Genome-Wide Analysis of Aromatase Inhibitor-Resistant, Tamoxifen-Resistant, and Long-Term Estrogen-Deprived Cells Reveals a Role for Estrogen Receptor

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Abstract

Acquired resistance to either tamoxifen or aromatase inhibitors (AI) develops after prolonged treatment in a majority of hormone-responsive breast cancers. In an attempt to further elucidate mechanisms of acquired resistance to AIs, MCF-7aro cells resistant to letrozole (T+LET R), anastrozole (T+ANA R), and exemestane (T+EXE R), as well as long-term estrogen deprived (LTEDaro) and tamoxifen-resistant (T+TAM R) lines were generated. This is the first complete panel of endocrine therapy–resistant cell lines, which were generated as multiple independent biological replicates for unbiased genome-wide analysis using microarrays. Although similarities are apparent, microarray results clearly show gene signatures unique to AI-resistance were inherently different from LTEDaro and T+TAM R gene expression profiles. Based on hierarchical clustering, unique estrogen-responsive gene signatures vary depending on cell line, with some genes up-regulated in all lines versus other genes up-regulated only in the AI-resistant lines. Characterization of these resistant lines showed that LTEDaro, T+LET R, and T+ANA R cells contained a constitutively active estrogen receptor (ER)α that does not require estrogen for activation. This ligand-independent activation of ER was not observed in the parental cells, as well as T+EXE R and T+TAM R cells. Further characterization of these resistant lines was performed using cell cycle analysis, immunofluorescence experiments to visualize ER subcellular localization, as well as cross-resistance studies to determine second-line inhibitor response. Using this well-defined model system, our studies provide important information regarding differences in resistance mechanisms to AIs, TAM, and LTEDaro, which are critical in overcoming resistance when treating hormone-responsive breast cancers. [Cancer Res 2008;68(12):4910–8]

Introduction

Breast cancer, in 60% of premenopausal patients and 75% of postmenopausal patients, is a hormone-dependent disease that relies on the mitogenic effects of estrogen to drive carcinogenesis. Estrogens are synthesized by a cytochrome P450 enzyme complex called aromatase, which uses an androgen substrate in the production of estrogen. The abnormally high expression of aromatase in breast cancer cells has been shown by aromatase activity measurement, immunohistochemical analysis, and reverse transcription-PCR analysis (1). In addition, the use of animal models have shown that tumor xenografts that express aromatase do synthesize estrogen in situ, which subsequently results in enhanced tumor growth (2, 3). The production of 17β-estradiol (E2) is suggested to mediate cell survival and proliferation of breast cancer cells via the transcriptional program of estrogen receptor (ER)α and its target genes (4, 5). In addition, nongenomic or membrane-initiated signaling events have also been implicated in tumor progression, due to ER-dependent signal transduction pathways that involve G-protein–coupled receptors and tyrosine kinase growth factor receptors (6, 7). Overall, ERα plays a pivotal role in the pathogenesis of breast cancer.

Treatment of hormone-responsive breast carcinomas has primarily relied on the use of tamoxifen (TAM), a selective estrogen receptor modulator (SERM) that antagonizes ER function. However, being a SERM, TAM does have partial estrogenic activity in the uterus and is associated with an increased incidence of endometrial cancer, thromboembolic events, and significant rates of disease recurrence (8–10). Moreover, aromatase inhibitors (AI), which block estrogen synthesis, have shown good efficacy, significant increase in disease-free survival, and lengthened time to disease recurrence in multiple clinical trials (11–13). The third-generation AIs show good specificity, potency, and oral activity and include two nonsteroidal inhibitors [letrozole (LET) and anastrozole (ANA)] and a steroidal inhibitor [exemestane (EXE)], which resembles and competes with the androgen substrate for active site binding (14).

After prolonged endocrine therapy, acquired resistance to AIs and TAM is expected to occur in a majority of breast cancer patients, which establishes the importance of elucidating the molecular characteristics of acquired resistance. Investigation of TAM resistance has revealed some mechanistic details and insights into this phenomenon. Early studies that looked at TAM resistance using an MCF-7 cell background described elevated levels of ERα in the cytosol in comparison with parental cells (15) and also report an increase in ERα transactivation potential of certain target genes (16). More recent analysis of TAM resistance implicates the epidermal growth factor family, primarily epidermal growth factor receptor (EGFR) and ErbB2 (HER2), in the enhanced activation of phosphatidylinositol 3-kinase (PI3K)/AKT, mitogen-activated protein kinases (MAPK), PKC, and Src kinase (17–21), in addition to increased ERα coactivator function (AIB1; ref. 22). In addition to TAM resistance, the long-term estrogen deprivation (LTED) system has been proposed as a model for AI resistance, due to its lack
of a hormone environment that resembles aromatase inhibition. Several laboratories have reported an activation of the growth factor signaling pathways in LTED cell lines, namely HER2 and insulin-like growth factor I receptor, which crosstalk with the ER signaling pathway resulting in an activation of various MAPKs and PI3K/AKT involved in cell survival and proliferation (23–25). In addition, a study by Jelovac and colleagues (26) is the first report to directly address LET-resistance using a mouse xenograft model. LET-resistant cells show an activation of HER2 growth factor signaling, activating a MAPK cascade responsible for proliferation of these resistant cells.

To further investigate resistance to Al, our laboratory has established cell lines that are resistant to LET (T+LET R), ANA (T+ANA R), and EXE (T+EXE R), and for mechanistic comparison, we have also generated LTEDaro and TAM-resistant (T+TAM R) lines. Using affymetrix microarrays, genome-wide analysis of gene expression profiles has established a clear division between Al-resistant lines that does not resemble LTEDaro or T+TAM R. Further experimental analysis was carried out to assess differences in ERα functionality that serve to divide these resistant lines into hormone-independent (constitutively active) and hormone-dependent categories. This is the first complete genome-wide study of LTEDaro, TAM, and Al-resistant cell lines, which can provide key insight into gene expression profiles involved in conferring resistance to endocrine therapy in breast cancer cells.

Materials and Methods

Cell culture, compounds, and proliferation assays. MCF-7 human breast cancer cells (ER+) that stably overexpress the aromatase gene (MCF-7aro) were previously generated in this laboratory and used for resistant cell line production (27). MCF-7aro cells were routinely cultured in MEM containing 10% fetal bovine serum (FBS), 2 mmol/L L-Glutamine, 1 mmol/L sodium pyruvate, 100 U/mL penicillin-streptomycin, and 100 μg/mL G418. Resistant cell lines were maintained in phenol red-free MEM containing 10% charcoal/dextran-treated FBS with identical supplements as parental MCF-7aro cells. One-way ANOVA was performed to select target genes that were differentially expressed between resistant cell lines and MCF-7aro cells, based on a fold-change criteria of 3.5-fold and a P value of <0.0001 equivalent to false discovery rate (FDR) of <1%. Hierarchical clustering using average linkage with Pearson's correlation was applied to the target genes to classify the resistant cell lines. Venn diagrams were generated using Partek Genomics Suite to examine the overlapping genes among resistant cell lines. In addition, estrogen inducibility was determined by two different estrogen-responsive gene databases, KBERG4 and ERTargetDB.

RNA isolation, cDNA synthesis, and real-time quantitative PCR. TRIzol reagent (Invitrogen) was used for RNA isolation from MCF-7aro and resistant cell lines. Five micrograms of total RNA was used for reverse transcription, using 100 ng random primer (Invitrogen), 0.5 mmol/L deoxynucleotide triphosphate mix, 40 U avian myeloblastosis virus-RT and supplied reaction buffer (Life Science, Inc.), and RNase Inhibitor (Promega) for 45 min at 42°C. Subsequently, 1 μL cDNA was used for real-time PCR using iQ5 SYBR Green Supermix (Bio-Rad) on an iCycler iQ5 PCR machine (Bio-Rad). All real-time PCR reactions were done in triplicate and the housekeeping gene β-actin was used as a reference. Gene-specific primer sequences are listed as Supplementary Data (Supplementary Table S1). ER reporter assays. To assess ERα transcriptional activity, resistant lines were seeded in 6-well plates at a density of 2 × 10^4 cells per well and incubated overnight in phenol red-free MEM containing 10% charcoal/dextran-treated FBS. Cells were transfected with 0.5 μg pGL3-ERE reporter plasmid containing 3 tandem estrogen response elements (ERE), using Lipofectin reagent (Invitrogen) according to manufacturer’s protocol. After 5 h, Opti-MEM (Invitrogen) transfection medium was replaced with phenol red-free MEM and supplemented with either DMSO or 10 μmol/L E2 for 24 h. Cells were lysed with 1× reporter lysis buffer (Promega), protein concentration was determined using the Bradford method (28), and 5 μg of protein was used for luciferase reporter assays, using the Luciferase Assay System (Promega).

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assay kits were purchased from Upstate Biotechnology. Cells were grown in phenol red-free MEM containing 10% charcoal/dextran-treated FBS without hormone or inhibitor for 5 d, serum starved for 24 h, and treated with either 100 μmol/L E2 or DMSO for 45 min. Cells were subsequently crosslinked with 1% formaldehyde (JT Baker), harvested, lysed in 1× SDS lysis buffer, and sonicated with a Branson 450 sonifier six times for 10 s at 30% amplitude. Cell lysates were precleared with 60 μL protein A agarose beads for 3 h, followed by immunoprecipitation (IP) with either 1 μg mouse ERα antibody or 1 μg normal mouse IgG (Santa Cruz Biotechnology) overnight. Immunocomplexes were collected with 80 μL protein A agarose beads for 3 h. Protein/bead complexes were eluted for 30 min at room temperature using 1% SDS and 0.1 mol/L NaHCO3. Eluates were reverse crosslinked in the presence of 5 mol/L NaCl at 65°C for 4 h. Proteinase K digestion was done at 45°C for 1 h, and DNA was subsequently purified using Phenol/Chloroform. PCR amplification of the p52 gene promoter, containing the ERE, was done using primers listed as Supplementary Data (Supplementary Table S1).

IP and Western blotting for phospho-ERK. For IP analysis, 5 mg of cell lysate were precleared with 60 μL protein A agarose beads (Roche) and subsequently subjected to IP with total ERα antibody (Santa Cruz Biotechnology). For Western blotting, equal amounts of protein were run on an SDS-polyacylamide gel and transferred to a Trans-blot nitrocellulose membrane (Bio-Rad) using a Transblot SD semidry transfer system (Bio-Rad). Membranes were blocked for 2 h in 5% milk and probed with primary antibody overnight. The following antibodies were used for Western blotting: phospho-ERK Ser18 (Cell Signaling), phospho-ERK Ser167, and total mouse ERα (Santa Cruz Biotechnology).

Cross-resistance studies and cell cycle analysis. Resistant cell lines were treated with testosterone in addition to LET, ANA, EXE, or TAM (with the above-mentioned concentrations), and cell proliferation was determined by total protein concentration and expressed as a mean of triplicate wells. Data were shown as percent growth relative to control, expressed as an average of three independent experiments. For cell cycle analysis, MCF-7aro or resistant cells were serum starved for 24 h and subsequently treated with DMSO, 1 μmol/L testosterone, or testosterone plus inhibitor for 96 h. Cells were harvested, washed with PBS, and fixed with 70% ethanol
overnight. Before cell cycle analysis, cells were washed with PBS and incubated for 1 h with propidium iodide (PI) solution (0.02 mg/mL PI, 0.2 mg/mL RNase A, and 0.1% v/v triton X-100 in PBS) at 37°C. Flow cytometry was performed on a CyAn ADP 9-color cytometer (Dako, Inc.).

**Immunofluorescence studies.** Cells were seeded onto 4-well chamber slides, grown until 30% to 70% confluent, serum starved for 24 h, and treated with DMSO or 1 nmol/L E2 for 10 min. Slides were washed twice with PBS and fixed with 3% paraformaldehyde for 20 min. Permeabilization of cell membranes was done with PBS containing 1:1,000 dilution of triton X-100, 1 mmol/L MgCl₂, and 0.1 mmol/L CaCl₂. Cells were stained with ERL primary antibody (Santa Cruz Biotechnology) and fluorescein-conjugated secondary antibody (Chemicon). Slides were washed with PBS, mounted with vectashield hardset mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories), and visualized with an upright LSM510 2-photon confocal microscope (Zeiss).

**Results**

**Generation of acquired resistance to AIs and TAM in vitro.** MCF-7aro cells, the only adequate aromatase+/ER- system for these studies, were used to generate T+LET R, T+ANA R, T+EXE R, and T+TAM R cell lines. The addition of AIs or TAM induced a selection process, whereby a large majority of cells initially underwent cell death until the ability to proliferate was regained (2–8 months, depending on inhibitor). The concentrations of inhibitors (see Materials and Methods) were chosen to assure that hormone-dependent cell proliferation was suppressed, based on findings from previous studies in our laboratory (29, 30). Six independent sets of each type of inhibitor-resistant line were generated, which are continuously cultured in the presence of the appropriate inhibitor and testosterone. As an additional control to the parental cells, MCF-7aro lines were cultured long-term in the absence of testosterone alone (T-Only cells). Besides the resistant lines, long-term estrogen-deprived cell lines were generated by prolonged culture of parental MCF-7aro cells in steroid-depleted medium, and are called LTEDaro. Cell proliferation assays were done to show acquired resistance to each drug. The resistant cell lines showed proliferation rates similar to the parental MCF-7aro cells, suggesting that acquired resistance had been gained (1).

The effect of acquired resistance on aromatase and ERL was determined, to eliminate the possibility of adverse changes in these cell lines, which may interfere with subsequent results. Real-time PCR as well as Western analysis showed that no major changes had occurred in aromatase or ERL mRNA and protein levels in the LTEDaro or resistant cell lines, compared with MCF-7aro, except aromatase protein levels in T+EXE R (Supplementary Table S2). Wang and Chen (31) have reported that in addition to its action as a mechanism based inhibitor, ERE is also an aromatase destabilizer on the protein level. In addition, in-cell aromatase activity assays showed that aromatase was still enzymatically active and responded to AI treatment in the resistant cell lines, again, with the exception of T+EXE R lines due to mechanism based inhibition (Supplementary Table S2). Lastly, the resistant cell lines were responsive to ICI treatment in cell proliferation assays, especially the LTEDaro lines that showed a high sensitivity (Table S2).

**Genome-wide analysis of LTEDaro and inhibitor-resistant lines using microarray.** Genome-wide analysis of gene expression changes is an unbiased approach to elucidate mechanistic differences among the four inhibitor-resistant lines and LTEDaro. MCF-7aro parental cell lines were used as baseline and a total of 264 genes that were up or down-regulated by >3.5-fold in the resistant cell lines were selected. Hierarchical clustering analysis revealed that the AI-resistant lines clustered together (T+LET R, T+ANA R, and T+EXE R), with the biological replicates of each type of resistant line grouping somewhat uniformly (Fig. 1). In contrast, the LTEDaro lines did not cluster with the AI-resistant lines, although LTED is proposed as a model system for AI resistance. Similarly, T+TAM R lines did not cluster with the AI-resistant lines.

In this cell culture system, hormone treatment with testosterone is converted to E2 by aromatase, resulting in the corresponding up-regulation or down-regulation of estrogen-responsive gene expression, as indicated in the T-only cell lines. As shown by Itoh and colleagues (29), addition of AIs or TAM counter-regulates the expression of these estrogen-responsive genes in AI/TAM-responsive cells. Importantly, we found that in resistant cell lines, the expression of most estrogen-regulated genes was not affected by AIs or TAM. Based on the clustering analysis in Fig. 1, three major types of estrogen-responsive genes were observed in our hierarchical clustering, up-regulated in all cell lines (e.g., CTSD, TFF1, CCND1, and BIRC5), up-regulated in all lines except LTEDaro (e.g., PGR, GJA1, GREB1, and PDZK1), and up-regulated in all lines except LTEDaro and T+TAM R (e.g., MGP, EGR3, AREG, and CA2). Based on microarray analysis, validation of transcript levels of estrogen-responsive genes was done to confirm these expression profiles, with select real-time quantitative PCR analysis shown (Fig. 2).

Based on hierarchical clustering, the top 20 up-regulated and down-regulated genes sorted by T-only cell lines, along with their Gene Ontology (GO) functions and subcellular localization, are shown (Supplementary Tables S3 and S4, respectively). These top 20 up-regulated genes were primarily estrogen-responsive, and showed high levels of expression in the AI-resistant lines, with minimal up-regulation or even down-regulation in the LTEDaro lines (Supplementary Table S3). Up-regulated genes sorted by T+TAM R were also primarily estrogen-responsive (Supplementary Table S5). T+TAM R gene expression profiles did share some similarity with the AI-resistant lines, but certain genes such as ASCL1, TEX14, and MPPED2 were unique to T+TAM R lines. Based on our previous microarray work with AI and TAM responsive cells, we have identified ASCL1 as a TAM-responsive gene (29). In addition, further hierarchical clustering analysis identified a group of 70 genes that were solely up-regulated in LTEDaro and 40% of which were estrogen-responsive, according to the above mentioned databases. The top 20 induced genes, from this LTEDaro 70-gene set, were listed (Table S6). These LTEDaro-specific estrogen-responsive genes were not up-regulated in the other resistant lines, suggesting a unique mechanism of ER-dependent transcriptional activation that differed in the LTEDaro lines.

Venn diagram analysis was performed to look at a more global gene expression profile of the resistant lines using results generated from one-way ANOVA analysis, where a fold-change of ≥2 and P value criteria of 0.001 were used. It was determined that the three AI-resistant lines (T+LET R, T+ANA R, and T+EXE R) contained the most overlap in terms of global gene expression, ~48% overlap (Fig. 3B). It is worth noting that among the AI-resistant lines, microarray data suggested that T+LET R and T+ANA R cells were very comparable (64%; Fig. 3A). Although these two AI-resistant lines were not completely identical in terms of gene expression profiles, the overall estrogen-responsive gene expression signatures were very similar. Genes that differed, not making the 2-fold cutoff, among the nonsteroidal AI-resistant lines included ITM2A, CDC27, COLA1, and PLS3 (only in T+LET R) and RAB31, SOX3, TFPI, and PMP22 (only in T+ANA R). In contrast, the
LTEDaro lines, compared with the T+LET R and T+ANA R lines, showed significantly less overlap (25%; Fig. 3D). Surprisingly, the T+TAM R lines contained more overlap with the nonsteroidal AI-resistant lines (35%; Fig. 3C) than LTEDaro. Additionally, correlation coefficients were calculated for all resistant lines and displayed as a similarity matrix (Supplementary Fig. S1). The AI-resistant lines correlate highly with each other, as indicated in red. T+TAM R lines only correlate highly with themselves, as well as LTEDaro that show no correlation with other resistant lines.

Functional analysis of the resistant cell lines was performed using Ingenuity Pathways Analysis (IPA), using a 1.2-fold change criteria and a \( P \) value of <0.01. Highly significant functions and canonical pathways, compared with MCF-7aro, are shown (Supplementary Table S7) as well as GO functions (Supplementary Tables S3, S4, S5, and S6). All resistant cell lines were found to have elevated DNA replication, recombination, and repair function. Similar profiles were seen in cell cycle control and the pyrimidine metabolism pathway, suggesting an enhanced survival mechanism in the resistant cells (Supplementary Table S7). According to GO, all resistant lines showed increased signal transduction, cell-cell signaling, extracellular matrix support, and cell growth pathways (Supplementary Tables S3 and S4). Of particular interest, changes were seen in cellular assembly and organization, including cell motility, morphology, and lamellipodia, which was especially relevant in the LTEDaro lines (Supplementary Tables S6 and S7). Additionally, T-only lines often had a fewer number of genes and lower \( P \) values, implying these survival functions/pathways, although shared by the resistant lines, are a unique property of acquired resistance.

**Constitutive activation of ER\( \alpha \) in the LTEDaro, T+LET R, and T+ANA R lines.** Based on estrogen-responsive gene profiles in Fig. 1, it was apparent that the transcriptional functionality of ER\( \alpha \) differed in our resistant cell lines. Therefore, endogenous ER\( \alpha \) transcriptional activity was assessed in the parental and resistant cell lines using a luciferase reporter assay. Comparison of DMSO and E2 treatment revealed an activation of ER\( \alpha \) transcriptional activity, without the addition of the ligand (E2), in the LTEDaro, T+LET R, and T+ANA R lines (Fig. 4A). In contrast, this E2-independent activation of ER\( \alpha \) was not seen in the parental MCF-7aro, T-only, T+EXE R, and T+TAM R lines.

Ligand-independent activation of ER\( \alpha \) in LTEDaro, T+LET R, and T+ANA R lines suggested the possibility of phosphorylation in the AF-1 region of the nuclear receptor. IP with ER\( \alpha \) was done to enrich total protein, followed by Western analysis, which determined that phosphorylation of serine 118 of ER\( \alpha \) was seen in LTEDaro, T+LET R, and T+ANA R lines (Fig. 4B). These results with serine 118 phosphorylation of ER confirm previous results observed in the LTED system (23). Much lower levels of serine 118 phosphorylation were noted in the T-only lines compared with the resistant lines, suggesting that phosphorylation is a mechanism for acquiring resistance.

**Figure 1.** Hierarchical clustering of resistant cell lines. