

Tamoxifen Resistance in Breast Tumors Is Driven by Growth Factor Receptor Signaling with Repression of Classic Estrogen Receptor Genomic Function

Suleiman Massarweh,^{1,2} C. Kent Osborne,^{3,4,5,6} Chad J. Creighton,^{3,4} Lanfang Qin,^{3,4} Anna Tsimelzon,^{3,4} Shixia Huang,^{4,6} Heidi Weiss,^{3,4} Mothaffar Rimawi,^{3,5} and Rachel Schiff^{3,4,6}

¹Department of Internal Medicine and ²Markey Cancer Center, University of Kentucky, Lexington, Kentucky and ³Lester and Sue Smith Breast Center, ⁴Duncan Cancer Center, and Departments of ⁵Medicine and ⁶Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas

Abstract

Not all breast cancers respond to tamoxifen, and many develop resistance despite initial benefit. We used an *in vivo* model of estrogen receptor (ER)-positive breast cancer (MCF-7 xenografts) to investigate mechanisms of this resistance and develop strategies to circumvent it. Epidermal growth factor receptor (EGFR) and HER2, which were barely detected in control estrogen-treated tumors, increased slightly with tamoxifen and were markedly increased when tumors became resistant. Gefitinib, which inhibits EGFR/HER2, improved the antitumor effect of tamoxifen and delayed acquired resistance, but had no effect on estrogen-stimulated growth. Phosphorylated levels of p42/44 and p38 mitogen-activated protein kinases (both downstream of EGFR/HER2) were increased in the tamoxifen-resistant tumors and were suppressed by gefitinib. There was no apparent increase in phosphorylated AKT (also downstream of EGFR/HER2) in resistant tumors, but it was nonetheless suppressed by gefitinib. Phosphorylated insulin-like growth factor-IR (IGF-IR), which can interact with both EGFR and membrane ER, was elevated in the tamoxifen-resistant tumors compared with the sensitive group. However, ER-regulated gene products, including total IGF-IR itself and progesterone receptor, remained suppressed even at the time of acquired resistance. Tamoxifen's antagonism of classic ER genomic function was retained in these resistant tumors and even in tumors that overexpress HER2 (MCF-7 HER2/18) and are *de novo* tamoxifen-resistant. In conclusion, EGFR/HER2 may mediate tamoxifen resistance in ER-positive breast cancer despite continued suppression of ER genomic function by tamoxifen. IGF-IR expression remains dependent on ER but is activated in the tamoxifen-resistant tumors. This study provides a rationale to combine HER inhibitors with tamoxifen in clinical studies, even in tumors that do not initially overexpress EGFR/HER2. [Cancer Res 2008;68(3):826–33]

Introduction

The estrogen receptor (ER) is a nuclear ligand-dependent transcription factor that is believed to communicate most of estrogen's mitogenic and survival stimuli in breast cancer via direct modulation of gene expression (1). In addition to this mode of ER

action (also known as genomic action or nuclear-initiated steroid signaling; ref. 2), substantial experimental evidence for nongenomic action of ER has also recently solidified (3). This nongenomic action of ER (also known as membrane-initiated steroid signaling (MISS; ref. 2) may be mediated by the interaction between the small fraction of ER that resides in or at the vicinity of the plasma membrane and may activate various components of growth factor receptor tyrosine kinase pathways, such as insulin-like growth factor receptor-IR (IGF-IR), epidermal growth factor receptor (EGFR; or HER1), and HER2 (4–6).

Tamoxifen is a selective ER modulator, with mixed ER agonist/antagonist activities, that is thought to work by competitive blockade of the ER, thereby inhibiting estrogen-dependent gene transcription and tumor growth (7). Despite its benefit in patients with all stages of ER-positive breast cancer (8), the major obstacle to its use is treatment resistance, which either occurs *de novo* or is later acquired after initial benefit. The underlying mechanisms for tamoxifen resistance are probably multifactorial but remain largely unknown. In recent years, however, compelling evidence suggests that increased growth factor signaling, in particular the EGFR/HER2 pathway, contributes to this resistance (9). Results from numerous, although not all, clinical studies suggest that breast cancers with overexpression of HER2 and/or EGFR are less likely to benefit from tamoxifen (10–13). Using a preclinical model of breast cancer, we have recently shown that overexpression of HER2 leads to increased cross talk between ER and the EGFR/HER2 pathways, as well as enhanced MISS activity of ER. MISS is activated by both estrogen and selective ER modulators, causing tamoxifen to behave as an estrogen agonist and to stimulate growth as the mechanism of *de novo* resistance in xenograft tumors (9). When these cells are grown in tissue culture *in vitro*, short-term tamoxifen treatment also functions as an agonist on estrogen-regulated genes (9).

Less is known about the mechanisms of acquired tamoxifen resistance in breast tumors with initially low or normal levels of EGFR/HER2. Because biomarker data from human tumors progressing on tamoxifen are limited, preclinical models are crucial tools to study mechanisms and new strategies to overcome resistance. The goal of the present study was to investigate tamoxifen resistance mechanisms *in vivo* by studying ER-dependent genomic and nongenomic functions, as well as growth factor signaling, which has been implicated in resistance to endocrine therapy. We studied an experimental model of tamoxifen resistance using ER-positive MCF-7 human breast cancer cells grown as xenografts in athymic nude mice (14). Tamoxifen treatment suppresses tumor growth for several months, but growth eventually resumes as the tumors become stimulated by tamoxifen (15). Here, we show that activation of EGFR/HER2 and IGF-IR signaling, at least in part via

Requests for reprints: Rachel Schiff, Breast Center, Baylor College of Medicine, Room N1230.02, One Baylor Plaza-BCM 600, Houston, TX 77030. Phone: 713-798-1676; E-mail: rschiff@bcm.tmc.edu or Suleiman Massarweh, Markey Cancer Center, cc452, 800 Rose Street, Lexington, KY 40536. Phone: 859-257-3608; E-mail: massarweh@uky.edu.

