2) are often treated with HER2-inhibitors (e.g., trastuzumab, lapatinib). HER2 overexpressing tumors display aggressive cancer progression [1] and account for approximately 25% of all breast cancer patients. Treatment with trastuzumab (a monoclonal antibody which binds HER2) and lapatinib (a dual tyrosine kinase inhibitor that binds both HER2 and the epidermal growth factor receptor) has been shown to prolong survival in patients with HER2 overexpressing breast cancers [2–4]. Due to the importance of choosing the correct treatment for breast cancer patients, breast tumors are routinely screened for expression of ER and HER2. Currently, ER and HER2 expression are determined by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH).

Unfortunately, approximately one-third of breast tumors that overexpress HER2 do not respond to trastuzumab and lapatinib therapy [4]. Similarly, only 57% of patients with ER-positive breast cancers respond to tamoxifen therapy [5]. However, there are reasons to remain optimistic, as novel therapeutics are in development to overcome clinical resistance to these therapeutic inhibitors [6]. Early identification of those cancers that respond to targeted...
therapies versus those that are resistant will expedite clinical decisions regarding the course of treatment and will improve the clinical outcomes of breast cancer patients.

Methods currently under development to determine tumor response to therapy include positron emission tomography (PET), x-ray computed tomography (CT) and magnetic resonance imaging (MRI) [7–9]. Evidence that tumor response to targeted inhibitors can be visualized was demonstrated with the use of fluoro-deoxyglucose (FDG)-PET, which is capable of detecting focused areas of high glucose uptake that are often seen in solid tumors. A preliminary clinical study of lapatinib-treated breast cancers showed changes in tumor metabolism after 1 month of lapatinib treatment [10]. Yet, these currently available technologies provide only low resolution images, are non-portable, and usually require the use of contrast agents. Given the high cost of these procedures, it is unlikely that these will be adopted as standard of care, underscoring the need for more efficient, accurate, and cost-effective methods of identifying receptor expression and therapeutic response.

Cellular metabolism is a potentially powerful biomarker for tumor analysis. Unlike normal cells that rely on oxidative phosphorylation to generate ATP, or that use glycolysis under anaerobic conditions, cancer cells often generate ATP through aerobic glycolysis [11]. Interestingly, signaling through the HER2 and ER pathways in breast cancer cells is thought to promote aerobic glycolysis. ER increases glucose transport and glycolysis [12,13]. Similarly, HER2 activated pathways may increase glucose transport into the cell and glycolysis [14,15]. HER2 signaling activates phosphatidyl inositol 3-kinase (PI3K) a major driver of aerobic glycolysis [16–19]. In mouse models of HER2 overexpressing breast cancer, trastuzumab and lapatinib inhibited PI3K activity and decreased glucose uptake as measured by FDG-PET imaging [20]. Conversely, breast tumor cells exhibiting resistance to HER2 inhibitors display aberrantly increased PI3K activity and active hypoxia signaling despite the presence of adequate oxygen [18]. These studies suggest that differences in aerobic glycolysis may reflect not only oncogene-driven metabolic characteristics of the tumor cell, but also the effect of therapeutic inhibitors on tumor metabolism, and could therefore be used to distinguish tumors that are responsive to therapeutic inhibitors from those that are resistant.

During oxidative phosphorylation, NADH is oxidized to NAD$^+$ and FAD is reduced to FADH$_2$. However, the process of glycolysis causes NAD$^+$ to be reduced to NADH. Therefore, the ratio of NADH to FAD is a measurement of the balance between glycolysis (seen in tumor cells) and oxidative phosphorylation (seen in untransformed cells). NADH and FAD can be measured in situ using autofluorescence optical imaging techniques. The optical redox ratio (NADH fluorescence intensity divided by FAD fluorescence intensity) is a proven method of probing cellular metabolism and has been used to differentiate cancerous from non-cancerous tissues in a variety of models including oral and breast cancer [21–27]. Ostrander et al. showed the optical redox ratio is also sensitive to ER expression in breast cancer cell cultures, and that treatment with tamoxifen decreased the optical redox ratio of tamoxifen-sensitive cells, but not tamoxifen-resistant cells [27].

