Clinical Cancer Research

Upregulation of ER Signaling as an Adaptive Mechanism of Cell Survival in HER2-Positive Breast Tumors Treated with Anti-HER2 Therapy

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Abstract

Purpose: To investigate the direct effect and therapeutic consequences of epidermal growth factor receptor 2 (HER2)-targeting therapy on expression of estrogen receptor (ER) and Bcl2 in preclinical models and clinical tumor samples.

Experimental design: Archived xenograft tumors from two preclinical models (UACC812 and MCF7/HER2-18) treated with ER and HER2-targeting therapies and also HER2+ clinical breast cancer specimens collected in a lapatinib neoadjuvant trial (baseline and week 2 posttreatment) were used. Expression levels of ER and Bcl2 were evaluated by immunohistochemistry and Western blot analysis. The effects of Bcl2 and ER inhibition, by ABT-737 and fulvestrant, respectively, were tested in parental versus lapatinib-resistant UACC812 cells *in vitro*.

Results: Expression of ER and Bcl2 was significantly increased in xenograft tumors with acquired resistance to anti-HER2 therapy compared with untreated tumors in both preclinical models (UACC812: ER $P = 0.0014$; Bcl2 $P < 0.001$ and MCF7/HER2-18: ER $P = 0.0007$; Bcl2 $P = 0.0306$). In the neoadjuvant clinical study, lapatinib treatment for 2 weeks was associated with parallel upregulation of ER and Bcl2 (Spearman coefficient: 0.70; $P = 0.0002$). Importantly, 18% of tumors originally ER-negative (ER^-) converted to ER^+ upon anti-HER2 therapy. In $ER^-/HER2^+$ MCF7/HER2-18 xenografts, ER reexpression was primarily observed in tumors responding to potent combination of anti-HER2 drugs. Estrogen deprivation added to this anti-HER2 regimen significantly delayed tumor progression ($P = 0.018$). In the UACC812 cells, fulvestrant, but not ABT-737, was able to completely inhibit anti–HER2-resistant growth ($P < 0.0001$).

Conclusions: HER2 inhibition can enhance or restore ER expression with parallel Bcl2 upregulation, representing an ER-dependent survival mechanism potentially leading to anti-HER2 resistance. *Clin Cancer Res; 21(17); 3995–4003. ©2015 AACR.*

Introduction

Breast cancer is a highly heterogeneous disease and multiple signaling pathways can mediate tumor initiation and progression. These same pathways can contribute to treatment resistance when the main driver is inhibited. Approximately 20% to 25% of breast tumors have overexpression and/or gene amplification of the human epidermal growth factor receptor 2 (HER2), which represents the dominant driver of tumor cell growth and survival. In breast cancer, HER2 is the leading member of the HER receptor family, which includes four tyrosine kinase receptors (HER1-4; ref. 1). HER2 positivity is typically associated with an aggressive tumor phenotype and shorter survival (2, 3). The introduction of effective HER2-targeting treatments in clinical practice dramatically improved patient outcome. The monoclonal antibody trastuzumab (T) represents the first agent successfully developed and approved for HER2 inhibition. More recently, additional effective anti-HER2 agents, including lapatinib (L), a dual HER1/2 tyrosine kinase inhibitor, pertuzumab (P), a monoclonal antibody that blocks HER2 heterodimerization, and trastuzumab emtansine (TDM1), an antibody–toxin conjugate, have also been approved for clinical use. Despite the remarkable efficacy of these anti-HER2 agents, treatment resistance remains a major clinical problem. Different molecular mechanisms have been suggested to cause anti-HER2 resistance (4–6). First, acquired resistance to T is associated in preclinical studies with reactivation of the redundant HER receptor layer (6). Indeed, a more complete blockade of the

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Translational Relevance

This study confirms previous evidence of enhanced ER signaling as an adaptive cellular response to effective HER2 inhibition, using a unique series of preclinical and clinical tumor specimens collected before and during the administration of different anti-HER2 therapies, in the absence of the confounding effect of co-administered chemotherapy or endocrine therapy. We also describe a parallel upregulation of Bcl2 as a mechanism of survival entirely dependent on ER activity, and potentially leading to anti-HER2 resistance. Altogether, our findings further emphasize the importance of combining endocrine with anti-HER2 therapy in $ER^+/HER2^+$ tumors, and suggest that therapeutic strategies aimed at targeting Bcl2 alone may not be beneficial to prevent/reverse anti-HER2 resistance. Moreover, the early changes in ER expression observed upon anti-HER2 therapy may support the need to reevaluate hormone receptor status, if feasible, during administration of anti-HER2 therapy in $ER^-/HER2^+$ tumors.

HER receptors, achieved with various combinations of HER inhibitors (i.e., L + T and T + P), results in more effective tumor growth inhibition both *in vitro* and *in vivo*, compared with T alone (6–8). Consistent results have been reported also in the clinical setting in multiple randomized trials (9–13).

Even in the presence of effective and sustained HER inhibition, HER2-positive ($HER2^+$) tumor cells can acquire treatment resistance. Here, treatment resistance arises from activation of various other escape pathways that can become alternative dominant drivers of cell growth and survival. Several preclinical studies suggest that one of these potential escape pathways is the estrogen receptor (ER) signaling network (6, 14). Indeed, we and others found that ER-positive (ER^+)/$HER2^+$ tumor cells with acquired resistance to L (LR) or L + T (LTR) show increased expression of ER as well as its downstream products such as the antiapoptotic protein Bcl2, compared with parental cells (6, 14). Of note, these resistant cells demonstrate persistent inhibition of the HER2 pathway (6). This suggests that activation of ER and Bcl2 may represent an alternative mechanism of cell survival in the presence of HER2 pathway blockade. Consistent with this preclinical evidence, ER positivity, reported in about half of all $HER2^+$ tumors, is associated with reduced response to HER2-targeting therapies in the clinical setting (9).

