Lapatinib Restores Hormone Sensitivity with Differential Effects on Estrogen Receptor Signaling in Cell Models of Human Epidermal Growth Factor Receptor 2–Negative Breast Cancer with Acquired Endocrine Resistance

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Abstract

Purpose: Acquired endocrine resistance in estrogen receptor (ER)α+/human epidermal growth factor receptor 2–negative (HER2−) breast cancer has been associated with modest adaptive increases in HER2, although exactly how aberrant HER2 signaling affects the ERα pathway is poorly understood. We investigated (a) whether the epidermal growth factor receptor/HER2 inhibitor lapatinib could restore endocrine responsiveness in cell models of acquired endocrine resistance with modest increases in HER2, and (b) the nature of ERα/HER2 cross-talk in this process.

Methods: Combination growth studies, ERα transcription, immunoblot, and gene expression assays were conducted in two models of acquired resistance to (a) estrogen deprivation (long-term estrogen-deprived cells) and (b) tamoxifen (long-term tamoxifen-treated cells), and in hormone sensitive controls. Changes in ERα, PgR, and HER2 were assessed in samples from patients treated with tamoxifen.

Results: Both cell models of acquired endocrine resistance showed modest adaptive upregulation in HER2, and lapatinib restored endocrine sensitivity in both. The effect of lapatinib on ERα signaling varied markedly depending on the nature of the HER2/ERα cross-talk. In long-term estrogen-deprived cells characterized by enhanced ERα function, lapatinib suppressed ERα genomic activity (as measured by pERSer118, ERα transcriptional activity, and PGR gene expression). In contrast, in long-term tamoxifen-treated cells with reduced ERα activation, lapatinib reactivated ERα genomic function. Twenty percent of tamoxifen-resistant patients relapsed with modest increases in HER2 and either suppressed or enhanced ERα/PgR expression.

Conclusions: Aberrant GFR signaling can augment or suppress ERα function. Regardless, interrupting the HER2/ERα cross-talk with lapatinib can restore endocrine sensitivity and should be investigated as a therapeutic strategy in combination with endocrine therapy in ERα+/HER2− patients with acquired endocrine resistance. Clin Cancer Res; 16(5); 1486–97. ©2010 AACR.

Over 70% of breast cancers express detectable levels of the estrogen receptor-α (ERα). These can be selected for anticancer strategies that target the receptor itself, such as the selective ER modulator, tamoxifen, or the pure ERα antagonist, fulvestrant (1, 2). Estrogen deprivation (ED) offers another therapeutic strategy and can be achieved by ovarian ablation in the premenopausal patient, or with aromatase inhibitors in the postmenopausal setting. These therapies are well tolerated and highly effective; adjuvant endocrine therapy has been shown to reduce mortality from ERα+ breast cancer to the same degree as adjuvant chemotherapy (3). Unfortunately some patients with ERα+ breast cancer show de novo resistance to endocrine therapy, whereas others initially benefit but ultimately relapse with acquired endocrine resistance. Modulating or restoring endocrine responsiveness remains an important clinical priority. There is increasing evidence implicating membrane growth factor receptors (GFR) in endocrine resistance. The human epidermal GFR-2 (HER2) is significantly overexpressed, or amplified in 10% of early ERα+ breast cancers and has been associated with poor prognosis and de novo resistance to both tamoxifen and aromatase inhibitors (4–7). Importantly, preclinical models have shown that the development of acquired endocrine resistance can be associated with more modest adaptive upregulation in growth factor signaling pathways (8–11).
The proposed biological mechanisms to explain how GFR signaling results in endocrine resistance are conflicting. Some have shown that GFR signaling may impair endocrine responsiveness by increasing ERα function either directly by phosphorylating and activating the AF-1 domain of the receptor (at Ser167 or Ser118; ref. 12), or indirectly by recruiting coactivators to the ERα (13, 14), resulting in increased ERα transcriptional activity. In contrast, others have shown that signaling through membrane receptors and downstream effectors such as extracellular signal-regulated kinase (ERK)1/2 may in fact suppress ERα expression (15, 16) and function (17) thereby promoting ERα independence, or conversion to an ER- phenotype (18), with resulting endocrine resistance. Given these conflicting observations, any studies investigating the benefit of combining GFR- and ERα-targeted therapies should assess their impact on ER expression and function.