The purpose of this study was to determine the effect of HER2 overexpression on the metabolism of breast cancer cells as measured by the optical redox ratio. The hypothesis that HER2 overexpression influences cellular redox ratios independently of ER expression was tested. Additionally, the impact of HER2 inhibition on the redox ratio in HER2 overexpressing breast cancer cells was measured. Finally, we determined if therapeutic resistance to HER2 inhibitors reflected on redox ratio measurements as a failure to reduce redox ratios in response to HER2 inhibition. HER2 overexpression was found to increase the redox ratio, independently of ER expression. While HER2 inhibition decreased the optical redox ratios in HER2 overexpressing cells, the HER2 inhibitor, trastuzumab, had no impact on redox ratios in trastuzumab resistant cells, despite continued overexpression of HER2.
2. Materials and methods

2.1. Cell culture

MCF10A cells were cultured in Mammary Epithelial Cell Growth Medium (MEGM, Lonza, Walkersville, MD) excluding the gentamycin-amphotericin B mix and supplemented with 100 ng/ml cholera toxin and 1% penicillin:streptomycin. The BT474, MDA-MB-231, MCF7, and SKBr3 cells were grown in DMEM (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum and 1% penicillin:streptomycin, hereafter referred to as DMEM+. The resistant cell lines were grown in the DMEM+ at levels of the corresponding drug to maintain resistance. The lapatinib resistant cells were grown at 1 µM lapatinib (LC Laboratories, Woburn, MA) concentration and the trastuzumab resistant cells were grown at 25 µg/ml.

For imaging, all cell lines were plated at a density of 1x10⁵ cells per 35 mm plate, 48 hours prior to imaging. Glass bottom dishes (MatTek Corporation, Ashland, MA) were used to allow live cell imaging on an inverted, confocal microscope. The trastuzumab and lapatinib resistant cell redox ratio was determined from cells grown in trastuzumab and lapatinib supplemented media. For the drug perturbation experiments, the responsive BT474 cells were fed the drug supplemented media 24 hours prior to imaging. For the trastuzumab perturbation of the trastuzumab resistant cells, the cells were fed the DMEM+ media for the first 24 hours after plating and the DMEM+ with trastuzumab for the second 24 hours. Drug concentrations of the media were selected to mimic therapeutic drug dosage in patients, 25 µg/ml for trastuzumab (VUMC Pharmacy, Nashville, TN) and 2 µM for tamoxifen (Sigma-Aldrich, St. Louis, MO) [20,28].

For the cyanide experiment to verify measurement of the redox ratio, MCF10A cells were plated at 1 x 10⁵ cells per plate, 48 hours prior to imaging. Cells were imaged before the addition of cyanide. After 3 images from a plate were acquired, the cell media was exchanged for growth media supplemented with 4 mM NaCN (Sigma-Aldrich, St. Louis, MO). The cells were given one minute to react with the cyanide; then, three different places of each plate were imaged.

Proliferation rates of MCF10A, MCF7, and BT474 cells were determined by antibody labeling of cells grown on parallel imaging plates. Mitotic cells were first marked using a Phospho-Histone H3 (Ser10) antibody (Cell Signaling Technology, Danvers, MA). Then, labeling of the primary antibody with Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA) allowed counting by flow cytometry of the highly fluorescent mitotic cells and the less-fluorescent unlabeled cells. The percentage of proliferating cells was determined by dividing the number of highly fluorescent cells into the total number of cells counted.

2.2. Verification of ER and HER2 expression

Cells were homogenized in ice-cold lysis buffer [50 mM Tris pH 7.4, 100 mM NaF, 120 mM NaCl, 0.5% NP-40, 100 µM Na3VO4, 1X protease inhibitor cocktail (Roche)], sonicated for 10 s at 4°C, 13,000 x g for 5 min. Protein concentration was determined using the BCA assay (Pierce). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 3% gelatin in TBS-T (Tris-buffered saline, 0.1% Tween-20) for 1 h, incubated in primary antibody in 3% gelatin for 2 h at room temperature, washed with TBS-T, incubated in HRP-conjugated anti-rabbit or anti-mouse IgG, washed with TBS-T, and then developed using ECL substrate (Pierce). The following primary antibodies were used: ErbB2 (HER2) (Neomarkers, InVitrogen; 1:2000); ER-alpha (Santa Criz Biotechnologies; 1:1000); beta actin (Sigma-Aldrich; 1:5000).