Here, we report that ER and Bcl2 expression are simultaneously increased in breast cancer xenografts treated with anti-HER2 therapies. We also show that neoadjuvant treatment with lapatinib leads to a rapid increase in ER and Bcl2 expression in patients with $HER2^+$ breast cancer and demonstrate that cotargeting ER or Bcl2 along with HER2-targeted therapy circumvents this type of resistance. We finally report that endocrine therapy delays tumor progression in the presence of restored ER expression in xenograft tumors treated with anti-HER2 therapy.

Materials and Methods

Protein extracts and immunoblots

Total protein fractions were extracted from fresh cell cultures and archived frozen xenograft tumors, for immunoblotting as previously described (6, 8). Antibodies against phosphorylated (p)-Tyr1248 HER2, total (t)-HER2, and β-actin were purchased from Cell Signaling Technology; anti-ERα was from Abcam; anti-PR and anti-Bcl2 were purchased from Santa Cruz Biotechnology. Biomarker expression levels evaluated by immunoblotting were quantified by measuring band intensity with the use of ImageJ and normalized by β-actin expression.

Xenograft studies

Animal care was in accordance with the Institutional Animal Care and Use Committee (IACUC). For evaluating ER, Bcl2, and PR protein levels by immunoblotting, UACC812 and MCF7 HER2-18 xenograft tumors, which were treated with vehicle or anti-HER2 therapy, collected and stored previously in two independent published studies (6, 8), were used. We performed two additional *in vivo* experiments using MCF7 HER2-18 xenograft models. In the first experiment, mice bearing MCF7 HER2-18 tumors were treated with estrogen deprivation (ED) by estrogen (E_2) pellet removal, starting from a tumor volume of ≈200 mm^3. At the time of ED resistance (≈70 days), treatment with the anti-HER2 regimen T + P + gefitinib (TPG) was started and tumors were harvested after 7 or 14 days for ER evaluation, assessed by immunohistochemistry (IHC), as described below. In the second experiment, mice bearing MCF7 HER2-18 tumor xenografts were treated with ED until the development of resistance. At that point, animals were randomized to receive TPG with or without continuing ED. In all the aforementioned experiments, tumor volume was measured weekly as previously reported (7).

Immunohistochemistry

Archived formalin-fixed paraffin embedded (FFPE) tissue specimens collected in a neoadjuvant L trial were organized into 2-mm core tissue arrays and processed as previously described (6, 7, 15, 16). Tissue sections were incubated with primary antibody against ER (Vector Labs), PR, Bcl2, and Ki-67 (Dako Cytomation), t-HER2 (Thermo Scientific/Neomarkers), or p-Tyr1221/1222 HER2 (Cell Signaling Technology). Immunodetection was performed with the EnVision+ System (Dako). ER and PR expression was assessed according to the Allred score (17); Bcl2 and Ki67 were reported as percentage of positive cells. Levels of t- and p-HER2 were measured as signal intensity (0–3).

Cell line culture conditions and drugs for *in vitro* studies

The human breast cancer cell line UACC812, originally purchased from the American Type Culture Collection, was cultured as previously reported (6). UACC812 cells resistant to lapatinib (LR) were derived from parental cells *in vitro* and maintained as previously described (6). Both parental and LR cell lines were authenticated by DNA sequencing and immediately frozen in our laboratory. Upon thawing, cell morphology, signaling, and sensitivity/resistance to L were maintained. L (LC Laboratories), and ABT-737 (Selleckchem) were dissolved in dimethyl sulfoxide (DMSO). Fulvestrant (Ful) (AstraZeneca) was prepared with ethanol.

Cell growth, apoptosis, and signaling assays

In vitro UACC812 cell growth was assessed in the presence of different treatments using a methylene blue assay, as previously reported (6). Apoptosis was evaluated by using the Annexin V-FITC Apoptosis Detection Kit (Abcam). UACC812 cells (20,000/well) were plated 24 hours before starting each treatment. Annexin V was measured 24 hours after treatment initiation,

according to the manufacturer's instructions. Cells positive for Annexin V were counted by using the Celigo Cytometer (Cyntellect). The results were expressed as fold change relative to control, and calculated by (number of Annexin V–positive cells/total number of cells) for each treatment, divided by (number of Annexin V–positive cells/total number of cells) for control. Both the cell growth and apoptosis assays were performed in quadruplicate. For signaling analysis, cells were harvested after 72 hours of treatment.

Statistical analysis

Normalized ER, Bcl2, and PR expression levels in UACC812 and MCF7 HER2 18 *in vivo* xenograft tumors were compared among different treatment arms by using one-way analysis of variance followed with Tukey–Kramer adjustment for multiple pairwise comparisons. In the neoadjuvant L study, clinical response rate was defined according to the response evaluation criteria in solid tumors (RECIST). Correlations among biomarkers at baseline and among biomarker changes were evaluated by Spearman rank correlation, whereas the association between biomarker changes and clinical response (complete or partial responders vs. no responders) was determined by the Wilcoxon rank sum test.

In vitro analysis, cell growth, and apoptosis in UACC812 parental and LR cells under various treatment conditions were assessed using one-way analysis of variance. Differences between groups were then determined by multiple pairwise comparisons with Tukey–Kramer adjustment. Error bars on plots represent standard error (SE).

Xenograft tumor growth curves were generated using the mean tumor volume at each time point. Error bars represented the standard error of the mean. Mice treated with anti-HER2 therapy (TPG) ± endocrine therapy were divided into responders and nonresponders. Response was defined as a decreased or stable tumor size after two consecutive measurements, compared with baseline. Tumor progression was defined as 1.5-fold increase of tumor volume compared with baseline. Time to progression was measured from the randomization day to the occurrence of tumor progression, and compared between treatment groups with Kaplan–Meier survival analysis and Generalized Wilcoxon test. Statistical analyses were done using SAS 9.2 (SAS institute Inc.).