Lapatinib is a dual epidermal GFR (EGFR)/HER2 tyrosine kinase inhibitor. Although there is increasing data to support that the antitumor effect of lapatinib alone or in combination with chemotherapy is likely limited to HER2-positive breast cancer, defined as 3+ by immunohistochemistry or positive by fluorescence in situ hybridization (HER2-3+/FISH+). In estrogen receptor (ER)+ breast cancer, we show in vivo that although the conversion to HER2-3+/FISH+ remains a rare event, modest adaptive increases in HER2 occur in ~20% of patients relapsing on tamoxifen. In cell models of HER2–, ER+ breast cancer with acquired endocrine resistance and modest adaptive increases in HER2, the combination of lapatinib with endocrine therapy resulted in synergistic growth inhibition. Our results support future trials investigating combined HER2-ER targeting in originally HER2– patients with endocrine-resistant breast cancer and demonstrable modest upregulation in HER2 expression or activation. Moreover, given the plasticity of endocrine-resistant breast cancer, treatment strategies should be based on the phenotype of the tumor at relapse rather than at diagnosis.

Translational Relevance

There is evidence that the efficacy of lapatinib is likely limited to HER2-positive breast cancer, defined as 3+ by immunohistochemistry or positive by fluorescence in situ hybridization (HER2-3+/FISH+). In estrogen receptor (ER)+ breast cancer, we show in vivo that although the conversion to HER2-3+/FISH+ remains a rare event, modest adaptive increases in HER2 occur in ~20% of patients relapsing on tamoxifen. In cell models of HER2–, ER+ breast cancer with acquired endocrine resistance and modest adaptive increases in HER2, the combination of lapatinib with endocrine therapy resulted in synergistic growth inhibition. Our results support future trials investigating combined HER2-ER targeting in originally HER2– patients with endocrine-resistant breast cancer and demonstrable modest upregulation in HER2 expression or activation. Moreover, given the plasticity of endocrine-resistant breast cancer, treatment strategies should be based on the phenotype of the tumor at relapse rather than at diagnosis.

Materials and Methods

**Cell lines.** Wild-type (WT) MCF7 cells were maintained in phenol red–free RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10 μg/mL insulin, and 1 mmol/L estradiol (E2). Long-term estrogen-deprived (LTED) cells were generated as previously described and were maintained in 10% dextran-coated charcoal (DCC)–stripped FBS (ref. 9; resulting in <3 pmol/L E2 by RIA; refs. 23, 24). Long-term tamoxifen-treated (LTam) cells were generated from cells kindly donated by A. Lykkesfeldt (Danish Cancer Society, Copenhagen, Denmark), initially generated by continuous growth of MCF7 cells in phenol red–free DMEM (Invitrogen) with 1% FBS, 6 ng/mL insulin, and 1 μmol/L tamoxifen for over 12 mo (25). In our laboratory, these cells were maintained in 2% FBS, insulin, and tamoxifen. Corresponding WT tamoxifen-sensitive controls were MCF7 cells maintained in 2% FBS and 6 ng/mL insulin. Cells were stripped for 4 d before the experiments in 10% DCC (MCF7 and LTED) or in 2% FBS (WT and LTam).

All cell culture products were obtained from Invitrogen unless otherwise stated. E2, androstenedione, tamoxifen, and 4-hydroxy-tamoxifen (4-OHT) were from Sigma-Aldrich. Lapatinib was obtained from GSK.

**Growth effect assays.** Assays were conducted as previously described (9). Cells were treated daily for 6 d and cell number was determined using a Z1 Coulter Counter (Beckman Coulter). Although LTam cells were generated and maintained in tamoxifen-containing medium, experiments were conducted using the more potent metabolite, 4-OHT. Graphs are representative of three independent experiments.

**Drug interaction analysis.** To determine the nature of the interaction between lapatinib and 4-OHT, combination studies were done using Chou and Talalay's constant ratio combination design (26). Cells were treated with increasing doses of lapatinib alone, 4-OHT alone, or a combination of equipotent doses of lapatinib and 4-OHT daily for 1 wk. Wherever possible, the IC₅₀ for each drug alone was selected to define the ratio of equipotent doses and the serial dilutions of the combination done spanning clinically relevant doses. The nature of the interaction was formally quantified using the Calcusyn software (BIOSOFT), based
on the combination index (CI) equation of Chou and Talalay. The CIs (mean and SD) for a growth inhibition of 25%, 50%, and 75% were obtained using mutually non-exclusive Monte Carlo CI simulations. Drug interaction is scored using the CI so that a CI = 1 is additive, CI < 1 is synergistic, and CI > 1 is antagonistic. Experiments were set up in triplicate.