2.3. Confocal imaging

Images were acquired using an Olympus FV-1000 Inverted Confocal Microscope with a 40X/1.3 NA oil-immersion objective. Confocal microscopy was chosen over widefield
fluorescence to reduce background noise for ease of NADH and FAD auto-fluorescence measurement. Additionally, the redox ratio is sensitive to measurement volumes and confocal microscopy ensures that the NADH and FAD signals can be co-registered to the same volume. For NADH fluorescence, the cells were excited at 405 nm and 410-510 nm emission was collected. FAD was excited at 488 nm and 500-600 nm emission was collected. The two images were acquired simultaneously, with NADH and FAD acquired sequentially for each pixel. A pixel dwell time of 2 µs was used. Each line was averaged 4 times to reduce noise. A single 1024x1024 pixel image required 39.0 s to acquire. To ensure the cells were not photobleaching, two images of the same field of view were acquired consecutively with no significant change in average pixel intensity. Settings for the gain, offset, and pinhole were maintained across all imaging sessions. To account for daily variations in laser power or instrumentation instability, the images were normalized to MCF10A cell measurements acquired during each imaging session. Each plate was imaged at 3 different, non-overlapping locations.

2.4. Image analysis

The optical redox ratio, NADH fluorescence intensity divided by FAD fluorescence intensity, was computed for each cell in the image using ImageJ software (NIH). The fluorescence signal of non-cellular regions, or background signal, was removed to ensure the fluorescence comparisons were made only for the cells. NADH and FAD used for cellular metabolism are contained within the cytoplasm and mitochondria, and the fluorescence signal from the nucleus is not involved in cellular metabolism. Therefore, the fluorescence signal from the nucleus was also removed to ensure isolation of metabolic NADH and FAD and eliminate the possibility of nuclear size as a confounding factor. Next, a NADH/FAD per pixel image was computed and the redox ratio for each cell in the image was determined. The average redox ratio value from all cells in each image was computed and normalized to the corresponding session's MCF10A measurement. Standard error was computed from the mean redox ratio value across all images from each cell line. A rank sum test was used for all statistical comparisons.

3. Results

The optical redox ratio of a panel of human breast-derived cell lines with varying expression of ER and HER2 were studied (Table 1, Fig. 1). MCF10A, a non-cancerous breast cell line with negligible or low expression of ER and HER2 was used as a control for each analysis. To verify the use of optical imaging to measure the redox ratio, cyanide was used to disrupt the electron transport chain, thus preventing the conversion of NADH to NAD\(^{+}\) [29]. As predicted, MCF10A cells treated with cyanide exhibited increased NADH fluorescence (p < 0.01, Fig. 2) and decreased FAD fluorescence (p < 0.05; Fig. 2). Therefore, the redox ratio

| Cell line | Cancerous | ER status | HER2 overexpression | n |
|-----------|-----------|-----------|---------------------|---|
| MCF-10A   | Non-cancerous | --/low    | --/low              | 30 |
| MDA-MB-231| Cancer     | Negative  | Negative            | 15 |
| MCF-7     | Cancer     | Positive  | Negative            | 15 |
| BT-474    | Cancer     | Positive  | Positive            | 15 |
| SKBr3     | Cancer     | Negative  | Positive            | 15 |
| HR6 (Trastuzumab Resistant BT-474) | Cancer | Positive | Positive | 15 |
| BT-LR (Lapatinib Resistant BT-474) | Cancer | Positive | Positive | 15 |
was increased in cyanide-treated MCF10A cells as compared to untreated cells (p < 0.005, Fig. 2). These results confirm that our optical imaging methods accurately reflect the balance between glycolysis and oxidative phosphorylation, and can be used to assess the metabolic state of tumor cells.

![Western blot analysis demonstrates overexpression of HER2 in the SKBR3 and BT474 cells and expression of ER in the MCF7 and BT474 cells.](image1)

**Fig. 1.** Western blot analysis demonstrates overexpression of HER2 in the SKBR3 and BT474 cells and expression of ER in the MCF7 and BT474 cells.

![Addition of CN to MCF10A cells (n = 6) results in an increase in redox ratio and NADH fluorescence and a decrease in FAD fluorescence. Bar height represents mean and error bars represent SE.](image2)

**Fig. 2.** Addition of CN to MCF10A cells (n = 6) results in an increase in redox ratio and NADH fluorescence and a decrease in FAD fluorescence. Bar height represents mean and error bars represent SE.