Results

Acquired resistance to anti-HER2 therapy is associated with increased expression of ER, Bcl2, and PR, in two xenograft models *in vivo*

To test whether upregulation and/or reactivation of ER signaling occurs at the time of acquired anti-HER2 resistance *in vivo*, we assessed ER expression levels and ER's downstream gene products Bcl2 and PR in archived xenograft tumors from two experiments previously performed in our laboratory. In the first experiment (6), mice bearing $ER^+/HER2^+$ UACC812 tumor xenografts were treated with $L + E_2$ or with E_2 alone (control). Tumor tissue was harvested at the time of treatment resistance. Expression of ER protein, measured by Western blotting, was significantly higher in $L + E_2$-treated tumors in comparison with E_2-treated tumors ($P = 0.0014$; Fig. 1A). Concordant changes were observed in Bcl2 and PR protein levels (Fig. 1A).

In the second experiment, $ER^+/HER2^+$ MCF7 HER2-18 xenografts were treated with a combination of $L + T$ (LT) $+ E_2$, or with E_2 alone (control). In this instance, tumor tissue was collected either after short-term treatment during the sensitive phase, or at the time of treatment resistance (8). ER expression levels were slightly increased in LT-sensitive tumors compared with E_2-treated tumors, though this difference was not statistically significant (Fig. 1B). However, ER upregulation was marked and statistically significant in LT-resistant tumors, confirming the data in the UACC812 model (p: 0.0007; Fig. 1B). The changes in the levels of Bcl2 and PR were consistent with those of ER, showing a nonsignificant increase in LT-treated tumors harvested in the sensitive phase and a higher and statistically significant increase in the LT-resistant tumors, compared with control (Fig. 1B).

These results confirmed *in vivo* our previously conducted *in vitro* studies showing increased ER expression and transcriptional activity at the time of acquired resistance to potent anti-HER2 therapies (6). In addition, the marginal increase in ER expression and activity found in LT-treated MCF7 HER2-18 tumors while still sensitive to treatment suggests that ER reactivation may occur early after starting anti-HER2 therapy.

Neoadjuvant treatment with lapatinib results in rapid upregulation of ER and Bcl2 in breast cancer patients

To evaluate in the clinical setting the effect of anti-HER2 therapy on ER expression and activity, we analyzed tumor samples collected in a phase II neoadjuvant clinical trial enrolling patients with $HER2^+$ primary breast cancer. Forty-nine patients were treated initially with L for 6 weeks, followed by trastuzumab plus docetaxel for 12 weeks, before surgery. For this study, tumor biopsies were performed at baseline and after 2 weeks of L treatment (Supplementary Fig. S1). The expression levels of ER, PR, Bcl2, Ki67, and p- and t-HER2 were assessed by IHC. The clinical results of this trial have been previously published (15). Thirty-five of the 49 (71%) tumors were available for baseline evaluation of both ER and Bcl2. Of those, 12 (34%) were ER^+ and 23 (66%) ER-negative (ER^-). As expected, a significant inverse correlation of ER expression with t- and p-HER2 and with Ki67 was found at baseline (Table 1). However, ER expression strongly and positively correlated with both Bcl2 and PR levels, not surprising given that Bcl2 and PR are estrogen-regulated genes (Table 1). Similarly, Bcl2 expression at baseline positively correlated with PR levels, and inversely correlated with t-HER2 expression (Table 1).

Subsequently, the changes in biomarker expression after 2 weeks of lapatinib treatment were analyzed. Twenty-three pairs of tumor biopsies (47%) were available for evaluation of ER and Bcl2 at both baseline and week 2. Of those, 6 (26%) were ER^+ and 17 (74%) were ER^- at baseline. At week 2, three of the 17 (18%) ER^- tumors converted to ER^+ (Fig. 2A). In addition, in the six tumors that were originally ER^+, ER expression increased further in three cases, remained stable in two, and decreased in only one case. All five tumors with stable or increased ER expression had an increase in Bcl2 levels, whereas the one tumor with ER reduction had a parallel decrease of Bcl2 expression (Fig. 2B). In addition, all three tumors converting from ER^- to ER^+ had a parallel increase in Bcl2 (Fig. 2B). However, among the 14 tumors that were ER^- both at baseline and at week 2, two had a decrease and only one had an increase in Bcl2 expression. The rest were $Bcl2^-$ at both baseline and week 2 (Fig. 2B).

Overall, Bcl2 expression increased in eight of the nine (89%) ER^+ tumors (including the three tumors converting to ER+), and increased in only one of the 14 (7%) tumors that were persistently

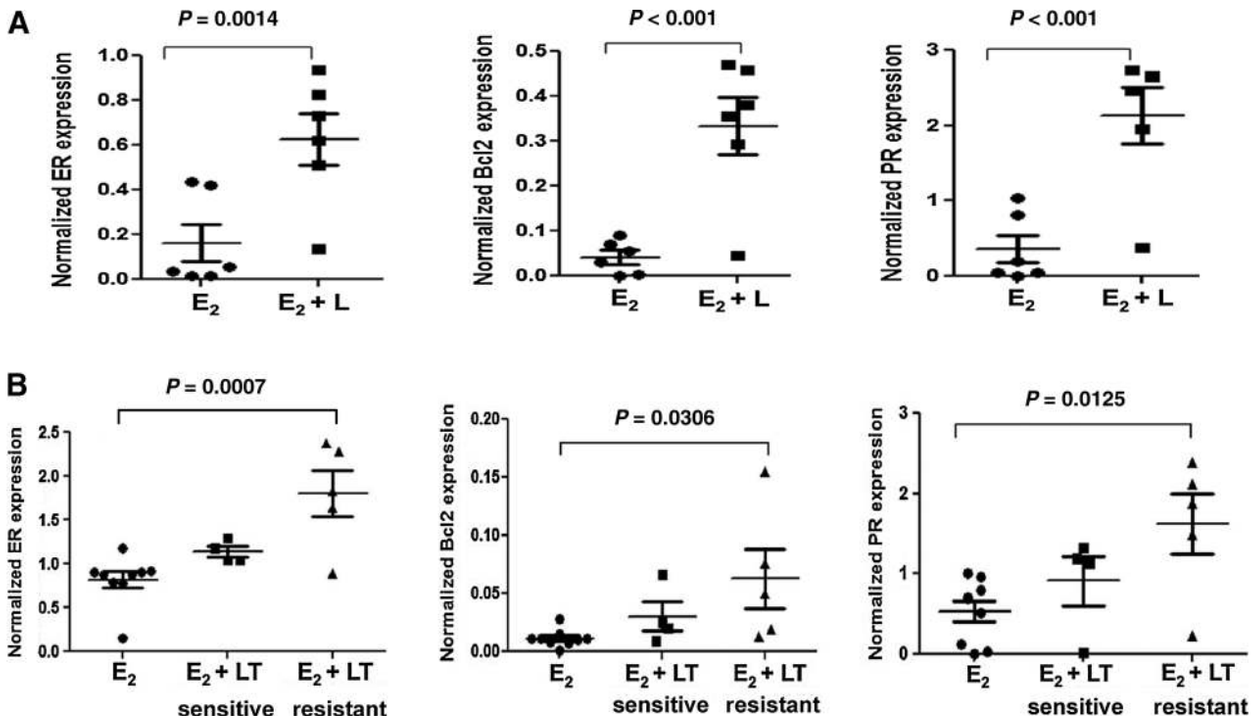


Figure 1.
Expression of ER and its downstream gene products Bcl2 and PR increases at the time of acquired resistance to HER2-targeting therapy in two different $HER2^+/ER^+$ preclinical models *in vivo*. A, expression levels of ER, Bcl2, and PR in UACC812 tumor xenografts grown only in the presence of estrogen (E_2; $n = 6$) and treated with lapatinib in the presence of E_2 ($E_2 + L$; $n = 6$). L-treated tumors showed a significant increase in the expression of ER, PR, and Bcl2, compared with tumors treated with vehicle. B, expression levels of ER, Bcl2, and PR in MCF7 HER2-18 tumor xenografts grown in presence of E_2 with ($n = 9$) or without ($n = 9$) lapatinib + trastuzumab (LT). Tumors treated with LT were harvested either when still sensitive to the treatment ($n = 4$) or at the time of acquired resistance ($n = 5$; ref. 8). LT-sensitive tumors showed a nonsignificant trend toward increase in ER expression, as well as in Bcl2 and PR levels, compared with control tumors (E_2). LT-resistant tumors showed a significant increase in ER, Bcl2, and PR expression compared with control.

ER^- (Fig. 2C). As expected, these changes in Bcl2 expression positively and significantly correlated with levels of PR expression (Table 2). No correlation between changes in either ER or Bcl2 and clinical response rate, evaluated after 6 weeks of L treatment, was observed.

We assessed ER expression levels also in archived tissue samples from a previously described phase II neoadjuvant trial with trastuzumab (16). Importantly, in these $HER2^+$ breast tumors treated with trastuzumab we found changes in ER expression that were consistent with those observed in the lapatinib trial (Supplementary Fig. S2). In this instance, conversion from ER^- to ER^+ was observed in one out of 18 cases (6%; Supplementary Fig. S2). Bcl2 expression was not assessed on tumor samples in that study due to insufficient tumor.

Altogether, these clinical results suggest that effective inhibition of the HER2 pathway can lead to rapid activation and restoration of ER expression and signaling and is associated with a parallel upregulation of Bcl2.

Table 1. Correlation among biomarkers at baseline

Marker	Correlation with ER Spearman coefficient	*P*
t-HER2 ($N = 36$)	−0.55	0.0005[a]
P-HER2 ($N = 36$)	−0.37	0.028[a]
Ki67 ($N = 32$)	−0.39	0.027[a]
PR ($N = 34$)	0.57	0.0004[a]
Bcl2 ($N = 35$)	0.75	<0.0001[a]

Marker	Correlation with Bcl2 Spearman coefficient	*P*
t-HER2 ($N = 35$)	−0.43	0.0097[a]
P-HER2 ($N = 35$)	−0.23	0.1932
Ki67 ($N = 32$)	−0.22	0.2229
PR ($N = 35$)	0.53	0.0015[a]

[a]Statistically significant.

ER signaling inhibition is required to overcome lapatinib resistance in an $ER^+/HER2^+$ preclinical model *in vitro*

To establish whether the parallel changes of Bcl2 and ER observed in patient tumors treated with L could contribute to anti-HER2 resistance, we tested the effect of Bcl2 and ER inhibition *in vitro* using the UACC812 cell line model. We have previously shown that L-resistant UACC812 cells show a sustained inhibition of HER2 and its downstream signaling molecules (6). It is noteworthy that these resistant cells display increased expression of ER and Bcl2, similar to what we observed in tumor samples from patients treated with L. The effects of Bcl2 and ER inhibition by ABT-737 and fulvestrant, respectively, on tumor cell growth and apoptosis were tested in parental versus L-resistant UACC812 cells. The Bcl2 inhibitor alone significantly, although modestly, reduced cell growth in L-resistant cells compared with control ($P = 0.0029$), whereas it had no effect in parental cells (Fig. 3A). In contrast, inhibition