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ER nongenomic mechanisms, mediates growth in the tamoxifen-resistant tumors, whereas classic ER genomic function remains suppressed. We also show that targeting HER signaling using the EGFR tyrosine kinase inhibitor (TKI) gefitinib (ZD1839, Iressa) can improve tamoxifen benefit and delay acquired resistance to therapy.

Materials and Methods

Reagents, hormones, and antibodies. Tamoxifen citrate was purchased from Sigma. Gefitinib was provided by AstraZeneca. Immunoblotting antibodies included total EGFR, total HER2, IGF-IR, phosphorylated IGF-IR (p-IGF-IR), Akt (Ser⁴³⁷), p42/44 MAPK (Thr²⁰²/Tyr²⁰⁴), total MAPK, and phosphorylated p38 (p-p38) from Cell Signaling Technology and ER α (H-184), progesterone receptor (A and B), insulin receptor substrate-1 (IRS-1), BCL-2, and cyclin D1 from Santa Cruz Biotechnology.

Xenograft studies. MCF-7 cells were maintained in culture as previously described (15). MCF-7 xenografts were established in ovariectomized 5-week-old to 6-week-old nu/nu athymic nude mice (Harlan Sprague Dawley) supplemented with 0.25 mg 21-day release estrogen pellets (Innovative Research) by inoculating s.c. 5×10^6 cells as previously described (16). When tumors reached the size of 150 to 200 mm³ (3–5 weeks), the animals were randomly allocated ($n = 12$ per group) to continued estrogen (E2), estrogen withdrawal alone (–E2, by removal of the estrogen pellets), estrogen withdrawal plus tamoxifen citrate (500 μ g/animal in peanut oil by s.c. injection 5 days/week), with either gefitinib (100 mg/kg, 5 days/week) or vehicle (1% Tween 80) given by gavage. Tumor growth was assessed, and tumor volumes were measured as described previously (16). Tumors were harvested for molecular studies after 3 weeks of treatment (sensitive tumors, $n = 3$ –4) and when they became resistant to treatment and reached the size of 1,000 mm³ ($n = 8$). Each tumor analyzed was from a different mouse; 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. In a separate xenograft experiment, MCF-7 HER2-18 cells (MCF-7 cells engineered to overexpress HER2; refs. 9, 17) were used to establish tumors in ovariectomized nu/nu athymic nude mice supplemented with estrogen pellets as previously described (9, 18). When tumors reached the size of ~ 200 mm³ (2–4 weeks), the animals were randomly allocated ($n = 12$ –16 per group) to receive continued estrogen supplementation alone (E2), estrogen supplementation plus tamoxifen (+E2 + TAM), estrogen withdrawal alone (–E2), or estrogen withdrawal plus tamoxifen (–E2 + TAM). Tumor growth curves were constructed as previously described (18).

Tumor extracts and immunoblots. Three to four individual 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 \times protease inhibitor cocktail (Roche Molecular Biochemicals). Tumor lysates were collected, sonicated (5 \times for 5 s on ice), and microcentrifuged at 15,300 $\times g$ for 20 min at 2 $^\circ\text{C}$. Supernatants of the lysates were aliquoted and stored at -70°C . Protein concentration was measured by the Bio-Rad protein assay kit (Bio-Rad Laboratories) according to manufacturer's directions. Equivalent amounts of protein from each sample were separated under denaturing conditions by electrophoresis on 8% to 10% polyacrylamide gels containing SDS-PAGE and transferred by electroblotting onto nitrocellulose membranes (Schleicher & Schuell). 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 with 0.3% Triton X-100 (pH 7.5) and 0.9% NaCl containing 0.1% Tween 20 (PBST)] for 1 h and then reacted with primary antibodies at dilutions per the manufacturer's directions for all phosphorylated antibodies in 5% bovine serum albumin in PBST overnight at 4 $^\circ\text{C}$ or otherwise in blocking buffer for 2 h at room temperature. The blots were washed thrice in PBST and then incubated for 1 h at room temperature in 5% nonfat dry milk in PBST and horseradish

peroxidase-labeled antirabbit (1:2,000) as secondary antibody (Amersham Pharmacia Biotech). The blots were then washed in PBST, after which the labeled protein was visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia) and exposure of the membranes on X-ray film (Kodak). All gels were run thrice (except for p-IGF-IR, which was run twice). Individual tumors for each shown marker were from gels that were run simultaneously. Representative blots are presented.

Affymetrix microarray analysis. Gene expression patterns of three estrogen-stimulated and four tamoxifen citrate-resistant/stimulated tumor xenografts were determined using U133plus2 Affymetrix Genechip Arrays; each profile was from a different tumor grown in a different mouse (seven profiles in all). RNA extraction was carried out in BioRobot EZ1 workstation using an EZ1 RNA universal tissue kit according to the manufacturer's instructions (Qiagen). cDNA synthesis, cRNA labeling, and array hybridization were performed as previously described (19). Arrays were normalized and compared using DNA Chip Analyzer software (dChip; ref. 20). Array data has been deposited in the public Gene Expression Omnibus database (accession GSE7327). Transcripts with average estimated expression in the bottom 50% of the dataset were removed from the analysis. Two-sample t tests using log-transformed data were performed as criteria for determining significant differences in mean gene mRNA levels between groups of samples. Fold changes between groups were estimated by taking the ratio between the average of the estimated expression values of the one group versus that of the other. Expression values were visualized as color maps using the Cluster and Java TreeView software (21, 22). Expression patterns from the xenograft tumor profile dataset were examined in the public profile datasets (23, 24) by joining the Affymetrix probe set identifier, and the statistical significance of the overlap between the gene sets was examined by a one-sided Fisher's exact.

