Mechanisms of Tumor Regression and Resistance to Estrogen Deprivation and Fulvestrant in a Model of Estrogen Receptor–Positive, HER-2/*neu*-Positive Breast Cancer

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Abstract

HER-2/*neu* in breast cancer is associated with tamoxifen resistance, but little data exist on its interaction with estrogen deprivation or fulvestrant. Here, we used an *in vivo* xenograft model of estrogen receptor (ER)-positive breast cancer with HER-2/*neu* overexpression (MCF7/HER-2/*neu*-18) to investigate mechanisms of growth inhibition and treatment resistance. MCF7/HER-2/*neu*-18 tumors were growth inhibited by estrogen deprivation and with fulvestrant, but resistance developed in 2 to 3 months. Inhibited tumors had reductions in ER, insulin-like growth factor-I receptor (IGF-IR), phosphorylated HER-2/*neu* (p-HER-2/*neu*), and phosphorylated p42/44 mitogen-activated protein kinase (p-MAPK). p27 was increased especially in tumors sensitive to estrogen deprivation. Tumors with acquired resistance to these therapies had complete loss of ER, increased p-HER-2/*neu*, increased p-MAPK, and reduced p27. In contrast, IGF-IR and phosphorylated AKT (p-AKT) levels were markedly reduced in these resistant tumors. The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib, which can block EGFR/HER-2/*neu* signaling, significantly delayed the emergence of resistance to both estrogen deprivation and fulvestrant. Levels of p-MAPK and p-AKT decreased with gefitinib, whereas high ER levels were restored. Eventually, however, tumors progressed in mice treated with gefitinib combined with estrogen deprivation or fulvestrant accompanied again by loss of ER and IGF-IR, increased p-HER-2/*neu*, high p-MAPK, and now increased p-AKT. Thus, estrogen deprivation and fulvestrant can effectively inhibit HER-2/*neu*-overexpressing tumors but resistance develops quickly. EGFR/HER-2/*neu* inhibitors can delay resistance, but reactivation of HER-2/*neu* and signaling through AKT leads to tumor regrowth. Combining endocrine therapy with EGFR/HER-2/*neu* inhibitors should be tested in clinical breast cancer, but a more complete blockade of EGFR/HER-2/*neu* may be optimal. (Cancer Res 2006; 66(16): 8266-73)

Introduction

Current endocrine therapies for breast cancer target the estrogen receptor (ER) by reducing its ligand-induced activation (ovarian ablation, aromatase inhibitors), by partially blocking its function [selective ER modulators (SERM)], or by more completely blocking ER functions and inducing its degradation (fulvestrant; refs. 1, 2). Although these therapies are effective in many patients with ER-positive tumors, *de novo* and acquired resistance remain major obstacles.

Laboratory and clinical studies suggest that breast cancers that overexpress membrane tyrosine kinase receptors of the c-erb-B (HER) family may be particularly less responsive to SERMs, such as tamoxifen (3, 4). The mechanisms by which HER-2/*neu* signaling causes resistance to tamoxifen in ER-positive tumors may involve molecular cross-talk between these two pathways (5). We have recently shown that HER-2/*neu* overexpression promotes tamoxifen resistance by enhancing the rapid membrane (nongenomic) effects of ER that may be triggered at the cell membrane, which in turn activate HER signaling in response to both estrogen and tamoxifen (6). Downstream HER-2/*neu* signaling then activates ER coactivator complexes, thereby increasing the estrogen agonist effects of tamoxifen in nuclear-initiated ER signaling. The net result is tamoxifen-stimulated growth as a mechanism of *de novo* resistance. Blocking HER-2/*neu* activation using the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) gefitinib inhibits this cross-talk, eliminates agonist effects of tamoxifen on both membrane and nuclear ER functions, and restores tamoxifen-inhibited growth.

Less is known about the role of HER-2/*neu* in resistance to other endocrine therapies, such as the aromatase inhibitors, which markedly reduce serum and tumor estrogen levels, or fulvestrant, a potent ER antagonist that also induces ER degradation. We hypothesized that these ER-targeted therapies would be more effective than tamoxifen in HER-2/*neu*-overexpressing tumors. Estrogen deprivation would reduce both the nuclear and membrane ER activities, whereas fulvestrant would do the same by inducing ER degradation. Both therapies would then inhibit two critical cell proliferation and survival pathways (ER and HER-2/*neu*) and thereby inhibit tumor growth. Here, we show potent antitumor effects of estrogen deprivation and fulvestrant in a xenograft model of HER-2/*neu*-overexpressing, ER-positive breast cancer, and we examine the mechanisms of acquired resistance to these therapies. Finally, we show the therapeutic potential of growth factor receptor–targeted strategies used to subvert this form of resistance.

Materials and Methods

Reagents, hormones, and antibodies. Tamoxifen citrate was purchased from Sigma (St. Louis, MO). Gefitinib was provided by AstraZeneca (Macclesfield, United Kingdom). Immunoblotting antibodies included phosphorylated HER-2/*neu* (p-HER-2/*neu*; Tyr^{1248}), insulin-like growth factor-I receptor (IGF-IR), phosphorylated AKT (p-AKT; Ser^{437}), p-AKT

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(Thr^{308}), total p42/44 mitogen-activated protein kinase (T-MAPK), phosphorylated MAPK (p-MAPK; Thr^{202}/Tyr^{204}; Cell Signaling Technology, Beverly, MA), ERα (H-184), and p27 (Santa Cruz Biotechnology, Santa Cruz, CA).