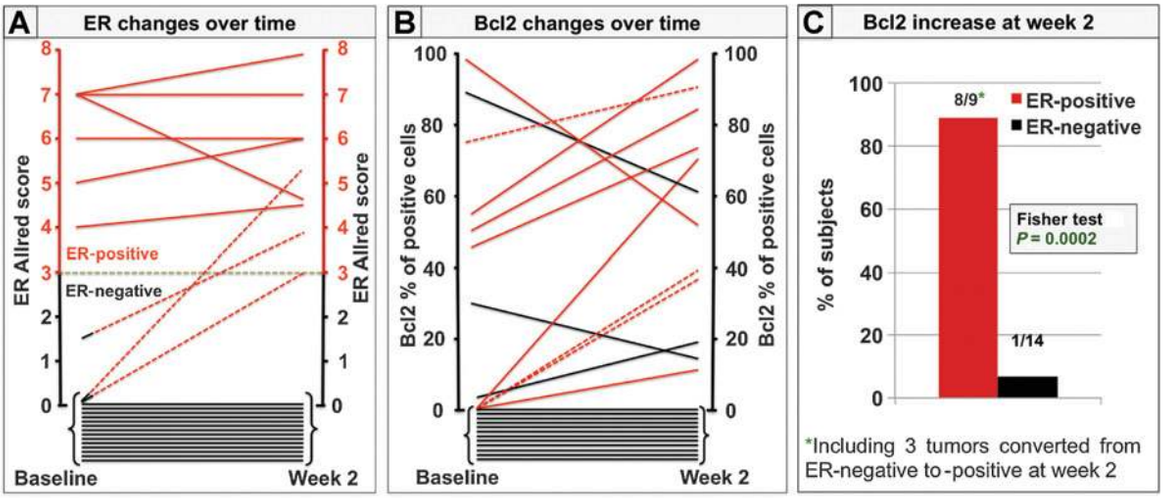


Figure 2.
ER and Bcl2 changes upon neoadjuvant treatment with lapatinib. A, each line represents changes in ER expression levels from baseline to week 2. ER levels are expressed as Allred scores. Solid red lines indicate tumors with ER^+ status at baseline (Allred score ≥ 3); dashed red lines indicate those three tumors that were ER^- at baseline (Allred score < 3) and became positive at week 2; solid black lines indicate tumors with ER^- status at baseline that remained negative at week 2 (14 tumors). B, each line represents Bcl2 expression changes from baseline to week 2. Bcl2 levels are expressed as percentage of positive cells. Solid red lines indicate Bcl2 changes in the 6 tumors with ER^+ status at baseline; dashed red lines indicate Bcl2 changes in the 3 tumors converting from ER^- to positive; solid black lines indicate Bcl2 changes in the 14 tumors that were ER^- at both baseline and week 2. C, percentage of tumors with a Bcl2 increase over time, according to ER status. Bcl2 increased at week 2 in 8 of 9 of the ER^+ tumors (including the three tumors converting to ER^+), and in 1 of 14 tumors that remained ER^-.

of ER signaling by fulvestrant led to complete growth arrest in L-resistant cells ($P < 0.0001$), but not in parental cells (Fig. 4A). The combination of ABT-737 with fulvestrant was not significantly superior to fulvestrant alone in L-resistant cells ($P = 0.7914$; Fig. 3A). Next, we assessed apoptosis in L-resistant and parental cells after 24 hours of treatment with either ABT-737 or endocrine therapy. ABT-737 showed a limited and nonsignificant increase in L-resistant cells compared with control ($P = 0.6492$), whereas no effect at all was observed in parental cells (Fig. 3B). In contrast, fulvestrant induced a significant increase in apoptosis in L-resistant cells compared with control ($P < 0.0001$), and no significant effect in parental cells (Fig. 3B). Finally, the combination of fulvestrant with ABT-737 resulted in a further increase in apoptosis in L-resistant cells compared with fulvestrant alone ($P = 0.0454$; Fig. 3B). Western blot analysis of protein extracts collected after 72 hours of treatment confirmed sustained inhibition of HER2 activation, as well as upregulation of ER signaling in L-resistant UACC812 cells (Fig. 3C). As expected, treatment with fulvestrant completely eliminated ER and Bcl2 upregulation. These results suggest that Bcl2 inhibition alone may have only limited efficacy in reverting anti-HER2 resistance, whereas blocking ER signaling with fulvestrant may be more effective. These data also suggest that ER-mediated growth involves multiple genes, and is not solely due to Bcl2 increase.

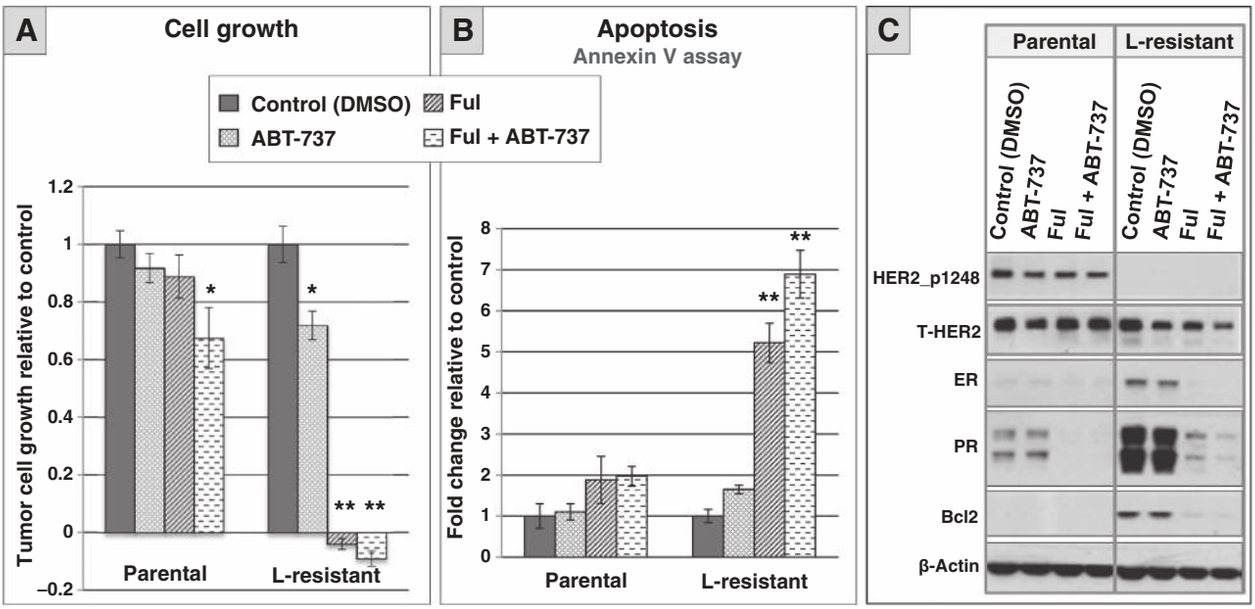
Table 2. Correlation among biomarker changes after 2 weeks of lapatinib treatment