Statistical analysis. Tumor growth curves were constructed using the mean tumor volume at each time point of measurement with error bars representing SEs. Median time to progression was calculated using Kaplan-Meier survival curves with 95% confidence intervals, and tumor volume tripling was used as the end point for progression for each tumor. Comparison among groups was accomplished using ANOVA. After significant differences among groups were established, contrasts were generated to perform pairwise comparisons between any two groups of \pm gefitinib with adjustment in P values due to multiple testing based on the permutation method. The two-sample t test was used for two-group comparisons. To compare the mean tumor volumes in the tamoxifen citrate group with those in the tamoxifen citrate + gefitinib group, the difference in tumor volume from each day (day 7, day 14, day 21, day 28, day 35, day 42, day 49) versus day 0 was calculated. A growth curve model on the log of this difference was fitted to compare slopes between tamoxifen citrate and tamoxifen citrate + gefitinib groups. All P values were two-tailed.

Results

Acquired tamoxifen resistance in MCF-7 tumors is associated with increased EGFR/HER2 expression. First, we examined the levels of EGFR and HER2 in tumors that were treated with tamoxifen (given along with estrogen withdrawal) and in tumors treated with estrogen withdrawal alone (Fig. 1). Compared with the control estrogen-stimulated tumors, in which EGFR was undetectable, levels of EGFR increased in the tamoxifen-sensitive tumors and increased markedly when tumors progressed, suggesting that this pathway may play a role in acquired tamoxifen resistance (Fig. 1A). In contrast, levels of EGFR were not increased in tumors resistant to estrogen withdrawal alone, suggesting that EGFR does not contribute to acquired resistance to this type of endocrine therapy in this model. Levels of HER2 were also very low or undetectable in estrogen-stimulated tumors, but upon estrogen withdrawal, whether alone or with tamoxifen, there was a modest induction of HER2 expression that persisted at the time of acquired resistance (Fig. 1B).

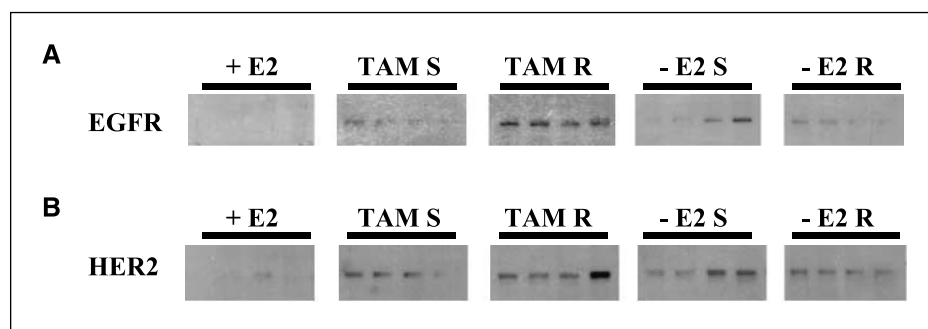


Figure 1. Acquired tamoxifen resistance in MCF-7 tumors is associated with increased EGFR/HER2 expression. *A*, immunoblot analysis of total EGFR levels in tumors stimulated with estrogen (+E2), tumor growth inhibited by tamoxifen plus estrogen withdrawal after 3 wk (TAM S), identically treated tumors at the time of resistance (TAM R), tumors responding to estrogen withdrawal alone at 3 wk (-E2 S), and identically treated tumors at the time of acquired resistance (-E2 R). The panel shows three different tumors from each of the respective groups. *B*, immunoblot analysis of total HER2 levels in the same tumors as *A*.

Gefitinib improves tamoxifen response and delays acquired resistance in MCF-7 tumors. Because acquired resistance to tamoxifen was associated with increased EGFR and HER2 levels, we hypothesized that an EGFR/HER2 pathway inhibitor might delay the emergence of resistance. Mice bearing MCF-7 tumors established in the presence of estrogen were randomized to receive continued estrogen supplementation (control), estrogen withdrawal alone, or estrogen withdrawal plus tamoxifen, each group either alone or in combination with gefitinib (an EGFR TKI that inhibits signaling from EGFR homodimers and heterodimers of EGFR with HER2 and HER3; Fig. 2; refs. 9, 25, 26). There was no growth inhibition using gefitinib as a single agent to treat estrogen-stimulated tumors, which is consistent with the undetectable levels

of EGFR in these tumors and suggests that estrogen-stimulated growth does not rely on EGFR signaling (Fig. 2A). Gefitinib significantly improved the antitumor effect of tamoxifen, however, as evidenced by the lower mean tumor volume in the combination group compared with the tamoxifen alone group (as measured by the difference in tumor volume on days 7, 14, 21, 28, 35, and 42, $P = 0.05$; see Materials and Methods). Importantly, the median time to development of resistance in the tamoxifen plus gefitinib group was significantly prolonged; 161 days with gefitinib [95% confidence interval (95% CI), 154–210] compared with 105 days (95% CI, 77–161) with tamoxifen alone ($P = 0.0149$; Fig. 2A).

Because EGFR expression is highest in resistant tumors, we investigated whether using gefitinib later would be more effective