**Estrogen response element transcriptional assays.** Cells were seeded (in 10% DCC-FBS or 2% FBS at 0.5 to 1 x 10^5/well) into 24-well plates and were transiently cotransfected in serum-free media using Eugene 6 (Invitrogen) with 0.1 μg of estrogen response element luciferase reporter construct (EREtkLUC) and 0.1 μg of pCH110 (β-galactosidase to normalize luciferase data). The next day, cells were pretreated with inhibitor alone for 30 min before treatment with E2 alone or in the presence of inhibitors at the indicated concentrations for 24 h. ERE-luciferase activity was measured using a luminometer and was normalized to β-galactosidase activity. When comparing activity across cell lines, ERE activity was measured on the same day, using the same luminometer sensitivity.

**Immunobots.** Cell monolayers were washed with cold PBS twice and were collected by scraping. Cell pellets were lysed in extraction buffer (1% (v/v) triton X-100, 10 nmol/L Tris-HCl (pH 7.4), 5 nmol/L EDTA, 50 nmol/L sodium fluoride, 2 nmol/L Na3VO4, and one tablet of protease inhibitor (Roche) per 10 ml buffer) and homogenized through a 27-gauge needle before centrifugation (14,000 rpm for 10 min at 4°C). Protein concentration was quantified using the Bio-Rad protein assay kit. Fifty micrograms of protein were resolved by SDS-PAGE and were transferred overnight to nitrocellulose membranes. Membranes were blocked for 1 h in PBS/0.05% Tween 20 (PBS-T) containing 5% dry milk, followed by 2 h of incubation in primary antibody (1:1,000) in PBS/0.05% Tween 20 containing 0.1% azide and 1 h of incubation in secondary antibody (1:2,000) in blocking solution on a shaker at room temperature. Immune complexes were detected using Ultra-Signal Chemiluminescent kit (Pierce). For studies using inhibitors, cells were serum deprived for 24 h and were treated with vehicle or inhibitor alone for 30 min before the indicated treatment in serum and hormones. Antibodies used were HER2 and pHER2 (Tyr1248) from Upstate, Akt and ERK1/2 from New England Biolabs, ER and PgR from Novacstra, pER (Ser118) from Epitomics, pER (Ser167) from Santa Cruz Biotechnology, pERK1/2 (Thr183/Tyr185) and actin from Sigma, and pAkt (Ser473) from DAKO. Secondary horseradish peroxidase-conjugated antibodies were from Amersham (Pharmacia Biotech). Quantification of immunobots was done using the NIH ImageJ software, and immunobots were normalized to actin.

**Quantitative reverse transcription-PCR.** RNA was extracted from whole cells using RNeasy minikit (Qiagen) according to the manufacturer’s recommendations, and was quantified by measuring absorbance at 260 nm by spectrophotometric analysis (NanoDrop). RNA was reverse transcribed using SuperScript III (Invitrogen), and random primers and 20 ng of resulting cDNA of each sample were analyzed in triplicates by reverse transcription-PCR using the ABI Perkin-Elmer Prism 7900HT Sequence detection system (Applied Biosystems). Taqman gene expression assays (Applied Biosystems) were used to detect genes of interest (ESR1, PGR, and TFF1) and were normalized to two housekeeping genes (KIAA and PUM1). These housekeepers were selected from a previously published list of appropriate reference genes for breast cancer (27) because their expression level was shown to be unaffected by E2 in the four cell lines used in this study (data not shown). Gene expression was quantified using a standard curve generated from serial dilutions of reference cDNA from a pool of breast cancer cell lines.

**Clinical samples.** Paired tumor samples were available from 80 patients who had their initial surgery at the Royal Marsden Hospital, London, United Kingdom for invasive breast cancer, who received adjuvant tamoxifen, and who had a tamoxifen-resistant invasive recurrence. This series is an update of that previously published by Gutierrez et al. (22). Triplicate tissue microarrays of 0.6-mm cores were constructed of the pairs. This study was approved by the hospital Local Research Ethics Committee. Tissue microarrays were stained for ERα (6F11, Vector Laboratories), and HER2 (HercepTest, DAKO) whole sections were stained for PgR (321, Vector Laboratories) due to the heterogenous nature of this marker. ERα+ was defined as H-score > 1, and PgR+ was defined as H-score > 20. HER-2 scoring was done as per the manufacturer’s instructions. Fluorescence in situ hybridization (FISH) of HER2 was done following the manufacturer’s instructions (Vysis PathVysion). For the purpose of this study, paired samples from patients with ERα−/PgR− breast cancer at diagnosis were excluded (n = 16), as were those for whom paired data were not available on ERα, PgR, and HER2 (n = 12, due to insufficient malignant tissue in one set of cores).