Xenograft studies. MCF7/HER-2/*neu*-18 cells were maintained in culture as described previously (4, 7). MCF7/HER-2/*neu*-18 xenografts were established in ovariectomized 5- to 6-week-old *nu/nu* athymic nude mice (Harlan Sprague Dawley, Madison, WI) supplemented with 0.25 mg 21-day release estrogen pellets (Innovative Research, Sarasota, FL) by inoculating the mice s.c. with 5×10^6 cells as described (7). When tumors reached the size of 150 to 200 mm^3 (in 2-4 weeks), the animals were randomly allocated (n = 12) to continued estrogen, estrogen deprivation alone (removal of the estrogen pellets), estrogen deprivation plus tamoxifen citrate (500 μg/animal s.c. in peanut oil, 5 days/wk), or estrogen deprivation plus ICI 182,780 (fulvestrant, Faslodex; 5 mg/mouse s.c. once weekly) with either gefitinib (100 mg/kg, 5 days/wk) or vehicle (1% Tween 80) given via gavage. Tumor growth was assessed and tumor volumes were measured as described previously (7). Tumors were harvested after 2 to 3 weeks when they were treatment sensitive (n = 3-4) or when they became resistant and reached the size of 1,000 mm^3 (n = 8). Tumor tissues were removed from each individual mouse and kept at −190°C for later analyses. Mice were maintained and sacrificed according to institutional guidelines.

Tumor extracts and immunoblots. Three to four frozen tumors from each of the different treatment groups were manually homogenized in lysis buffer (Cell Signaling Technology) supplemented with 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Tumor lysates were collected, sonicated (five times for 5 seconds on ice), and microcentrifuged at 15,300 × g for 20 minutes at 2°C. Cell supernatants were aliquoted and stored at −70°C. Protein concentration was measured by the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's directions. Equivalent amounts of protein (25 μg) from each sample were separated under denaturing conditions by electrophoresis on 8% to 10% polyacrylamide gels containing SDS and transferred by electroblotting onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The blots were first stained with Ponceau S to confirm uniform transfer of all samples and confirm equivalent loading and then immunoblotted with the specific antibodies according to the manufacturer's directions. Briefly, blots were blocked with blocking buffer [5% (w/v) nonfat dry milk in PBS containing 0.1%, Tween 20 (PBST)] for 1 hour and then reacted with primary antibodies at dilutions according to the manufacturer's directions: for all antibodies to phosphorylated proteins in 5% bovine serum albumin in PBST overnight at 4°C, or otherwise in blocking buffer for 2 hours at room temperature. The blots were washed thrice in PBST and then incubated for 1 hour at room temperature in 5% nonfat dry milk in PBST and horseradish peroxidase–labeled anti-rabbit as secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were then washed in PBST, after which the labeled protein was visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) and exposure of the membranes on X-ray film (Kodak, Rochester, NY). T-MAPK levels served as a control for equal loading. Representative blots are presented.

Immunohistochemistry. A piece of tumor was fixed in 4% neutral-buffered paraformaldehyde overnight before processing and paraffin embedding. One 3-μm-thick section was examined by H&E staining to verify the adequacy of tumor tissue. A 4-mm core of the tumor was selected and assembled in a multitissue paraffin block. Arrangement of tumors in the tissue array paraffin block was made according to a randomly generated map. Immunohistochemistry was done on 4-μm-thick sections from these paraffin blocks. Briefly, after deparaffinization, endogenous peroxidase was blocked by 3% H_2O_2 and endogenous avidin and biotin by avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA). Two primary antibodies were used to measure ER. A mouse monoclonal antibody 6F11 (Novocastra, Newcastle upon Tyne, United Kingdom) at 1:200 dilution and a rabbit polyclonal antibody Ab-16 (Neomarkers, Fremont, CA) at 1:500 dilution were used. For ER-6F11, Animal Research kit (DakoCytomation, Carpinteria, CA) was used to decrease nonspecific binding of mouse antibody to mouse tissue. The detection system otherwise included biotinylated secondary antibody, labeled streptavidin peroxidase, and 3,3′-diaminobenzidine as chromogen (DakoCytomation). A light hematoxylin was used as counterstain. p27 was assessed using the ab7961 rabbit polyclonal antibody (Abcam, Cambridge, MA) at 1:200 dilution. Tumors were scored using the Allred score according to the estimated proportion of tumor cells that were positively stained (0-5) and according to intensity of staining (0 = none, 1 = weak, 2 = intermediate, 3 = strong). Scoring criteria, based on the estimated fraction of positively staining cells, were as follows: 0 = none; 1 < 1/100; 2 = 1/100-1/10; 3 = 1/10-1/3; 4 = 1/3-2/3; and 5 > 2/3.

Statistical analysis. Tumor growth curves were constructed using the mean tumor volume at each time point of measurement with error bars representing the SE. Median time to progression was calculated using Kaplan-Meier survival curves with 95% confidence intervals (95% CI), and tumor volume doubling was used as the end point for progression for each tumor. Comparison among groups was accomplished using a generalized Wilcoxon test. The two-sample t test was used for two-group comparisons. All statistical tests were two sided.

Results

Estrogen deprivation and fulvestrant inhibit MCF7/HER-2/*neu*-18 tumor growth. We have previously shown that MCF7 cells stably transfected with the HER-2/*neu* oncogene (MCF7/HER-2/*neu*-18) rapidly form tumors in nude mice in the presence of estrogen and are *de novo* resistant to tamoxifen compared with the wild-type MCF7 tumors (4, 6). We have now compared the sensitivity of these MCF7/HER-2/*neu*-18 tumors to estrogen deprivation, estrogen deprivation plus fulvestrant, estrogen deprivation plus tamoxifen (6), and control estrogen treatment (Fig. 1*A*). Tumor growth was strikingly inhibited by estrogen deprivation either alone or in combination with fulvestrant. As we have shown recently, estrogen deprivation plus tamoxifen stimulates tumor growth due to the enhanced estrogen agonist activity of the drug derived from its ability to stimulate the rapid membrane activities of ER (6).