Changes in	Correlation with Bcl2 changes Spearman coefficient	*P*
ER ($N = 23$)	0.70	0.0002[a]
PR ($N = 20$)	0.57	0.0076[a]

[a]Statistically significant.

Endocrine therapy delays tumor progression in presence of restored ER expression in xenograft tumors treated with anti-HER2 therapy

Anti-HER2 therapy with lapatinib or trastuzumab was associated in our neoadjuvant studies not only with upregulation of ER signaling in ER^+ tumors, but also, in some cases, with conversion of ER expression from negative to positive. In turn, this restored ER activity, if left uninhibited, may provide alternative proliferation and survival signals to escape anti-HER2 therapy. We tested this hypothesis using an *in vivo* model mimicking the ER plasticity observed in our clinical specimens. Specifically, mice bearing $ER^+/HER2^+$ MCF7 HER2-18 tumor xenografts ($N = 15$) received endocrine therapy with ED (to mimic aromatase inhibitors) until development of endocrine resistance and complete loss of ER expression, as previously published (18). These $ER^-/HER2^+$ xenograft tumors were then treated with the potent anti-HER2 combination of T, P, and the HER1 inhibitor G (TPG), in association with continued ED (Fig. 4A). As expected, the addition of anti-HER2 to endocrine therapy determined tumor size reduction in a substantial fraction of mice (8 of the 15, 53%), whereas the remaining tumors were *de novo* resistant (Fig. 4A). Also, adding TPG therapy induced a progressive restoration of ER levels. This ER reexpression was limited to tumors that showed early response to TPG (within the first 2 weeks; Fig. 4A). Subsequently, we sought to test whether, despite an initial response to treatment, the observed reexpression of ER could mediate tumor survival and proliferation, and ultimately result in anti-HER2 resistance, in the absence of endocrine therapy. To test this, we randomized mice bearing ED-resistant ER^- MCF7 HER2-18 tumors ($N = 30$) to receive TPG in the presence ($N = 16$) or absence ($N = 14$) of continued endocrine therapy with ED (Fig. 4B). Notably, in the presence of an initial response to treatment, where restored ER expression was observed, TPG combined with continued ED was associated with a significant delay in tumor progression,



* $P < 0.05$
** $P < 0.0001$

Figure 3.
Effects of pharmacologic inhibition of Bcl2 and ER in $ER^+/HER2^+$ cells with acquired resistance to lapatinib *in vitro*. A, tumor cell growth relative to control (DMSO) evaluated by methylene blue assay after 6 days of treatment with DMSO, ABT-737 (1 μmol/L), fulvestrant (Ful; 0.1 μmol/L), or the combination of ABT-737 with Ful, in parental and lapatinib (L)-resistant UACC812 cells. B, evaluation of apoptosis by annexin V assay after 24 hours of treatment with either DMSO (control), ABT-737 (1 μmol/L), Ful (0.1 μmol/L), or the combination of ABT-737 with Ful, in parental and L-resistant UACC812 cells. C, Western blot evaluation of phosphorylated (p)-HER2 and total (t)-HER2, and ER and its downstream gene products PR and Bcl2, in parental and L-resistant UACC812 cells after 72 hours of treatment with either DMSO, ABT-737 (1 μmol/L), Ful (0.1 μmol/L), or the combination of ABT-737 with Ful.

compared with TPG + E_2 ($P = 0.018$; Fig. 4B). However, within the group of mice that did not experience initial response to treatment (i.e., cases in which ER expression was not restored), no difference in time to tumor progression was observed between treatment arms (Fig. 4B).

Discussion

Compelling preclinical evidence (6, 8, 19) and results from multiple neoadjuvant trials (10–12, 20) demonstrate the superiority of anti-HER2 combinations over single-agent therapy in $HER2^+$ breast cancer. However, the pathologic complete response (pCR) rates achieved by the combination anti-HER2 treatment strategies in these trials were remarkably lower in the ER^+ compared with the ER^- subset of patients (9–12, 20). We hypothesized that ER signaling, if left uninhibited, can become an alternative driver of cell growth and survival in $ER^+/HER2^+$ tumors in the presence of sustained HER2 inhibition, reducing the effect of anti-HER2 therapy. In this study, we confirm in two different *in vivo* xenograft models our previous *in vitro* analyses (6) showing increased ER expression and/or activity in the presence of acquired resistance to sustained HER2 inhibition in $ER^+/HER2^+$ cells. We also found a trend towards increased ER expression in LT-treated MCF7/HER2 xenograft tumors during the early sensitive phase of treatment. This may suggest that ER upregulation can occur soon after starting anti-HER2 therapy as a result of HER2 pathway inhibition, becoming more pronounced as treatment resistance evolves. Our data are in agreement with previous studies showing an inverse relationship between ER expression and HER pathway activity in breast tumors (21). As previously reported, hyperactivation of protein kinases downstream of HER receptors, such as p42/44 mitogen-activated protein kinase (MAPK) and PI3K/Akt, results in substantial reduction or complete loss of ER expression and activity (21–27). Although the molecular mechanisms causing this negative regulation are not completely clear, it has been hypothesized that HER pathway activity mediates inhibition of ER gene transcription and reduced ER protein stability (23–27). For example, Akt inactivates the Forkhead box protein FOXO3a that represents a key regulator of ER gene transcription (23). Likewise, hyperactive p44/42 MAPK signaling has been shown to downregulate ER at both mRNA and protein levels, via mechanisms involving epigenomic proteins and NF-κB (26, 28–30).