**Statistical analysis.** Two-tailed t tests were used to assess the difference between two means. One-way ANOVA was used to assess the difference between more than two treatment groups; if a significant difference was detected among all groups, unpaired t tests were done to confirm significance. Differences were considered significant at P < 0.05. For clinical samples, the difference between mean H-scores was assessed by unpaired Mann-Whitney tests. All analyses were done using the GraphPad prism software (GraphPad Software). For combination assays, a statistical test was done to examine whether the CI differed significantly from 1. This was based on calculating the P value for the standard normal deviate calculated to test whether 1 − CI differed significantly from 0.

**Results**

**Lapatinib restores endocrine sensitivity in LTED cells.** Although parental MCF7 cells are very sensitive to E2 deprivation, going into cell cycle arrest, LTED cells proliferate in the absence of detectable E2 [25-fold increase in cell number after 6 days in DCC-stripped–FBS; data not
As such, LTED cells provide a useful *in vitro* model for postmenopausal breast cancer with acquired resistance to ED, e.g., an aromatase inhibitor. The molecular phenotype of LTED cells was consistent with our previously published work (9): LTED cells showed 2.7-fold increase in HER2 expression and increases in pHER2, HER3, and downstream pAkt/pERK compared with parental MCF7 controls (Fig. 1A). LTED cells also showed an increase in total ER and pERSer118. No pERSer167 was detected in LTED or MCF7 cells under basal conditions (data not shown). Despite increases in HER2 expression and signaling, LTED cells were no more sensitive than MCF7 cells to the antiproliferative effects of lapatinib alone (IC50 = 3-5 μmol/L; Supplementary Fig. S1).

To determine the effect of lapatinib in combination with ED on growth, cells were treated daily with medium containing postmenopausal levels of E2 (10 pmol/L; control), lapatinib in the presence of E2, E2-deprived media, or E2 deprivation + lapatinib for 6 d before cell counting. Columns, mean fold change in cell number over E2 control from triplicate wells; bars, SEM. Results are representative of three independent experiments. The difference between treatments was assessed by one-way ANOVA, and significance was confirmed by unpaired two-tailed t test. Not significant (NS), *P* > 0.05.

C, dose-response curve to 4-OHT in the absence or presence of 1 μmol/L lapatinib (LAP). Cells were treated daily with E2 and increasing 4-OHT for 6 d in the presence of vehicle or a fixed dose of lapatinib. Point, mean fold change in cell number over E2 ± lapatinib; bars, SEM. The difference between curves was assessed by paired t test: *, *P* = 0.03 for vehicle versus lapatinib in LTEDs; **, *P* > 0.05 for vehicle versus lapatinib in MCF7 cells.

Fig. 1. Lapatinib enhanced the sensitivity of LTED cells to ED and 4-OHT. A, molecular phenotype of LTED cells. Exponentially growing MCF7 and LTED cells in their basal media were harvested for immunoblot analyses of the indicated total and phosphorylated proteins. Band intensity was normalized to actin and was expressed relative to MCF7 controls. Results are representative of three independent experiments. B, growth inhibition with lapatinib, E2 deprivation, or the combination in LTED versus MCF7 cells. Cells were treated daily with medium containing postmenopausal levels of E2 (10 pmol/L; control), lapatinib in the presence of E2, E2-deprived media, or E2 deprivation + lapatinib for 6 d before cell counting. Columns, mean fold change in cell number over E2 control from triplicate wells; bars, SEM. Results are representative of three independent experiments. The difference between treatments was assessed by one-way ANOVA, and significance was confirmed by unpaired two-tailed t test. Not significant (NS), *P* > 0.05.
to that observed in MCF7 cells. In contrast, 1 μmol/L lapatinib had no effect in parental hormone-sensitive MCF7 cells in the presence or absence of 4-OHT (Fig. 1C). The dose of 1 μmol/L lapatinib selected for these studies is equal to the plasma concentration achieved using the recommended daily dose of 1,500 mg/day (28).

The antiproliferative effects of the combination were scored by generating the CIs at 25%, 50%, and 75% growth inhibition (CI25 = 0.17 ± 0.19, CI50 = 0.07 ± 0.02, and CI75 = 0.4 ± 0.12; Supplementary Table S1; ref. 26). The CIs achieved in LTED cells were all significantly less than 1 (P < 0.0001; Supplementary Table S1), showing that lapatinib and 4-OHT acted synergistically to inhibit proliferation in the resistant model. Importantly, the doses of lapatinib required to achieve synergy spanned clinically relevant doses (<1.5 μmol/L; ref. 28). In contrast, in hormone-sensitive MCF7 cells, the CI25 and CI50 were >1, consistent with antagonism, whereas CI75 was <1, but required a dose of >3 μmol/L lapatinib, which is not achieved in the clinic (Supplementary Table S1).