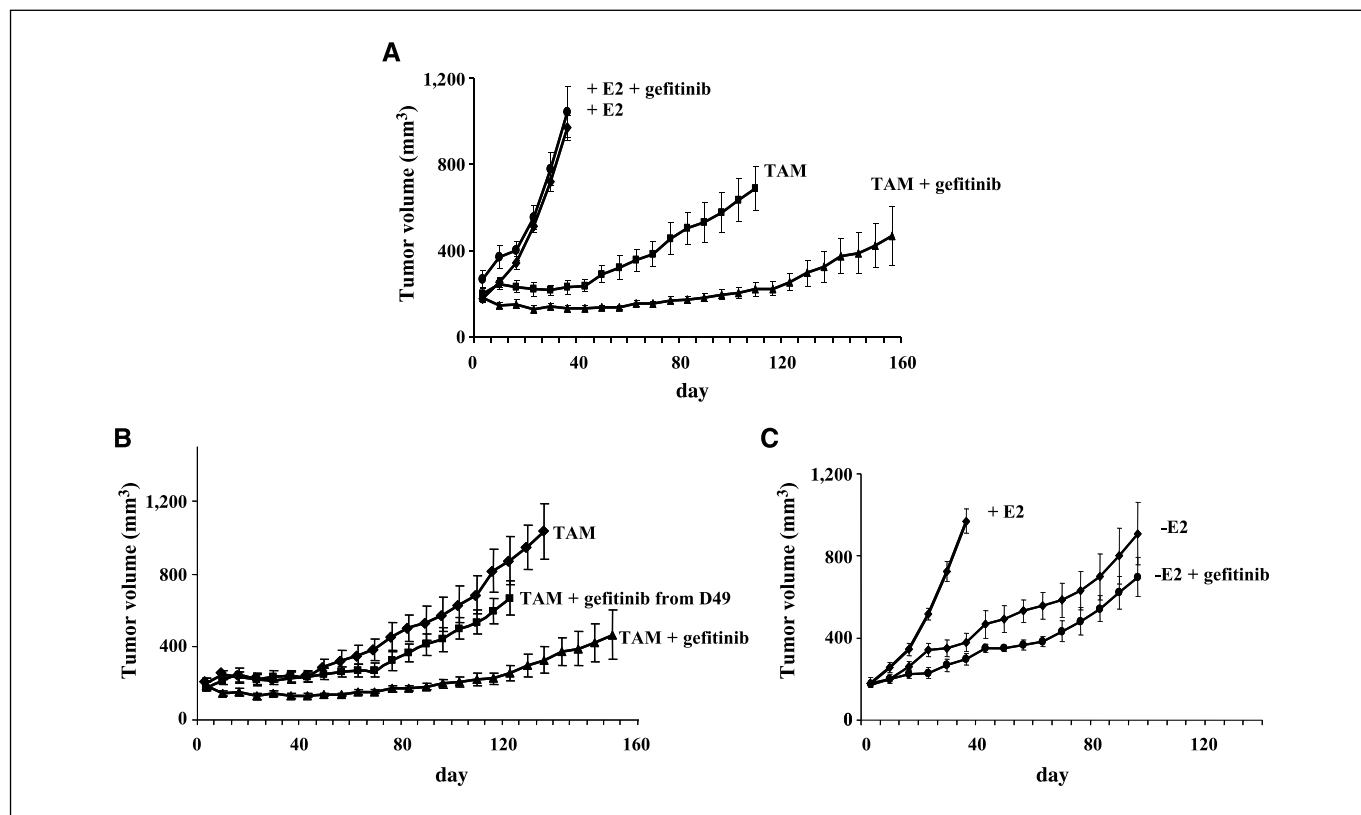


Figure 2. Gefitinib improves tamoxifen response and delays acquired resistance in MCF-7 tumors. Mice bearing established MCF-7 tumors were randomized (on day 1) to receive continued estrogen supplementation (+E2), estrogen withdrawal alone (-E2), estrogen withdrawal plus tamoxifen (TAM), each group either alone or in combination with gefitinib (+E2 + gefitinib, TAM + gefitinib, -E2 + gefitinib, respectively). An additional group of mice (in the same experiment) received estrogen withdrawal plus tamoxifen, and gefitinib was added later after 49 d of treatment at the approximate time of anticipated resistance (TAM + gefitinib from D 49). *A*, tumor growth curves showing +E2, +E2 + gefitinib, TAM, and TAM + gefitinib. *B*, tumor growth curves showing TAM, TAM + gefitinib, and TAM + gefitinib from D 49. *C*, tumor growth curve showing control +E2, -E2, and -E2 + gefitinib.

than earlier when EGFR is lower. One group of mice was treated with tamoxifen alone, and then gefitinib was added on day 49 (tamoxifen plus late gefitinib group; Fig. 2B). Although there was a trend for delayed resistance in the late gefitinib group compared with tamoxifen alone (median time to resistance of 122 days; 95% CI, 91–147), this difference was not statistically significant ($P = 0.5296$). Median time to the development of resistance in this tamoxifen plus late gefitinib group, however, was statistically significantly shorter than the group receiving tamoxifen and gefitinib from the outset ($P = 0.0137$). When gefitinib was combined with estrogen withdrawal, there was a trend for delayed resistance compared with estrogen withdrawal alone with a median time to resistance of 84 days (95% CI, 70–89) versus 42 days (95% CI, 21–133; Fig. 2C), but the difference did not reach statistical significance ($P = 0.1911$).

Gefitinib inhibits phosphorylation of EGFR/HER2 downstream signaling kinases in tamoxifen-treated MCF-7 tumors.

To explore the potential mechanisms by which gefitinib may delay the EGFR-mediated tamoxifen resistance, we examined the levels of phosphorylated MAPK (p-MAPK) 42/44, phosphorylated AKT (p-AKT), and the stress signaling intermediate p-p38 MAPK, all of which are activated downstream of EGFR/HER2 (Fig. 3A). Whereas p-MAPK 42/44 was increased in the tamoxifen-resistant tumors compared with the tamoxifen-sensitive ones, p-AKT was only minimally changed at the time of resistance. P-p38 MAPK, on the other hand, increased slightly in the tamoxifen-sensitive tumors compared with the control estrogen-stimulated tumors and increased further at the time of acquired resistance to tamoxifen as previously shown (Fig. 3A; ref. 27). Gefitinib reduced the levels of p-MAPK 42/44 and p-AKT both in the control estrogen and in the tamoxifen-treated tumors. Gefitinib also reduced the level of p-p38 in the tamoxifen-treated tumors.

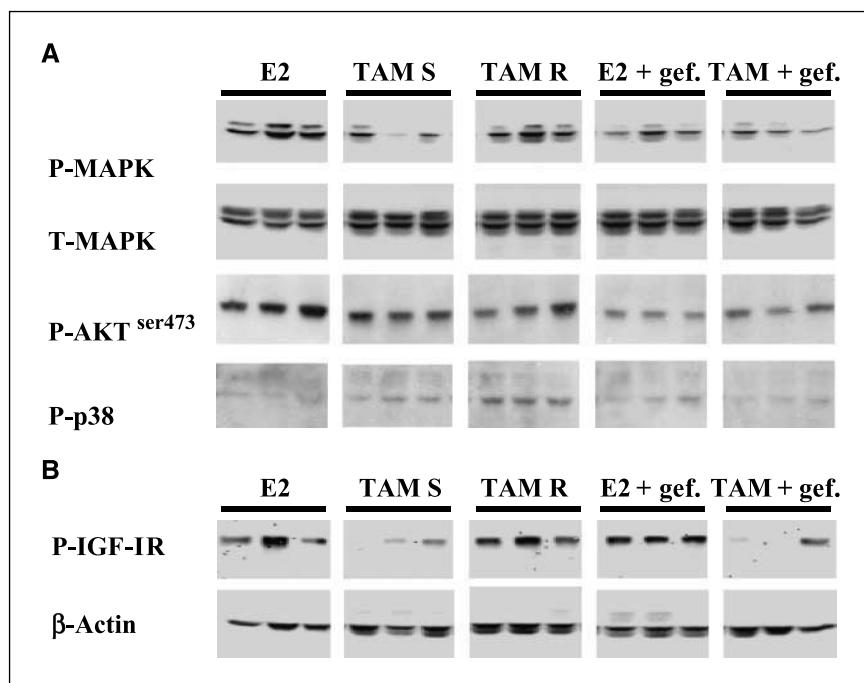
In view of recent data suggesting that the IGF-IR, via dimerizing with or indirectly activating of EGFR, may play a role in mediating ER activation of EGFR (6), we next measured levels of p-IGF-IR in

the different tumor groups (Fig. 3B). P-IGF-IR levels were reduced in the tamoxifen-inhibited tumors compared with the control estrogen-stimulated tumors, suggesting that activation of IGF-IR is mainly dependent on ER in these MCF-7 tumors. Indeed, gefitinib had little effect on phosphorylation of IGF-IR in the estrogen-stimulated tumors (Fig. 3B). Interestingly, however, p-IGF-IR levels were increased in the tamoxifen-resistant tumors compared with the tamoxifen-sensitive group, although total IGF-IR levels were markedly reduced as shown in the next figure (Fig. 4A).

Tamoxifen activates growth factor-regulated transcription but continues to suppress ER genomic function at the time of resistance. We have previously shown that MCF-7 xenografts that become resistant to tamoxifen continue to express high levels of ER (28). Interestingly, when we studied the expression levels of proteins known to be regulated by ER (progesterone receptor, IGF-IR, IRS-1, BCL-2, cyclin D1) in these resistant MCF-7 tumors, we found that levels continued to be effectively suppressed at the time of acquired resistance to tamoxifen, except for cyclin D1, which is also known to be up-regulated by growth factor signaling (Fig. 4A; ref. 29). The markedly reduced levels of total IGF-IR suggest that IGF-IR is dependent on ER for expression and is therefore reduced upon ER inhibition. However, despite the very low total IGF-IR level at the time of acquired tamoxifen resistance, remaining IGF-IR is strongly phosphorylated and therefore activated, suggesting that it may contribute to the development of resistance in this model.

To determine whether the observed down-regulation of selected ER-inducible gene targets in tamoxifen resistance was a result of a systematic shutdown of the estrogen signaling pathway, we compared global gene expression profiles of tamoxifen-resistant MCF-7 xenograft tumors with estrogen-supplemented tumors. Analysis of the data revealed 1,113 RNA transcripts that were differentially expressed between the two xenograft groups ($P < 0.01$, fold change > 1.5 ; Fig. 4B, panel 1). A set of 369 RNA transcripts, representing ~249 unique-named genes, had consistently higher

Figure 3. Gefitinib reduces phosphorylation of downstream signaling kinases in tamoxifen-treated MCF-7 tumors. *A*, immunoblot analysis of p-AKT (Ser⁴⁷³), phosphorylated (Thr²⁰²/Tyr²⁰⁴) p42/44 MAPK, total MAPK, and p-p38 in three individual tumor extracts from Fig. 1, treated by estrogen (E2), estrogen plus gefitinib (E2 + gef.), tamoxifen alone (TAM S), or in combination with gefitinib (TAM + gef.) at 3 wk of treatment, and of tamoxifen alone at the time of acquired resistance (TAM R). *B*, immunoblot analysis of p-IGF-IR in the same tumor extracts as *A*. β -Actin is shown as the control for equivalent loading.



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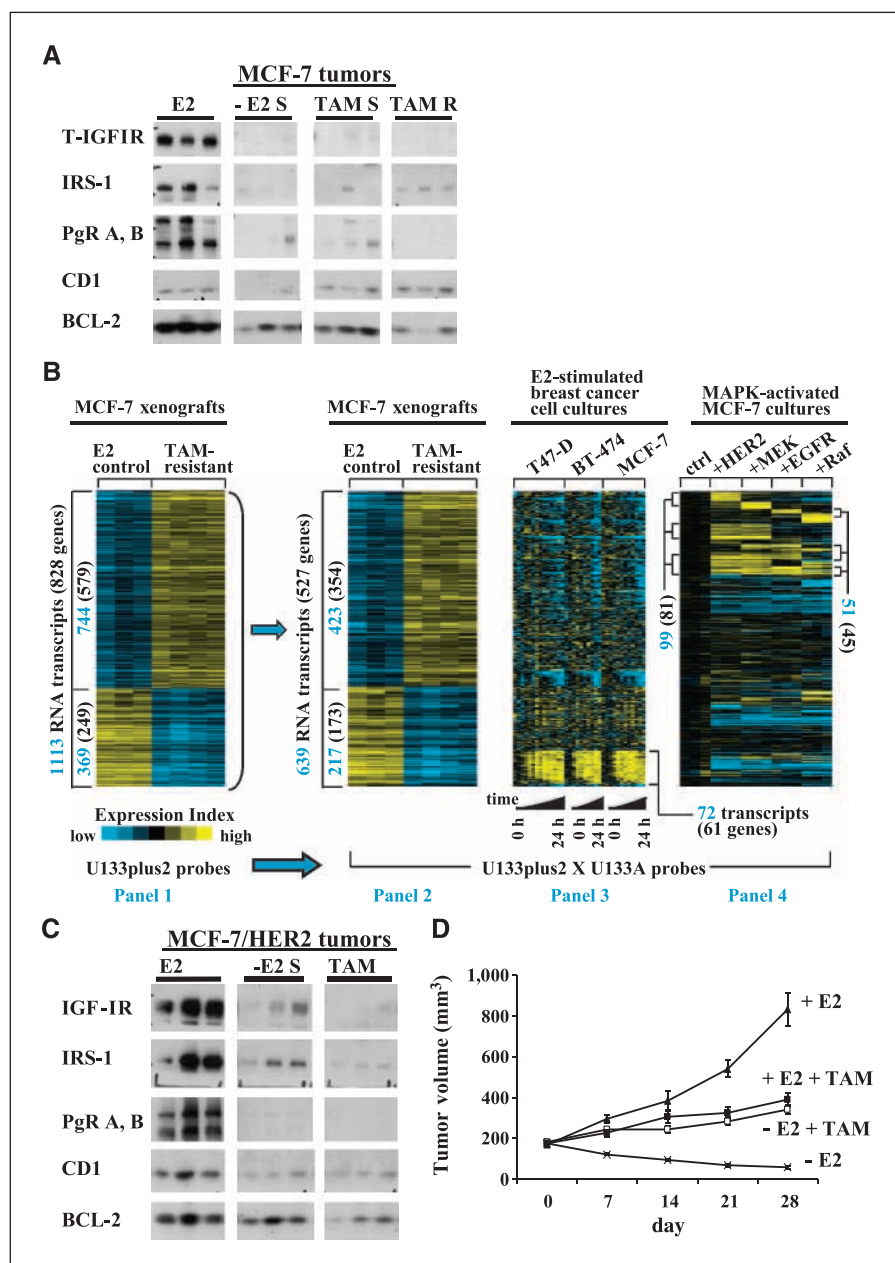


Figure 4. Tamoxifen activates transcriptional programs associated with growth factor signaling at the time of resistance but continues to suppress ER-dependent genomic functions and can antagonize estrogen-stimulated growth of resistant tumors. *A*, immunoblot analysis of total IGF-IR (*T-IGFIR*), IRS-1, progesterone receptor (*PgR A, B*), cyclin D1 (*CD1*), and BCL-2 in three individual MCF-7 tumors stimulated by estrogen (*E2*), sensitive to estrogen withdrawal alone (*-E2 S*) or to estrogen withdrawal plus tamoxifen (*TAM S*) at 3 wk of treatment, and at the time of acquired resistance to tamoxifen (*TAM R*). *B*, expression patterns for genes differentially expressed ($P < 0.01$, fold change >1.5) in MCF-7 tumors resistant to tamoxifen compared with tumors with continued estrogen supplementation (*E2*) using the U133plus2 Affymetrix Chip array (*panel 1*). *Panel 2* includes a subset of the 1,113 transcripts from *panel 1* that are also present on the U133A array to allow comparison with previous data sets. Xenograft patterns were examined in a profile dataset of estrogen-stimulated breast cancer cell cultures (*panel 3*; ref. 23) and in another dataset of MCF-7 cultures with activated oncogenes associated with growth factor receptor signaling and the MAPK pathway (*panel 4*; ref. 24). Expression patterns are represented as a color map; yellow, high expression. Each row of a color map represents a gene; each column represents a profiled sample. Numbers of transcripts and corresponding genes are shown in blue and black in brackets, respectively. The order of the genes is the same across *panels 2* to *4*, allowing one to observe where distinct expression patterns of interest overlap. Expression data presented here is available in Supplementary Fig. S1. *C*, immunoblot analysis of IGF-IR, IRS-1, progesterone receptor (A and B), cyclin D1, and BCL-2 in three individual MCF-7 HER2 tumors stimulated by estrogen, sensitive to estrogen withdrawal, or *de novo* stimulated by tamoxifen. *D*, mice bearing established MCF-7/HER2 tumors were randomized on day 1 ($n = 8$) to receive continued estrogen supplementation, estrogen withdrawal, or tamoxifen either in the presence of estrogen or with estrogen withdrawal. (The +E2 and -E2 groups were the same as in Fig. 1*B* previously published (18), and all the treatment groups were done as part of one xenograft experiment.)

expression in the estrogen-stimulated tumors compared with the tamoxifen-resistant tumors.

To further characterize these differentially expressed genes for the recognition of molecular pathways associated with tamoxifen resistance, we identified a subset of these 1,113 transcripts that were commonly represented in expression array platforms used in previous studies of similar and relevant breast cancer models (23, 24) and included 639 transcripts. Two hundred seventeen of these 639 transcripts (Fig. 4*B*, *panel 2*), representing ~ 173 unique-named genes, were overexpressed in the estrogen-stimulated tumors. Of these genes, 61 were also found to be rapidly induced by estrogen in breast cancer cell cultures in a previously published dataset (ref. 23; Fig. 4*B*, *panel 3*); this overlap between the two gene sets was highly significant ($P < 1e-20$; one-sided Fisher's exact test). These genes included those for which the protein products are displayed in Fig. 4*A*, except for cyclin D1, which was

significantly reduced at the mRNA level (by 1.4-fold change), although it did not reach the predefined level of 1.5-fold change. In addition to the dataset of estrogen targets *in vitro* (23), we considered two independently derived profile datasets of estrogen-inducible genes, one from profiling MCF-7 xenografts deprived of estrogen for 24 to 48 h *in vivo* (653 genes with $P < 0.001$; ref. 23) and one of 151 up-regulated estrogen-responsive genes found to be adjacent to ER-binding sites (30). Both of these gene sets were also highly enriched within the 249 genes down-regulated in our *in vivo* tamoxifen-resistant set, with 52 genes of the first dataset (23) overlapping at P of $<1e-30$ by one-sided Fisher's exact test and 40 genes of the second dataset (30) overlapping at P of $<1e-40$.