To confirm our preclinical observation in the clinical setting, we used a unique series of tumor specimens collected in a neoadjuvant clinical trial using L monotherapy. The design of this study allowed us to confirm the presence of early changes in ER expression and signaling induced by HER2 pathway inhibition, in the absence of the confounding effects of co-administrated chemotherapy or endocrine therapy. Although these molecular changes have been previously described in patient tumors (14), our study represents the first prospective clinical trial reporting these findings in a serial collection of tissue specimens. Consistent with the upregulation of ER observed in patients treated with L, we found similar results in a different set of $HER2^+$ tumors treated

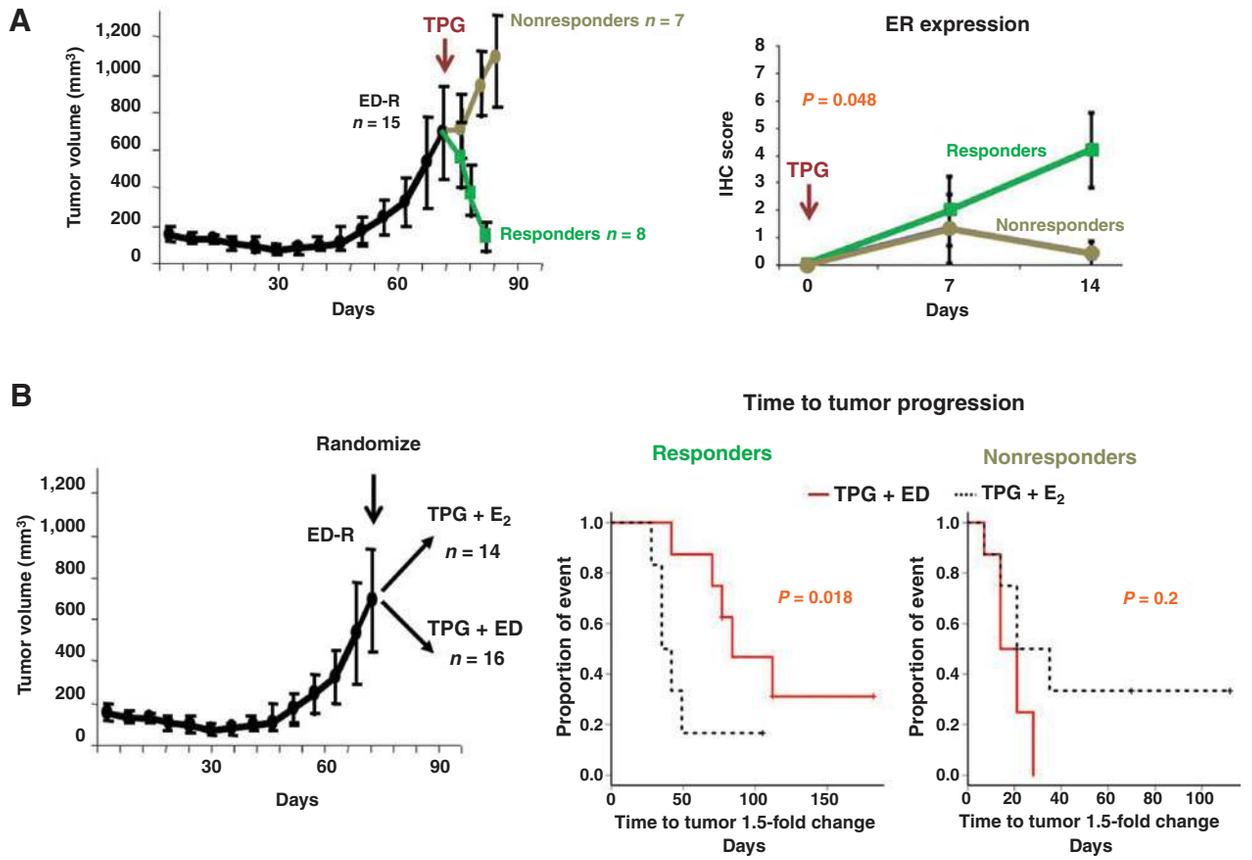


Figure 4.
Endocrine therapy delays tumor progression in presence of restored ER expression in tumor xenografts treated with anti-HER therapy. A, mice bearing MCF7 HER2-18 tumor xenografts were subjected to ED until development of resistance, which was associated with complete loss of ER expression (18). At that time, the combination of trastuzumab, pertuzumab, and gefitinib (TPG) was added to ED. Anti-HER therapy with TPG induced tumor regression in 53% of mice (responders), whereas the remaining tumors continued to grow despite the inhibition of both ER and HER pathways. Restoration of ER expression was significantly associated with tumor response. B, mice bearing MCF7 HER2-18 tumor xenografts were randomized at the time of ED resistance to receive TPG with or without continuing endocrine therapy (ED). The combination of TPG + ED was able to significantly delay tumor growth compared with TPG + E2 in the responders, but not in the nonresponders.

with neoadjuvant T, suggesting that ER upregulation can be induced also by other therapies targeting the HER2 pathway, such as T and its combination with P. This scenario might be the reason for a pCR rate of only 6% achieved in the Neo-Sphere trial by the combination of T + P (in the absence of chemotherapy chemo- and endocrine therapy) in ER^+ patients, compared with 29% in those with ER^- disease (10). In contrast, in two recent neoadjuvant trials, in patients with significantly larger ER^+ tumors L + T combined with endocrine therapy, again in the absence of chemotherapy, overall achieved higher pCR rates (20, 31). Although cross-trial comparisons have limited value, these differences are consistent with preclinical data from our and other groups, and provide intriguing hypothesis for further clinical investigation.