In LTED cells, acquired endocrine resistance is associated with enhanced ER function and lapatinib reduces ER genomic activity. LTED cells are characterized by upregulated ERα expression, and activation as measured by pER-Ser118 (Fig. 1A). ERα-mediated transcriptional activity was also significantly higher in LTED versus MCF7 cells under basal conditions (P = 0.01; Fig. 2A), whereas the expression of the ER-responsive gene, PGR, was over 10-fold greater (P = 0.0001; Fig. 2B). Treatment with lapatinib alone reduced ERα transcriptional activity by over 50% in LTED cells (P = 0.009), while having no significant effect in MCF7 cells (Fig. 2A). Lapatinib also more modestly, but significantly, reduced PGR gene expression by 20% in LTED cells (P = 0.04; Fig. 2B), while having no effect in MCF7 controls. There was no change in ESR1 gene expression with lapatinib treatment (data not shown). These results suggest that in LTED cells, ERα genomic activity is augmented by the HER2-mediated activation of the ERα.

Either lapatinib or 4-OHT on their own reduced pER-Ser118, but the combination of lapatinib + 4-OHT was...
more effective than either treatment alone (Fig. 2C). Similarly, a combination of lapatinib + ED resulted in greater reduction in pERSer118 than either strategy alone. Total ERα protein levels were upregulated with either 4-OHT or ED, but no significant changes were detected with lapatinib treatment. Lapatinib+4-OHT also resulted in greater inhibition of ERα transcriptional activity than either lapatinib (P = 0.05) or 4-OHT alone (P = 0.006; Fig. 2D), and lapatinib + ED reduced ER transcription significantly more than either ED (P = 0.009) or lapatinib (P = 0.03). The addition of lapatinib to ED also resulted in a small, but statistically significant decrease in the transcription of the ERα-responsive gene, PGR (data not shown).

These data suggest that in LTED cells, endocrine resistance is associated with the GFR-mediated enhancement of ER function, and that the synergy between lapatinib and endocrine therapy may be attributable to cooperative inhibition of ER signaling.

**Lapatinib enhances tamoxifen sensitivity in LTam cells.** Immunoblots of relevant molecular markers along the GFR and ERα transduction pathways were done to characterize the adaptive phenotypic changes associated with acquired endocrine resistance in LTam cells. LTam cells showed increases in total and phosphorylated HER2, HER3, and downstream pAkt and pERK compared with WT parental cells (Fig. 3A). Similar to LTED cells, despite the adaptive upregulation in HER2 and downstream signaling, LTam cells were no more sensitive to lapatinib than WT controls (Supplementary Fig. S1). However, the addition of a fixed dose of 1 μmol/L lapatinib significantly increased 4-OHT sensitivity in LTam cells (P = 0.04), while having no effect on the dose-response curve to 4-OHT in WT cells (Fig. 3B). Fixed ratio combination assays were done as previously described. Using clinically relevant doses of both drugs, the combination resulted in CI50 and CI75 significantly <1 in LTam cells showing that lapatinib and 4-OHT interacted synergistically to inhibit growth in LTam cells (CI50 = 0.38 ± 0.14 and CI75 = 0.33 ± 0.14; P < 0.0001; Supplementary Table S2). In contrast the CIs were consistent with antagonism in 4OHT-sensitive WT cells (Supplementary Table S2).

In LTam cells, acquired endocrine resistance is associated with suppressed ER activity and lapatinib reactivates ER genomic function. In contrast to LTED cells characterized by heightened ERα function, LTam cells showed no change in ERα levels compared with WT controls, and a complete loss of PgR protein expression (Fig. 3A). Basal ERα transcriptional activity in LTam cells was markedly downregulated compared with WT controls (P < 0.0001; Fig. 4A). Because PgR was almost completely lost at a protein and gene level in LTam cells (Supplementary Fig. S2; Fig. 4B), the expression of another well-described ER-responsive gene, TFF1 (data not shown), was measured by reverse transcription-PCR. TFF1 gene expression was significantly lower in LTam cell compared with WT (P = 0.04; Fig. 4C), offering further evidence that in LTam cells, the development of endocrine resistance is associated with a downregulation of ERα genomic function.