![Representative NADH, FAD, and redox ratio images for MCF10A, MDA-231, MCF7, BT474 and SKBr3 cells. Optical redox ratio increases with ER and HER2 expression.](image3)

**Fig. 3.** Representative NADH, FAD, and redox ratio images for MCF10A, MDA-231, MCF7, BT474 and SKBr3 cells. Optical redox ratio increases with ER and HER2 expression.
Redox ratio is elevated in cells overexpressing HER2.

We used optical imaging to capture NADH and FAD fluorescence in five breast derived cell lines (Fig. 3): MCF10A (untransformed breast epithelial cells), MDA-MB-231 (ER-/HER2-), MCF-7 (ER+/HER2-), SKBR3 (ER-/HER2+), and BT474 (ER+/HER2+). The fluorescence intensities were used to calculate the redox ratio per cell for each cell line (Fig. 4). While ER-/HER2- MDA-MB-231 cells displayed a redox ratio that was similar to what was seen in untransformed MCF10A cells, redox ratios were elevated in all other breast cancer cell lines (p < 0.005). ER+/HER2- breast cancer cell lines displayed elevated redox ratios (p<0.005), but not to the extent seen in HER2+ breast cancer cell lines (p<0.001).

Controlling for differences in rates of proliferation among the MCF10A, MCF7, and BT474 cells did not reduce the significance of the redox ratio results shown in Fig. 4. In fact, controlling for proliferation (redox ratio divided by proliferation rate for each cell line) increases the differences between these three cell lines (Fig. 5).

The redox ratio of both the trastuzumab resistant cell line, HR6, and the lapatinib resistant cell line, BT-LR, was lower than the redox ratio for the responsive parental BT474 cells (Fig. 6A). The HR6 cells are BT474-derived cells that continue to grow in the presence of trastuzumab [30]. Likewise, the BT-LR cells are BT474-derived cells that were selected in culture with increasing concentrations of lapatinib [31]. To investigate the contributions of the

![Graph: Normalized Redox Ratio](image1)

![Graph: Redox Ratio divided by Proliferation Rate](image2)
ER and HER2 signaling pathways to the redox ratio, BT474 cells (ER+/HER2+) were exposed to the ER antagonist tamoxifen and the anti-HER2 monoclonal antibody trastuzumab, (Fig. 6B). Trastuzumab significantly decreased the redox ratio in ER+/HER2+ breast cancer cells (p<0.001; Fig. 6B). Similarly, tamoxifen resulted in a decrease in redox ratio in the same cells (p<0.001, Fig. 6B). The combination of tamoxifen with trastuzumab decreased the redox ratio in BT474 cells (ER+/HER2+) to a greater extent than single agent tamoxifen (p<0.001) or trastuzumab (p<0.001; Fig. 6B). Interestingly, treatment of HR6 cells with trastuzumab did not cause any alterations in the redox ratio (Fig. 6A). It is difficult to fully eliminate the influence of lapatinib on the BT-LR cells; and therefore, outside the scope of this paper to robustly perform a comparison of pre- and post- lapatinib treatment in these cells.

4. Discussion

The selective use of aerobic glycolysis over oxidative phosphorylation by cancer cells has been known for over a century. We and others have shown that this characteristic of cancer cells can be monitored in situ using optical imaging to measure the redox ratio, or the ratio of glycolysis to oxidative metabolism. Furthermore, we show that “driver” oncogenes in breast cancer cells also drive aerobic glycolysis in these cells. Specifically, HER2 activity was required for maximal aerobic glycolysis in breast cells overexpressing HER2, while ER signaling was required for aerobic glycolysis in ER+ breast cancer cells. These results demonstrate the effect of the HER2 and ER signaling pathways on cancer cell metabolism, as measured by the optical redox ratio. These data support the conclusion that optical imaging of redox ratios can be used to measure the tumor cell response to therapeutic inhibitors in situ. Finally, using HER2 overexpressing breast cancer cells with acquired resistance to HER2 inhibitors, we demonstrated that loss of oncogene dependence was reflected in the optical redox ratio measurement, such that HER2 inhibitors failed to decrease the redox ratio in a resistant cell line. Therefore, these findings could be applied to future studies using optical
imaging of breast cells and tumors to predict their response to HER2 inhibitors, a development that would significantly inform clinical decisions regarding therapeutic strategies used to treat patients with HER2-positive breast cancers.