In contrast, from the gene expression profile data, 744 transcripts, representing ~ 579 unique genes, were consistently higher in the tamoxifen-resistant MCF-7 xenograft tumors compared with the estrogen-stimulated tumors (Fig. 4*B*, *panel 1*). The expression

patterns for these genes were also examined in a published profile dataset of MCF-7 cultures, with stably overexpressed EGFR or constitutively activated HER2, Raf, or MAP/extracellular signal-regulated kinase kinase, which had resulted in these cell lines exhibiting hyperactivation of MAPK and estrogen-independent growth (Fig. 4B, panel 4; ref. 24). Interestingly and in agreement with our data from the Western blot analysis (Fig. 1A), genes showing up-regulation by tamoxifen treatment in our xenograft model shared extensive overlap with genes that were activated *in vitro* by the HER growth factor receptor signaling. Of the 354 tamoxifen-induced genes represented in the cell culture dataset (Fig. 4B, panel 2), 81 showed up-regulation ($P < 0.01$) by HER2 and 45 showed up-regulation by EGFR (Fig. 4B, panel 4). The extent of gene overlap was highly significant in each case ($P < 1e-11$ and $P < 1e-6$, respectively; one-sided Fisher's exact test). In addition, extensive overlap was observed between the genes down-regulated in the tamoxifen-resistant tumors and genes down-regulated by growth factor receptor signaling (Fig. 4B, panel 4). Collectively, data from this model suggest that acquired tamoxifen resistance occurs relatively independent of classic ER-regulated genomic function and is largely mediated by activation of growth factor receptor signaling, possibly through a nongenomic ER-driven mechanism that is inhibited by the HER inhibitor gefitinib.

Previously, we reported that when HER2 is overexpressed in MCF-7 cells, the resultant derivative clone (MCF-7 HER2/18) develops enhanced bidirectional cross talk between ER and HER2, resulting in activation of both genomic and nongenomic ER-mediated signaling upon tamoxifen treatment *in vitro* (9). To investigate whether HER2 augments ER-dependent classic transcription functions *in vivo*, we measured the levels of the same ER-dependent proteins from Fig. 4A in MCF-7 HER2/18 xenografts, which we have previously shown are *de novo* stimulated by tamoxifen (Fig. 4C). Surprisingly, even with marked overexpression of HER2 in these xenografts, levels of these classically ER-dependent genes remained markedly suppressed in tamoxifen-stimulated tumors, similar to the tumors that are inhibited by estrogen withdrawal. This suggests that tamoxifen has no demonstrable agonist effect on these ER-dependent classic transcription functions and indeed may continue to function as a partial estrogen antagonist for these genes even in tumors with marked HER2 overexpression.

To investigate whether tamoxifen can still inhibit estrogen-stimulated growth of tumors that overexpress HER2, we next randomized mice bearing established MCF-7/HER2-18 tumors to receive either continued estrogen alone, estrogen withdrawal alone, tamoxifen plus estrogen, or tamoxifen plus estrogen withdrawal (Fig. 4D). Remarkably, whereas tamoxifen showed agonist activity on tumor growth as we have previously shown (9), it effectively inhibited estrogen-stimulated tumor growth, thus exhibiting simultaneous agonist and antagonist effects on ER function. Based on our data, tamoxifen's agonist effect *in vivo* is largely mediated by activation of growth factor receptor signaling driven by ER nongenomic function or perhaps other mechanisms, whereas tamoxifen maintains its antagonist effect on at least some ER-dependent genomic activities even at the time of therapeutic resistance.

Discussion

Unraveling the mechanisms of resistance to tamoxifen and other forms of endocrine therapy could help predict patient benefit from

such treatment and serve to identify new targets to overcome resistance. Data from cell culture models suggest that EGFR plays a role in tamoxifen resistance *in vitro*, where it may provide alternate survival mechanisms that reduce cancer cell dependence on ER (31). Other published work in a model of long-term tamoxifen treatment and tumor retransplantation also supports a role for the HER family members in acquired tamoxifen resistance (32). EGFR is not infrequently expressed in clinical breast cancer and may indeed predict reduced benefit from tamoxifen (11, 33–35). Inhibition of EGFR can reverse tamoxifen resistance in breast cancer cells *in vitro* (36, 37), suggesting a role for EGFR as a target to modulate endocrine response.

In this paper, we report, using an *in vivo* model of ER-positive breast cancer, that acquired resistance to tamoxifen is associated with elevated levels of EGFR and activation of its downstream kinase signaling and gene expression program. We further reveal that this phenotype of tamoxifen's agonist effect on tumor growth occurs while it retains its antagonist effect on classic ER-mediated gene expression, and suggest that nongenomic ER-mediated activation of EGFR may be a predominant mechanism of acquired resistance. Moreover, our new observation that ER-mediated gene expression remains suppressed by tamoxifen *in vivo* even in the *de novo* resistant HER2-overexpressing MCF-7 xenografts supports the idea that *in vivo* tamoxifen resistance/stimulated growth in these two models is driven by nongenomic actions of ER. Of significant note, there is currently no direct evidence or complete molecular details portraying the nongenomic ER action responsible for tamoxifen resistance *in vivo*. However, previous observations showed that these EGFR-overexpressing tamoxifen-resistant MCF-7 tumors retain expression of ER and remain sensitive to subsequent endocrine therapy with fulvestrant (15), suggesting that resistance cannot be driven solely by EGFR (or HER2) and that ER must share a role in the resistance mechanism. This concept is supported by parallel *in vitro* cell culture models of tamoxifen resistance (5, 9).