To confirm the antiestrogenic effect of fulvestrant, mice bearing MCF7/HER-2/*neu*-18 tumors were randomized in a second experiment to receive estrogen supplementation, estrogen deprivation either alone or with fulvestrant, or fulvestrant with continued estrogen supplementation (Fig. 1*B*). Fulvestrant antagonized estrogen-stimulated tumor growth while showing no intrinsic agonist effect of its own in the presence of estrogen deprivation. Thus, unlike the SERM tamoxifen, fulvestrant is a more complete antagonist because it blocks both nuclear and membrane ER functions. Interestingly, fulvestrant did not completely antagonize the effects of estrogen even when it was administered twice rather than once weekly (data not shown).

Resistance develops to estrogen deprivation and fulvestrant accompanied by activation of HER-2/*neu* signaling. Despite the striking initial tumor growth suppression achieved by estrogen deprivation alone or with fulvestrant, treatment resistance developed after an average of 2 to 3 months and tumor growth resumed (Fig. 2*A*). The duration of growth inhibition by fulvestrant in these MCF7/HER-2/*neu*-18 tumors was particularly short compared with the prolonged growth-inhibitory effects of fulvestrant in the parental MCF7 tumors, which have low levels of HER-2/*neu* expression (7). These data suggest that HER-2/*neu* signaling plays a role in the rapid development of resistance to fulvestrant and possibly to estrogen deprivation.

To better understand the pathways activated when tumors progressed, we next analyzed protein extracts from sensitive and resistant tumors for p-HER-2/*neu*, total IGF-IR (an ER-dependent

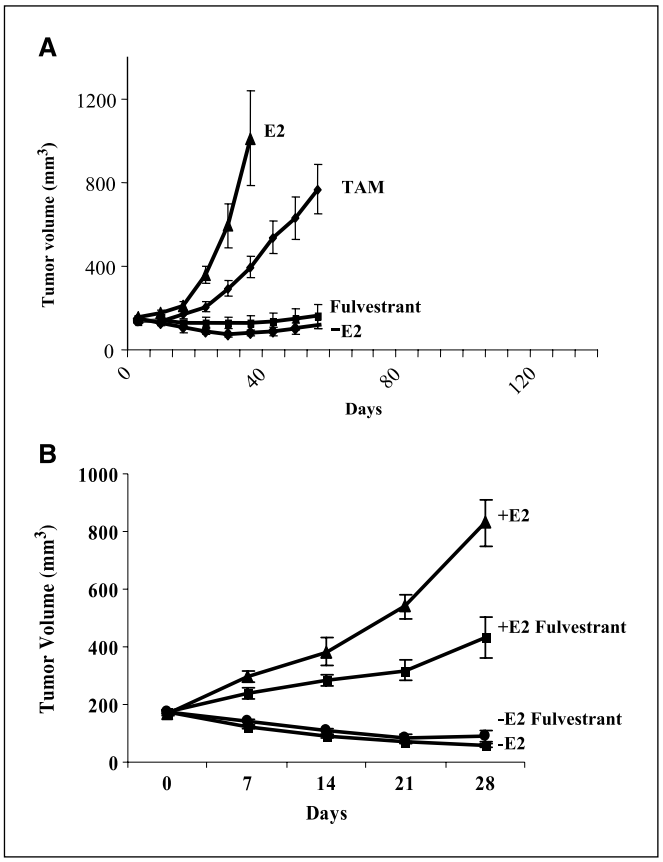

Figure 1. Estrogen deprivation and fulvestrant inhibit MCF7/HER-2/*neu*-18 tumor growth. *A,* MCF7 cells stably transfected with HER-2/*neu* (MCF7/HER-2/*neu*-18 clone) were established as xenografts in ovariectomized athymic nude mice in the presence of estrogen. Mice were then randomly assigned (day 1) in groups of eight to continued estrogen supplementation (*E2*), estrogen deprivation (*−E2*), estrogen deprivation plus the antiestrogen tamoxifen (*TAM*), or estrogen deprivation plus fulvestrant, and tumor volumes were followed over time. *Points,* mean of tumor volumes in each treatment group; *bars,* SE. *B,* mice bearing established MCF7/HER-2/*neu*-18 tumors in a second experiment were randomized on day 1 to continued estrogen supplementation, estrogen deprivation alone, fulvestrant in the continued presence of estrogen, or fulvestrant in the absence of estrogen. *Points,* mean of tumor volumes in each treatment group; *bars,* SE.

gene product), and the signaling kinases p-AKT and p-MAPK that are activated downstream from HER-2/*neu* and IGF-IR (Fig. 2*B* and *C*). Compared with the estrogen-stimulated tumors and the tamoxifen *de novo*–resistant/stimulated tumors (shown here as control), both estrogen-deprived and fulvestrant-inhibited tumors had markedly reduced levels of p-HER-2/*neu*. Treatment with estrogen deprivation and fulvestrant also markedly reduced levels of the downstream signaling molecule p-MAPK, whereas T-MAPK levels were unchanged (Fig. 2*B*). In tumors that were stimulated by tamoxifen and in those that acquired resistance to estrogen deprivation or fulvestrant, however, there was a marked up-regulation of p-HER-2/*neu* and the downstream signaling molecule p-MAPK, suggesting that this pathway may mediate the resistant phenotype. Interestingly, levels of p-AKT Thr^{308}, another signaling molecule downstream of HER-2/*neu*, were markedly reduced in the tamoxifen-stimulated tumors but only slightly reduced in the estrogen-deprived and fulvestrant-sensitive tumors (Fig. 2*C*). Levels of IGF-IR, which is known to regulate AKT phosphorylation, were markedly reduced in the estrogen-deprived and fulvestrant-sensitive tumors. Interestingly, IGF-IR nearly disappeared and levels of p-AKT Thr^{308} were also markedly reduced in both the estrogen-deprived and fulvestrant-sensitive tumors as well as in the tamoxifen-resistant/stimulated tumors, indicating that the IGF-IR/AKT pathway is not involved in the resistant growth (Fig. 2*C*). p-AKT Ser^{473} showed similar findings to p-AKT Thr^{308} (data not shown).

p27, a cyclin-dependent cell cycle regulator, was increased in tumors inhibited by estrogen deprivation, consistent with its role as a mediator of cell cycle arrest. Fulvestrant, however, had an inconsistent effect on p27. In contrast, its levels were markedly reduced in the tamoxifen-stimulated and in the rapidly growing tumors harvested at the time of resistance to both estrogen deprivation and fulvestrant (Fig. 2*C*). p27 has been shown in other systems to inversely correlate with HER-2/*neu* (8), suggesting that its down-regulation may contribute to tumor growth stimulation by growth factor signaling. Staining for p27 by immunohistochemistry showed nuclear localization with little or no cytoplasmic staining and with no apparent changes in subcellular localization between the various treatment groups (data not shown).