Lapatinib treatment increased ERα transcriptional activity by over 2.5-fold relative to vehicle in LTam cells (P < 0.001), while having no significant effect in WT cells (Fig. 4A). Similarly, lapatinib had no effect on TFF1 gene expression in WT cells. However, in LTam cells, lapatinib increased TFF1 mRNA levels 1.7-fold (P = 0.05; Fig. 4C), essentially restoring TFF1 expression to the level observed in WT controls. PGR gene expression remained barely detectable even with lapatinib treatment in LTam cells (Supplementary Fig. S2). Whether this gene is transcriptionally silenced by hypermethylation in LTam cells is under investigation. There was no significant difference in ESR1 gene expression in LTam versus WT cells, and lapatinib actually decreased ESR1 levels in both cell lines.
In an effort to ascertain the degree of sustained ER dependence of LTam cells, studies were conducted using the ERα downregulator fulvestrant. Despite effective ER degradation as shown by immunoblots, fulvestrant had no effect on the proliferation of LTam cells (Supplementary Fig. S3). However, a fixed dose of lapatinib restored sensitivity to the antiproliferative effects of fulvestrant, suggesting that lapatinib not only reactivated ER function in LTam cells but also restored ER-dependent growth (Supplementary Fig. S3).

Given the evidence that lapatinib alone reactivated ERα signaling in LTam cells, the effect of lapatinib in combination with 4-OHT on ERα activity was investigated next. 4-OHT achieved complete reversal of the lapatinib-induced increase in ERα transcriptional activity in LTam cells (P = 0.0001; Fig. 4C). The addition of 4-OHT also reversed lapatinib-induced increase in TFF1, and the combination resulted in greater inhibition of TFF1 than either treatment alone (P = 0.02 versus lapatinib alone and P = 0.054 versus 4-OHT alone; Supplementary Fig. S4). Because of the enhancement of ERα signaling by lapatinib, it was important to determine whether lapatinib inhibited growth factor–mediated signaling. There was no change in HER2 phosphorylation (Tyr1248 and Tyr1221; data not shown) with lapatinib treatment in LTam cells, which may be attributable to the fact that HER2 can be phosphorylated on several other sites. However, lapatinib reduced levels of both pAkt and pERK1/2, two key signaling molecules downstream of membrane tyrosine kinase receptors, in LTam cells (Fig. 4D), and the most effective inhibition of pAkt was achieved with a combination of lapatinib+4-OHT.

These results suggest that in LTam cells, acquired endocrine resistance is associated with GFR-mediated downregulation of ERα genomic activity and lapatinib may restore endocrine sensitivity by reactivating the functional target for endocrine therapy.

**Clinical samples.** ERα, PgR, and HER2 immunohistochemistry results from 52 patients at diagnosis and relapse following tamoxifen treatment were assessed to determine (a) whether a proportion of HER2− patients showed modest increases in HER2 expression with endocrine...
resistance, and (b) if so, how ERα expression and ERα genomic activity (as measured by PgR expression) changed within this subset?

Matching FISH data for HER2 was available on 48 pairs. In 61.5% of cases (32 of 52), there was no change in HER2 expression with relapse, whereas 10 cases showed some loss of HER2 expression (Fig. 5A) and 1 case was associated with a conversion from FISH+ to FISH-. There were separate 10 cases (19.2%) who showed increasing HER2 expression in the relapsed tumor (Fig. 6A); 2 of the patients who converted to 3+ by immunohistochemistry also converted from FISH− to FISH+, whereas the other 8 patients remained FISH−. Although the difference in HER2 phenotype was only rarely associated with a true conversion of HER2 using standard cutoffs (+ to - in 2.1% and - to + in 5.7% of cases), modest changes in HER2 protein expression were more frequent (10 of 52, i.e., 19.2%; Fig. 6A).

Within the subset with stable or decreasing HER2 (n = 42), relapse on tamoxifen was associated with a significant decrease in ERα expression (mean H-score = 103.9 versus 54.1; P = 0.0003) and a significant decrease in PgR expression (mean H-score = 102.8 versus 47.5; P = 0.0004; Fig. 5B). When considering expression changes in individual patients, 79% and 76% relapsed on tamoxifen with suppressed ER or PgR expression, respectively (Fig. 5C). In contrast, when focusing on the subset of patients with increasing HER2 (n = 10), the mean ER and PgR H-scores showed no significant change (mean ER = 106 versus 81.3; P = 0.37; and mean PgR = 92.1 versus 90.5; P = 0.99; Fig. 6B). When examining changes in individual patients within the subset relapsing on tamoxifen with increasing HER2 (n = 10), there was a more balanced split between tumors relapsing with upregulation versus downregulation in ER/PgR expression (Fig. 6C). The proportion of patients relapsing with increasing PgR levels was significantly greater among the group with increasing HER2 (50%; Fig. 6C) than among the group with stable or decreasing HER2 (17%; Fig. 5C; 50% versus 17%, P = 0.038 by Fisher’s exact test).

**Discussion**

The only previously published study using lapatinib in an effort to overcome endocrine resistance *in vitro* used an MCF7-derived cell line with partial tamoxifen resistance (MCF7p; ref. 29). The investigators showed that a combination of lapatinib and tamoxifen was more effective at inducing cell cycle arrest (29), but no formal...
evaluation of synergy was done. In addition, the MCF7PF
cells used in that study showed no change in HER2 or
EGFR expression, prompting the authors to conclude that
in the setting of tamoxifen resistance, lapatinib plus ta-
moxifen may be effective irrespective of HER2/EGFR ex-
pression (29). In contrast, both models of endocrine
resistance used in our studies, LTED and LTAm cells,
showed modest adaptive increase in HER2 expression
(3- and 1.5-fold, respectively), and increased activation
of the pathway as measured by pHER2 and downstream
pAkt and pERK1/2. These changes are likely to have had
significant input on the effects of lapatinib seen in our
studies. Neither cell lines showed an increase in total
EGFR, and phosphorylated EGFR was undetectable (data
not shown). It is therefore unlikely that EGFR signaling
contributed to the endocrine-resistant phenotype in our
models.

Neither LTED nor LTAm cells achieved HER2 expression
levels comparable with HER2 amplified breast cancer;
HER2 amplified BT474 cells are known to have 100-fold
higher HER2 mRNA and protein levels than MCF7 cells
(19, 30). This together with the demonstration that LTED
and LTAm cells are no more sensitive to the antiprolifera-
tive effects of lapatinib alone than parental controls sug-
gest that these cells have not shifted completely over to
HER2-driven proliferation. Rather, our results suggest that
cross-talk develops between the HER2 and ERα pathways
as part of the endocrine resistance phenotype: lapatinib
alone had a significant impact on ERα genomic activity:
clinical cancer research
and continued dependence on E\(\alpha\) for survival. Importantly enhanced E\(\alpha\) genomic function in LTEDs is augmented by HER2 signaling, as supported by the observation that lapatinib reduced pER\(\alpha\) Ser118, E\(\alpha\)-mediated transcription and the expression of the E\(\alpha\)-responsive gene, PGR. The transcriptional activity of E\(\alpha\) is regulated by two activating function (AF) domains: AF-2 is located at the carboxy terminus and requires E2 binding, and AF-1 is located at the NH\(_2\) terminus (31, 34). AF-1 can be activated through phosphorylation at Ser118 or Ser167 by effectors downstream of HER2, and cooperate with AF-2 to increase the efficiency of E\(\alpha\) transcriptional activity (31, 34). In LTED cells, the synergy between lapatinib and endocrine therapy is likely due to the cooperative inhibition of E\(\alpha\) genomic function: either lapatinib alone or endocrine therapy alone reduce E\(\alpha\) activity, but not enough to result in growth inhibition; simultaneous targeting of both E2-mediated activation of AF-2 and GFR-mediated activation of AF-1 is required to achieve the effective suppression of E\(\alpha\) genomic function and result in synergistic growth inhibition.

In contrast, LTam cells are characterized by adaptive upregulation in HER2 and suppressed E\(\alpha\) signaling compared with WT cells. In LTam cells with GFR-mediated suppression of E\(\alpha\), lapatinib reactivated E\(\alpha\) genomic function. Despite this agonist effect on E\(\alpha\) activity, lapatinib alone did not stimulate growth in LTam cells. This may be attributable to the fact that while activating E\(\alpha\), lapatinib still inhibited mitogenic signaling through the Akt and mitogen-activated protein kinase (MAPK) pathways, thus resulting in an overall mild antitumor effect. However, these findings would suggest that if over time lapatinib treatment may restore endocrine responsiveness by reactivating E\(\alpha\) function and dependence, it is imperative that lapatinib be combined with concurrent E\(\alpha\) blockade in the clinical setting of endocrine-resistant breast cancer with GFR suppression of E\(\alpha\).

Hyperactive signaling through EGF, HER2, or ras, and downstream MAPK has been shown to repress E\(\alpha\) in vitro and result in the complete loss of E\(\alpha\) expression (16). Critically, inhibition of EGF or HER2 using gefitinib or trastuzumab has been shown to reinduce E\(\alpha\) expression in ER− breast cancer and restore response to endocrine therapy, in vitro and in vivo (32, 35). Similarly, a recent report also showed that lapatinib increased ER expression in HER2-amplified BT474 cells and clinical samples (15). In contrast, LTam cells did not show loss of ER expression relative to tamoxifen-sensitive WT controls, and lapatinib did not induce ER expression. In most studies, loss of E\(\alpha\) expression was associated with very high levels of GFR signaling, such as those achieved with endogenous HER2 amplification (15, 32), or in cell lines engineered to express constitutively active HER2, MAPK, or Ras (16). LTam cell only show modest increases in HER2. Exactly how GFR signaling represses E\(\alpha\) function in LTam cells is unclear, but cannot be attributed to the loss of E\(\alpha\) expression. The level of HER2 or MAPK activation may dictate whether ER expression or its activity is suppressed.

Possible mechanisms implicate changes in the levels of transcription factors such as the forkhead box transcription factor, FOXO3A (15), or NF-κB (36).

Consistent with our previous publication, we have shown in an updated series of paired biopsies from patients relapsing on tamoxifen that conversion from HER2− to HER2+ remains a rare event (5-6%) when using conventional cutoffs (22). However, ~20% of tamoxifen-resistant patients relapsed with modest adaptive upregulation in HER2 and either suppressed or enhanced E\(\alpha\) expression and signaling in vitro. This shows that the phenotypical alterations acquired during endocrine resistance in vitro can be observed in vivo in a subset of patients with endocrine resistance. These are retrospective observations in a few patients and do not allow any assessment of causality between changes in HER2 and E\(\alpha\)/PGR expression but do suggest that the interactions described in our cells models may be relevant to a proportion of patients with acquired endocrine resistance. Although modest upregulation in HER2 may not predict for benefit from HER2-targeted therapy alone, these adaptive changes may be relevant in the setting of acquired endocrine resistance and justify a combined ER/HER2-targeted approach. This is in part supported by the results of the recently reported EGF30008 trial of letrozole alone or in combination with lapatinib in hormone receptor–positive metastatic breast cancer (37). Although there was no benefit to the addition of lapatinib to letrozole in the hormone receptor–positive/HER2− group as a whole, there was a trend toward a benefit in the endocrine-resistant hormone receptor–positive/HER2− subset (defined as relapse within 6 months of tamoxifen). Biological correlates are awaited and may shed light on the molecular phenotype of the tamoxifen-resistant tumors that responded to the combination.

Our clinical sample data are limited by the lack of information about HER2 pathway activation. Measurements of phosphorylated markers such as pHER2 and downstream pAkt and pMAPK were not available; differing time intervals from sample acquisition to fixation made the interpretation of phosphomarkers unreliable. However, analyses of other key membrane receptor tyrosine kinases and surrogate markers of downstream signaling are under way and will be reported separately.

These models serve to illustrate the possibility that over time the acquisition of endocrine resistance may be associated with either positively or inversely related dynamic changes in the HER2 and E\(\alpha\) pathways. Although the growth factor–mediated enhancement of ER function through AF1 has been well characterized, the molecular interaction accounting for the growth factor–mediated downregulation of ER expression or function is less well described. Results presented here are largely observational and require further mechanistic studies to delineate exactly how HER2 signaling can repress the ER, and perhaps more importantly identify the intracellular signals that control whether HER2 activation results in the upregulation or downregulation of the ER pathway.
These results underscore the highly plastic and heterogeneous nature of endocrine-resistant breast cancer and the importance of making treatment decisions on the basis of the molecular profile of treated tumors rather than on the original phenotype. True conversion from HER2– to HER2+ (as defined by HER2 3+ or FISH+) is a rare event. However, given this observation, many clinicians are already performing biopsies of relapsed disease whenever possible so that patients who convert to HER2+ breast cancer (using standard cutoffs) can be offered therapies such as trastuzumab or lapatinib. Our results suggest that modest gains in HER2 occur more frequently and may be relevant in the context of acquired endocrine resistance. Patients with originally ER+/HER2– breast cancer who relapse on endocrine therapy with marginal increases in HER2 expression would be unlikely to benefit from lapatinib alone, but may benefit from a combined ER-HER2–targeted approach. This merits further investigation within a clinical trial with compulsory biopsies of relapsed disease.

Disclosure of Potential Conflicts of Interest

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