In addition to increased ER expression upon HER2 inhibition in baseline ER^+ tumors, we also found cases of baseline ER^- tumors, which converted to ER^+. Consistently, preclinical studies showed that inhibition of overactive HER pathway downstream kinases results in restoration of ER expression and endocrine sensitivity in breast cancer cells (21, 24, 25). To further confirm our intriguing clinical observations and the role of restored ER expression as a resistance mechanism for anti-HER2 therapy, we used an *in vivo* experimental $ER^-/HER2^+$ MCF7-HER2-18 xenograft model. Interestingly, an initial response to anti-HER2 combination regimen was significantly correlated with restoration of ER expression after two weeks of treatment. Although this finding could appear to contradict a role of ER in anti-HER2 resistance, we hypothesized that the observed ER reexpression may occur as an adaptive response to the effective blockade of HER2 signaling. However, in the tumors resistant *ab initio* to anti-HER2 therapy, the absence of ER increase may indicate that different signaling pathways mediate growth of these tumors. Importantly, addition of ED to anti-HER2 therapy in the presence of initial anti-HER2 treatment response and ER reactivation significantly delayed tumor progression compared with anti-HER2 therapy alone in the absence of ED. Collectively, these results may suggest that upregulation and/or restoration of ER represent an indicator of effective HER2 inhibition, but are also responsible for the activation of survival mechanisms that ultimately mediate anti-HER2 resistance.

The changes in ER expression levels observed in our preclinical models and in patient tumors treated with L were accompanied by increased expression of the ER target gene products PR and Bcl2. The latter has been implicated in the development of anti-HER2 resistance (32–34). In a recent study, reverse phase protein array (RPPA) analysis of *in vivo* $HER2^+$ preclinical tumors revealed a L-induced upregulation of Bcl2 family members (35, 36). However, in that study, the association of Bcl2 upregulation with ER was not assessed. Importantly, both our preclinical and clinical studies revealed that the upregulation of Bcl2 observed upon anti-HER2 treatment was entirely dependent on ER upregulation or restoration. Indeed, the changes in Bcl2 completely paralleled those in ER expression, and no discordance in the variation of these two biomarkers was observed in either xenograft or patient tumors. Moreover, in our *in vitro* UACC812 L-resistant cells with upregulation of both ER and Bcl2, inhibition of Bcl2 alone by ABT-737 only partially arrested resistant cell growth. In contrast, ER signaling blockade by fulvestrant completely reverted the resistant phenotype and abolished Bcl2 upregulation. Finally, the addition of ABT-737 to fulvestrant did not significantly enhance cell growth inhibition, compared with fulvestrant alone. These preclinical results imply that the prosurvival role of Bcl2 in anti-HER2 resistance is likely to be completely dependent on ER activity, and that additional ER-dependent survival and proliferation genes must be involved in anti-HER2 resistance. Our findings are clinically relevant as they suggest that inhibition of ER signaling may be needed in order to effectively overcome anti-HER2 resistance, whereas a Bcl2 inhibitor alone may not be effective in this setting.

The main limitation of our study is the relatively small number of available tumor biopsies for biomarker analyses. However, these tumor specimens, collected before and during the administration of only anti-HER2 therapy, are unique and represent the ideal setting to clinically confirm the direct effects of HER2 inhibition on ER and its transcriptional activity. We recognize that the upregulation of ER and Bcl2 observed in our study may at least partly be due to intratumor heterogeneity. If our findings were merely caused by tumor heterogeneity, we would expect to see upregulation as well as downregulation of these markers. However, we observed only upregulation in ER and Bcl2, suggesting that heterogeneity may not have a major contribution to our findings. Conversely, our observation may be the result of more rapid elimination of ER^- subset of tumor cells by anti-HER2 therapy, resulting in selection of ER^+ tumor cells that are more resistant to HER2-targeting treatments in absence of endocrine therapy.

Overall, our results emphasize the importance of combining endocrine with anti-HER2 therapy in $ER^+/HER2^+$ tumors. In $ER^-/HER2^+$ disease, the rapid ER reexpression and the resulting Bcl2 upregulation observed in clinical and preclinical tumors upon HER2 inhibition suggest the need to reevaluate ER status, if feasible, during administration of anti-HER2 therapy, and to cotarget ER if the tumor converts to ER^+. Our data also suggest that the increase in cell apoptosis, obtained by adding anti-HER2 treatment to chemotherapy, may be jeopardized by an ER-dependent Bcl2 upregulation, if endocrine therapy is not given simultaneously. Therefore, the dogma that endocrine therapy cannot be given simultaneously with anti-HER2 therapy in the presence of chemotherapy, due to potential antagonism with the latter, should be rechallenged in $HER2^+/ER^+$ breast cancer, a concept currently being explored in the NRG B-52 trial (37).

Disclosure of Potential Conflicts of Interest

S. Lopez-Tarruella is a consultant/advisory board member for Roche. M.F. Rimawi reports receiving commercial research grants from Glaxosmithkline. R. Schiff reports receiving speakers bureau honoraria and commercial research grants from AstraZeneca and GlaxoSmithKline, and is a consultant/advisory board member for AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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