While this is the first study to correlate HER2 overexpression and activity to the redox ratio, a previous study similarly determined that ER+ breast cells exhibited a higher optical redox ratio than ER-negative cells [27]. The previous study differs from the results presented herein because the previous study suggested that MCF7 cells (ER+/HER2-) exhibited a similar redox ratio to BT474 cells (ER+/HER2+). However, our results suggest that BT474 cells have significantly higher redox ratios than MCF7 cells. Several experimental factors could explain the differing results, including different excitation wavelengths for NADH, differing emission filters, and different growth media. Ostrander et al. used media supplemented with 5% FBS versus 10% used in our studies, which may have increased cellular proliferation in our experiments versus those described earlier, thus affecting tumor cell metabolism [32]. However, when controlling for cell proliferation, the difference in the redox ratio between MCF7 and BT474 cells increased in the current study (Fig. 5). Different emission filters capture different areas of the NADH and FAD emission curves, affecting absolute fluorescence intensity which may change the redox ratio. The results of the cyanide experiment ensure measurement of NADH and FAD fluorescence for this study (Fig. 2).

The results show that redox ratios in triple-negative MDA-MB-231 cells were similar to what was seen in MCF10A cells, which was less than the redox ratio seen in HER2+ or ER+ breast cancer cells. Triple-negative breast cancers (TNBC) lack HER2, ER, and PR, so they are difficult to target and no molecular targeted therapies exist for these breast cancers. Several FDG-PET studies in TNBC, ER+, and HER2+ tumors report high variability in the glucose uptake of triple negative cells [33,34]. Note that FDG-PET imaging illuminates different aspects of cellular metabolism (glucose uptake) than the optical redox ratio (relative concentrations of NADH and FAD, end products of metabolism). However, these previous reports indicate that the metabolism of TNBC is complex and heterogeneous, so a full characterization of this tumor subtype will likely require a more concentrated study.

The highest redox ratios were measured in breast cancer cell lines with HER2 overexpression. The higher redox ratio of HER2 overexpressing cells suggests that the HER2 pathway may affect cellular metabolism. Indeed, studies have found increased concentrations of glucose transporters and glycolytic enzymes present in HER2 overexpressing cells versus cells with low expression of HER2 [14,15].

We found that HER2 inhibition with trastuzumab decreased the redox ratio of HER2 overexpressing breast cancer cells (p< 0.001, Fig. 6B). This is consistent with several studies reporting reduced glucose uptake, decreased lactate secretion, and decreased glycolysis, in responsive HER2 overexpressing breast cancer cells treated with trastuzumab [35,36]. Treatment with both trastuzumab and tamoxifen decreased the redox ratio of responsive BT474 cells to levels seen in the non-cancerous MCF10A cell line (Fig. 6B). These results suggest that both ER and HER2 signaling affects tumor cell metabolism in breast cancer cells. Mechanistic studies of receptor expression support this result. Cells expressing ER overexpress glucose transporters and have higher reported rates of glycolysis [12,13]. Similarly, HER2 overexpression is linked with increased glucose transport into cells and increased glycolysis [14,15].

Innate and acquired resistance to HER2 inhibitors limits their current clinical success. Nearly all HER2-amplified breast cancers treated with trastuzumab or lapatinib will ultimately develop resistance to these targeted inhibitors. We examined trastuzumab-resistant cells, demonstrating that trastuzumab failed to reduce the redox ratio in the resistant cells (Fig. 6A). These results are the first of its kind, but are consistent with an FDG-PET study of trastuzumab responsive and non-responsive breast cancer xenographs, demonstrating that HER2 inhibitors failed to reduce FDG uptake in those tumors whose growth was unaffected by trastuzumab [37]. Interestingly, the redox ratio of the trastuzumab and lapatinib resistant
cells are different from the pre-treatment redox ratio value of responsive BT474 cells, p<0.001 (Fig. 6A). These results open up the possibility of screening HER2-inhibitor resistance with the optical redox ratio.

Future in vivo studies are needed to verify the influence of ER and HER2 expression on cellular metabolism. Increasing research is elucidating the importance of metabolism in cancer cells and its relationship with drug resistance. Here, upon treatment with receptor targeted therapies, the change in metabolism of resistant cells is shown to be negligible, while the metabolism of responsive cells is significant. Additionally, basal redox ratios are different in resistant versus responsive cells. Therefore, cellular redox ratios may prove an invaluable tool for research and clinical identification of receptor expression, tumor resistance to targeted therapies, and for monitoring treatment efficacy.

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