Next, we asked whether resistance to estrogen deprivation or fulvestrant was related to changes in levels of ER itself (Fig. 2*D*). When examined by Western blot analysis, ER content was reduced in tumors whose growth was blocked by estrogen deprivation and fulvestrant and it was almost completely lost in the resistant tumors. The reduction in ER by fulvestrant was only modest, perhaps because tumors were harvested only a short time after starting treatment (2-3 weeks) and before adequate tissue levels were achieved. Using immunohistochemistry, we also found that the reduced quantitative levels of ER were caused by a marked reduction in the proportion of ER-positive cells and not by a decrease in the intensity of staining (Fig. 2*D*). Confirming the Western blot analysis, ER nuclear staining by immunohistochemistry was totally lost at the time of treatment resistance when tumors were rapidly progressing.

EGFR/HER-2/*neu* tyrosine kinase inhibition using gefitinib delays resistance to estrogen deprivation and fulvestrant. Because development of resistance to estrogen deprivation and fulvestrant in the MCF7/HER-2/*neu*-18 tumors was marked by increased p-HER-2/*neu*, activation of MAPK, and loss of ER, we rationalized that inhibition of EGFR/HER-2/*neu* signaling might delay acquired resistance to these endocrine modalities. When gefitinib, a receptor TKI selective for EGFR that blocks signals from homodimers and heterodimers (6, 9–11), was combined either with estrogen deprivation alone or with fulvestrant, there was a marked delay in the emergence of acquired resistance that was statistically significant (Fig. 3*A*). Median time to progression (defined as doubling of tumor volume) was 175 days (95% CI, 112-210 days) in the gefitinib plus estrogen deprivation group versus 91 days (95% CI, 70-140 days) in the estrogen deprivation alone group (P = 0.036). Median time to progression in the gefitinib plus fulvestrant group was 161 days (95% CI, 98 to >190 days) versus 73 days (95% CI, 63-119 days) in the fulvestrant alone arm (P = 0.003; Fig. 3*B*).

Molecular analysis revealed that gefitinib further reduced p-MAPK when combined with estrogen deprivation alone or with fulvestrant, whereas p-HER-2/*neu* remained barely detectable (Fig. 3*C*). Gefitinib treatment also reduced levels of p-AKT but was associated with increased levels of IGF-IR compared with tumors treated by estrogen deprivation and fulvestrant alone (Fig. 3*D*). When tumors later developed resistance to the combination of

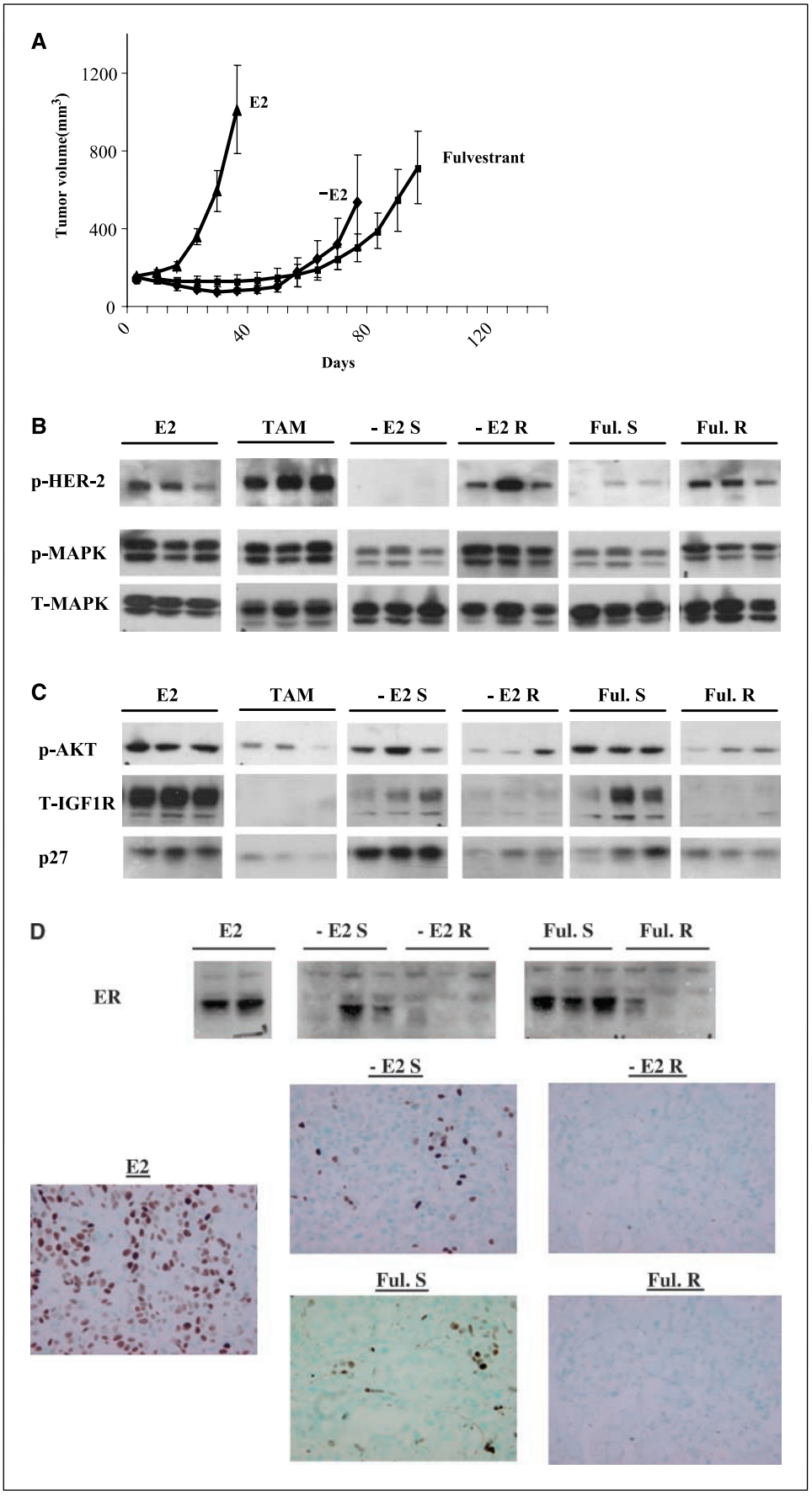

Figure 2. Resistance develops to estrogen deprivation and fulvestrant accompanied by activation of HER-2/*neu* signaling. *A,* MCF7/HER-2/*neu*-18 tumor volumes from the experiment in Fig. 1*A* were followed until tumor progression was observed. *Points,* mean of tumor volumes in each treatment group; *bars,* SE. *B,* immunoblot analysis of p-HER-2/*neu* Tyr^{1248} (*p-HER-2*), p-MAPK Thr^{202}/Tyr^{204}, and T-MAPK in three individual tumor extracts from the different treatment groups in (*A*) after 2 to 3 weeks of treatment and at the time of acquired resistance. T-MAPK levels were unchanged in the different treatment groups and also served as a control for equivalent protein loading. *E2,* estrogen-stimulated tumors; *TAM,* tamoxifen-stimulated tumors; *−E2 S,* estrogen-deprived tumors harvested at growth-inhibited phase; *Ful. S,* fulvestrant-treated tumors harvested at growth-inhibited phase; *−E2 R,* tumors harvested at the time of resistance to estrogen deprivation; *Ful. R,* tumors harvested at the time of resistance to fulvestrant. *C,* immunoblot analysis of p-$AKT^{Thr.308}$, total IGF-IR (*T-IGF1R*), and p27 in the same three individual tumor extracts from the different treatment groups as in (*B*). *D,* immunoblot analysis of total ER levels in two control estrogen-stimulated tumors and three representative individual tumor extracts from the different treatment groups and immunohistochemistry of ER nuclear staining in one representative tumor from each group.

gefitinib plus either estrogen deprivation or fulvestrant, there was in both cases an increase in p-HER-2/*neu* and a marked increase in p-MAPK. Interestingly, levels of p-AKT were now also increased in these resistant tumors, whereas IGF-IR levels remained markedly reduced (Fig. 3*D*). Hence, despite the presence of gefitinib, tumor regrowth was associated with reactivation of HER-2/*neu*, MAPK, and, in contrast to tumors that developed resistance to estrogen deprivation or fulvestrant alone, AKT.

p27 tumor levels did not seem to be increased further in mice treated by estrogen deprivation or fulvestrant combined with gefitinib, which was consistent with the complete tumor growth arrest seen with the endocrine treatment alone even without the use of gefitinib. In contrast, when tumors developed resistance to estrogen deprivation or fulvestrant plus gefitinib, levels of p27 were reduced, again consistent with the increased HER-2/*neu* activation and the resumption of tumor growth.

Gefitinib transiently restores ER expression, but resistance is marked by eventual loss of ER. These changes in growth factor signaling were associated with a striking effect on tumor ER levels (Fig. 4). In tumors treated for 2 to 3 weeks by estrogen deprivation or fulvestrant combined with gefitinib, ER levels were increased due to a much higher fraction of cells expressing ER. However, despite the initial preservation of ER in tumors treated with gefitinib and estrogen deprivation or fulvestrant, when these

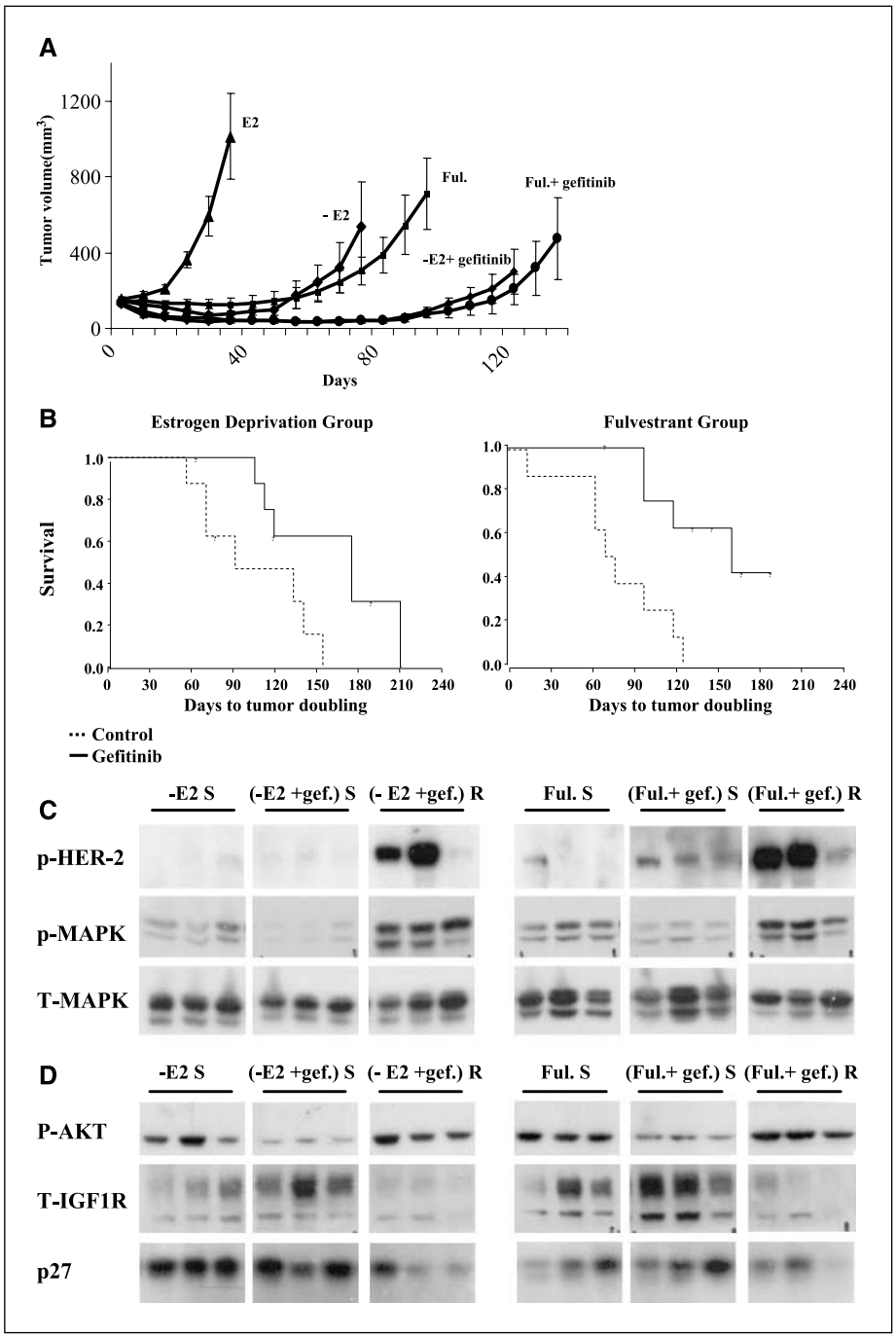

Figure 3. EGFR/HER-2/*neu* tyrosine kinase inhibition using gefitinib delays resistance to estrogen deprivation and fulvestrant. *A,* mice bearing established MCF7/HER-2/*neu*-18 tumors were randomized (on day 1) to receive estrogen deprivation or estrogen deprivation plus fulvestrant, each group either alone or in combination with gefitinib. *Points,* mean (n = 8) of tumor volumes in each group; *bars,* SE. Mice randomized to estrogen supplementation alone as a control arm. *B,* Kaplan-Meier survival curves of treatment groups in (*A*) showing time to treatment failure as defined by doubling of tumor volume relative to the lowest post-treatment value. *C,* immunoblot analysis of p-HER-2/*neu* Tyr^{1248}, p-MAPK Thr^{202}/Tyr^{204}, and T-MAPK in three individual tumor extracts from the different treatment groups in (*A*). *(−E2+gef.) S,* tumors are growth inhibited by estrogen deprivation plus gefitinib; *(Ful.+gef.) S,* tumors are growth inhibited by fulvestrant plus gefitinib; *(−E2+gef.) R,* tumors are harvested at the time of resistance to estrogen deprivation plus gefitinib; *(Ful.+gef.) R,* tumors are harvested at the time of resistance to fulvestrant plus gefitinib. T-MAPK levels were unchanged in the different treatment groups and used as a control for equivalent loading. *D,* immunoblot analysis of p-$AKT^{Thr.308}$, total IGF-IR, and p27 in the same three individual tumor extracts from the different treatment groups as in (*C*).

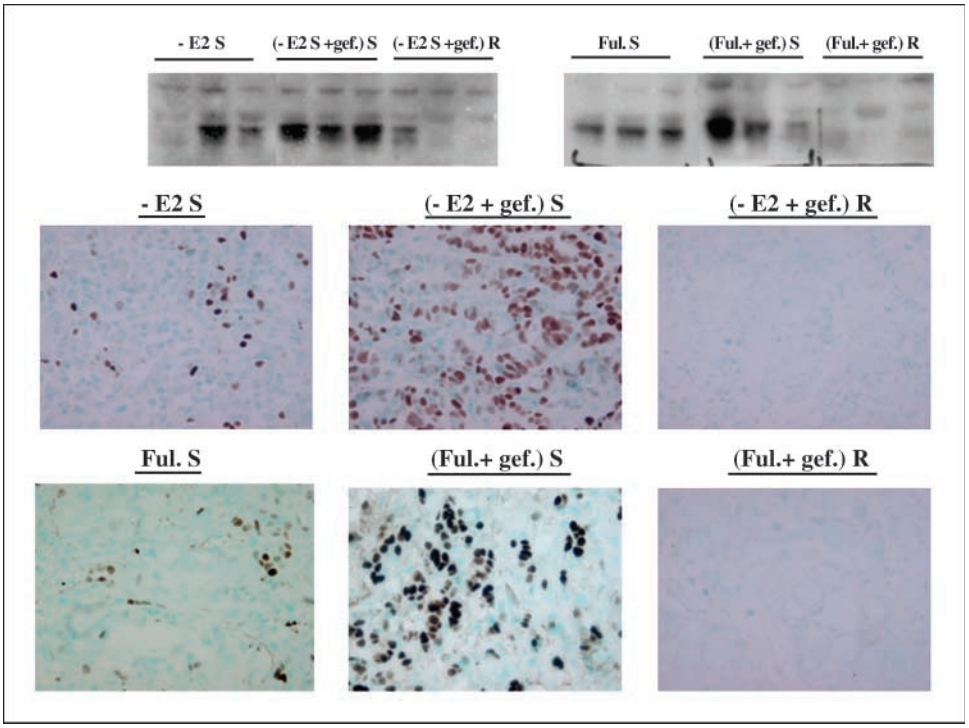

Figure 4. Gefitinib transiently restores ER expression, but resistance is marked by eventual loss of ER. Immunoblot analysis of total ER levels in three individual tumor extracts from the different treatment groups taken after 2 to 3 weeks of treatment and at the time of acquired resistance and immunohistochemistry of ER nuclear staining in one representative tumor from each group after 2 to 3 weeks of treatment and at the time of acquired resistance.

tumors eventually developed resistance, there was again a complete loss of ER similar to the observed ER loss in tumors resistant to endocrine therapy alone (Fig. 4).

Discussion

ER in breast cancer regulates tumor growth in a ligand-dependent fashion via its classic action to regulate gene transcription in the nucleus and also by activities thought to originate outside the nucleus, perhaps in the plasma membrane or cytoplasm, that have been termed its nongenomic or membrane-initiated effects (6, 12, 13). Nuclear activity increases the expression of genes regulated by ER binding to estrogen response elements or by ER tethering to other transcription factors, such as activator protein-1, in the promoters of genes that are important for cell proliferation and/or survival (12, 14). Membrane ER bound by estrogen activates growth factor signaling pathways, such as those mediated by the EGFR/HER-2/*neu* family of tyrosine kinase receptors (13, 15, 16).

Studies in cell lines and xenograft models of human breast cancer suggest that these nongenomic activities of ER may be most relevant in tumors that overexpress EGFR/HER-2/*neu* (6). In this setting, SERMs such as tamoxifen activate the nongenomic ER pathways similar to estrogen itself. Nongenomic ER signaling then activates the growth factor signaling cascade. As a consequence, downstream kinases, such as AKT and MAPK, then phosphorylate ER and its coactivator proteins, which completes the network cross-talk and further enhances classic ER activity. The net result is tamoxifen-stimulated growth as a mechanism of *de novo* resistance. Clinical studies support the idea that tamoxifen may be less effective in tumors that overexpress HER-2/*neu* (17, 18), in those that express high levels of EGFR (19, 20), or in those expressing high levels of HER-2/*neu* and the ER coactivator AIB1, a protein that is functionally activated by downstream kinases in the HER-2/*neu* pathway (21).

We hypothesized that HER-2/*neu*-overexpressing ER-positive tumors would be highly dependent on estrogen because the hormone would activate both genomic and nongenomic ER functions and also activate the EGFR/HER-2/*neu* pathway. ER-targeted therapies designed to block both ER functions would then be more effective in inhibiting tumor growth than SERMs, such as tamoxifen. Aromatase inhibitors markedly reduce the levels of estrogen available to activate the genomic and/or nongenomic ER, and fulvestrant, which has little or no intrinsic estrogen agonist activity, degrades ER. The data presented here support this hypothesis. Both estrogen deprivation (similar mechanistically to aromatase inhibition) and fulvestrant were very effective growth inhibitors of MCF7/HER-2/*neu*-18 tumors. This result confirms the highly estrogen-dependent nature of these HER-2/*neu*-overexpressing tumors, and it suggests that estrogen, acting through nongenomic ER activity, is the dominant effector of HER-2/*neu* activation in this model. Estrogen deprivation and fulvestrant coincidentally reduced p-HER-2/*neu* and p-MAPK levels in these tumors presumably by inhibiting the nongenomic as well as the genomic effects of ER, which are activated in mice receiving estrogen and tamoxifen.

Three recently completed neoadjuvant trials also suggest that estrogen deprivation therapy is very effective in the subset of patients with tumors amplified for HER-2/*neu* (22–24). Although the HER-2/*neu*-positive subgroups in each study had small numbers of patients, they all show very high response rates to the aromatase inhibitors letrozole and anastrozole in the HER-2/*neu*-positive subset and much lower response rates to tamoxifen. In fact, the response to aromatase inhibitors was numerically higher in the HER-2/*neu*-positive subsets than in the HER-2/*neu*-negative subsets, suggesting that HER-2/*neu* overexpression does not adversely affect response to these endocrine therapies despite the somewhat lower quantitative ER levels typically found in such tumors (25). There are no clinical trial data that comment on the role of HER-2/*neu* in fulvestrant response, but our results suggest

that, similar to aromatase inhibitors, fulvestrant would be effective in treating HER-2/*neu*-positive breast cancer.

Because each of these trials used a neoadjuvant design with treatment given for only 3 or 4 months before surgical resection of the primary tumor, the effect of HER-2/*neu* expression on long-term disease control could not be assessed. In our model system, the duration of response was relatively short. Although estrogen deprivation and fulvestrant were initially very effective in blocking growth of MCF7/HER-2/*neu*-18 xenografts, acquired resistance developed more quickly than in the parental MCF7 cells that have low HER-2/*neu* expression (7). The resistance was accompanied by increased HER-2/*neu* and MAPK activation and by a striking loss of ER and IGF-IR, an ER-regulated protein. We do not yet know if this ER loss is due to the selection of an ER-negative clone of cells from a heterogeneous mixed population, to reduced ER expression because of enhanced HER-2/*neu* signaling, or to increased protein degradation. The marked ER loss after only 2 to 3 weeks of treatment when tumor volumes remained stable argues against cell selection, although the decline in the proportion of ER-positive cells, but not in staining intensity in the positively staining cells, argues for it. This loss of ER is in distinct contrast to acquired resistance to estrogen deprivation in the parental MCF7 cells, which develop very high levels of ER and increased sensitivity to estrogen when resistance develops both *in vivo* and *in vitro* (26, 27). ER loss has previously been reported with estrogen deprivation in other cell lines *in vitro* (28). An inverse relationship between ER and HER-2/*neu* expression in human breast cancers and down-regulation of ER expression by HER-2/*neu* signaling in cell lines have been reported (25, 29), and reduced ER levels were linked to enhanced MAPK and nuclear factor-κB activation as one mechanism for ER down-regulation (29). Further studies to examine the mechanisms of ER loss in this model are currently in progress.