Our finding that classic ER function remains suppressed in tamoxifen-resistant tumors was shown both at the level of protein expression of selected classically ER-dependent genes and by global gene expression microarray studies. The comparison of global gene expression profiles between the tamoxifen-resistant tumors and the estrogen-stimulated ones clearly showed that whereas classic estrogen-induced gene expression profiles were absent in the tamoxifen-resistant tumors, other growth factor pathway-dependent expression profiles, especially of the EGFR/HER2 pathway and its downstream Raf/Mek signaling, now became established. This is in contrast to our previous observations *in vitro* involving HER2-overexpressing MCF-7 cells in which tamoxifen induced both classic genomic and nongenomic ER functions (9). Our new findings suggest that tamoxifen resistance *in vivo* is predominantly mediated by nongenomic mechanisms and that classic ER genomic action does not play a major role, although other nonclassic ER genomic mechanisms could also potentially play a role in this system (38). The relative contribution of genomic versus nongenomic ER functions to tamoxifen resistance may therefore involve a spectrum rather than a single mechanism, and this may depend on multiple factors, including the tumor milieu and the duration of tamoxifen exposure (9, 32, 39).

Confirming the functional role of EGFR in acquired tamoxifen-stimulated growth, the EGFR TKI gefitinib markedly delayed acquired resistance when used in combination with tamoxifen in this *in vivo* model. Of note, gefitinib improved the antitumor effect

of tamoxifen from the outset, suggesting that the intrinsic agonist effect of tamoxifen, driven in part by EGFR even when this receptor is expressed at relatively low levels, is manifest from the very start. This agonist growth-stimulating effect of tamoxifen increases over time as EGFR/HER2 expression and signaling increase until it eventually surpasses its estrogen antagonistic effects on genomic ER activity leading to tumor progression. Because tamoxifen induces expression of EGFR and HER2 from the beginning of treatment, it is tempting to rationalize that tamoxifen may actually generate a target for HER inhibitors, in contrast to the estrogen-stimulated tumors which have very low EGFR expression and are therefore not sensitive to gefitinib. This finding is consistent with the limited efficacy of gefitinib when used as monotherapy in unselected patients with advanced breast cancer (40).

Of potential clinical relevance, gefitinib had only a modest effect when combined with estrogen deprivation in our model, in which tumor resistance was not accompanied by an increase in EGFR levels. HER2, on the other hand, seems to be modestly increased in the estrogen-deprived tumors, similar to reported data from other models (41), suggesting that HER2 may be preferentially up-regulated by estrogen deprivation. Although no specific clinical data exist about HER2 up-regulation in patients progressing on estrogen deprivation therapies, there is evidence that HER2 levels can increase over time in patients given endocrine therapy in general (42, 43). Interestingly, preclinical studies, as well as recent clinical studies, support combining aromatase inhibitors with growth factor pathway inhibitors in the subset of breast cancers overexpressing HER2 (18, 44), whereas using growth factor pathway inhibitors in combination with aromatase inhibitors was not beneficial in unselected patients with breast cancer (45, 46).

An interesting observation in this model is the dissociation between the level of total IGF-IR and that of its phosphorylated form (p-IGF-IR) in the tamoxifen-resistant tumors. Because IGF-IR is an ER-dependent gene product, it is not surprising that tamoxifen, which effectively inhibits classically ER-dependent transcription, can effectively reduce its expression. Even at the time of acquired resistance to tamoxifen, IGF-IR levels remain suppressed by tamoxifen, similar to previously reported observations from *in vitro* cell systems (47, 48). In contrast, p-IGF-IR, although initially reduced in the tamoxifen-sensitive tumors, is increased at the time of resistance. This suggests that IGF-IR becomes activated at the time of acquired resistance and then augments important cell survival pathways. A recent model proposed by Song et al. (49) suggests that ER outside the nucleus, which can be activated by estrogen and tamoxifen, may associate with IGF-IR in the cell membrane as part of a receptor complex that includes Shc and EGFR. Subsequently, activation of metalloproteinases leads to

release of heparin-bound epidermal growth factor, which in turn activates EGFR and downstream signaling through MAPK and possibly other signaling intermediates. Thus, activated IGF-IR may mediate the interaction between ER and EGFR and could be responsible for tamoxifen resistance in this model. Recent *in vitro* data in MCF-7 cells suggest that gefitinib can inhibit MAPK phosphorylation induced by IGF-I (50), presumably by blocking the interaction between IGF-IR and EGFR (48, 51), supporting a potential role for IGF-IR in cross-activation of EGFR. Further work is needed to clarify this ER/IGF-IR/EGFR interaction *in vivo* and the potential role of IGF-IR inhibitors in modulating tamoxifen resistance.

Another interesting finding in our model is the increase in p-p38 levels that begins during the tumor growth inhibition phase and intensifies at the time of acquired tamoxifen resistance. Gefitinib reduces p-p38 levels in these tamoxifen-sensitive tumors, suggesting that this kinase is regulated by EGFR and that it may contribute to the development of the tamoxifen agonist effect. In other model systems, p38 has been reported to phosphorylate both the ER and its coactivator AIB1 *in vitro* (52, 53), enhance the agonist activity of tamoxifen-bound ER on a specific subset of genes, and contribute to tamoxifen resistance (54). Recent clinical reports of increased p-p38 and HER2 in tumors progressing on tamoxifen also support a role for p38 in tamoxifen resistance (42). Phosphorylated p42/44 MAPK, which is elevated in the tamoxifen-resistant tumors, may contribute to tamoxifen's agonist effect, and protein kinase C δ may also play a role (55), suggesting that multiple and redundant intracellular signaling pathways may contribute to tamoxifen resistance, especially in advanced stages of the disease.

In summary, data from this *in vivo* breast tumor model provide strong evidence that tamoxifen resistance is mediated by activation of HER family signaling, at least partly through ER-driven nongenomic mechanisms, whereas classic ER genomic functions remain largely suppressed by tamoxifen at the time of resistance. These data add new insights about the mechanisms of breast cancer resistance to tamoxifen and provide a strong rationale for using HER pathway inhibitors to improve tamoxifen response and delay emergence of resistance.

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