Long considered a relatively stable phenotype in human breast cancer, loss of ER is not a common mechanism of resistance to endocrine therapy (30), but there is little data on changes in ER level in HER-2/*neu*-positive tumors before and after endocrine therapy. Based on our data, assessment of ER levels on progression after endocrine therapy, especially in patients with HER-2/*neu*-positive tumors, is a clinically relevant question. ER loss, if it were to occur frequently in that situation, would preclude the use of subsequent endocrine manipulation. On the other hand, therapies targeting HER-2/*neu* might be used together with estrogen deprivation therapy to maintain ER and to prevent or delay acquired resistance.

We also observed loss of IGF-IR in tumors resistant to estrogen deprivation or fulvestrant. Down-regulation of IGF-IR coincident with ER loss is not surprising because this receptor protein is an ER-regulated gene product (31, 32). The loss of detectable IGF-IR expression coupled with low levels of p-AKT suggests that the IGF-IR pathway does not contribute to resistance to these endocrine therapies in this model. IGF-IR expression is also not associated with resistance to gefitinib-based therapy in our model, in contrast to prior reports implicating IGF-IR in resistance to HER-2/*neu* and EGFR-targeted therapy in other tumor model systems (33–35). The reduction in p-AKT coincident with loss of ER and IGF-IR in tumors resistant to estrogen deprivation and fulvestrant suggests that initially the IGF-IR pathway may have been the predominant signal for AKT activation in these tumors.

Because resistance to estrogen deprivation and fulvestrant was associated with reactivation of HER-2/*neu* and MAPK, we asked whether inhibition of this pathway would prevent or delay resistance. Resistance was delayed with gefitinib, an EGFR-selective TKI that blocks downstream signals coming from EGFR homodimers and EGFR/HER-2/*neu* or HER-3 heterodimers (9, 10). p-MAPK was reduced and high ER levels were restored after 2 weeks of gefitinib treatment during which tumor size remained stable. Whether the reemergence of detectable ER is due to clonal selection or increased expression of ER associated with MAPK inhibition (29) requires further study and is currently being investigated.

Eventually, tumors in our model system progress despite estrogen deprivation or fulvestrant combined with gefitinib. These "double-resistant" tumors have again lost ER and IGF-IR expression. Both HER-2/*neu* and MAPK are reactivated despite continued gefitinib treatment. One striking difference between these double-resistant tumors and tumors progressing after estrogen deprivation or fulvestrant alone is an increase in the level of p-AKT. Although the underlying mechanism for this resistance needs further clarification, one potential mechanism, given that the development of resistance seems to be independent of IGF-IR signaling, is that HER-2/*neu* is now being activated by an alternative pathway with different downstream signals. Signal activation in these gefitinib-resistant tumors could come from compensatory HER-2/*neu* homodimers or HER-2/*neu*/HER-3 heterodimers, which would not be affected by a drug that only blocks EGFR dimers. Indeed, HER-2/*neu*/HER-3 heterodimers or HER-2/*neu* homodimers may be more productive than EGFR/HER-2/*neu* heterodimers in activating the phosphatidylinositol 3-kinase/AKT pathway (36, 37). Our preliminary studies, which show that more complete blockade of HER-2/*neu* signaling in combination with endocrine therapy can lead to eradication of MCF7/HER-2/*neu*-18 tumors (38), further support the idea that reactivation of the HER-2/*neu* pathway is the most likely underlying mechanism for the resistance to endocrine therapy combined with gefitinib in this model system. However, because not all the tumors with resistance to combined gefitinib and endocrine therapy had an unequivocal increase in p-HER-2/*neu* levels, additional study is needed to determine if other mechanisms may be responsible for AKT activation and for the resistance to gefitinib. These data provide the rationale for a more complete blockade of the HER family of receptors than what is provided by an EGFR TKI alone in the treatment of ER-positive, HER-2/*neu*-positive tumors in combination with endocrine therapy.

A consistent finding in our model is the reciprocal relationship between p27 levels and HER-2/*neu* activation. p27, a cyclin-dependent cell cycle inhibitor, can also promote apoptosis and has a role in cell differentiation (39). Down-regulation of p27 is known to correlate with HER-2/*neu* overexpression through enhanced ubiquitin-mediated degradation (8). Lower p27 expression also correlates with increasing HER-2/*neu* activity in clinical breast cancer (40). Interestingly, phosphorylation of p27 by AKT impairs its nuclear import *in vitro,* and cytoplasmic p27 in conjunction with AKT activation is correlated with poor patient prognosis (41, 42). These data indicate that AKT itself, independent of HER-2/*neu*, may also contribute to tumor cell proliferation by phosphorylation and cytosolic retention of p27. We did not observe cytoplasmic staining of p27 by immunohistochemistry when p-AKT was elevated in gefitinib-resistant tumors, but total p27 was markedly reduced in these tumors, perhaps rendering any cytoplasmic immunohistochemistry signal undetectable in these rapidly growing tumors.

In summary, our data, using a unique experimental model of HER-2/*neu*-overexpressing breast cancer, show that inhibition of EGFR/HER-2/*neu* signaling may delay the emergence of resistance to treatment with estrogen deprivation and fulvestrant just as it overcomes *de novo* tamoxifen resistance in the model (6). These and other data provide the rationale for clinical testing of combined ER-targeted therapies and growth factor inhibitors especially in patients whose tumors overexpress HER-2/*neu*. Studies of tissue samples obtained before and after treatment with aromatase inhibitors and fulvestrant are urgently needed to confirm whether the resistance mechanisms observed in this model operate in patients with ER-positive, HER-2/*neu*-positive breast cancer. It will also be important to determine whether restoration of ER levels by potent HER-2/*neu* blockade also restores sensitivity to ER-targeted therapies.

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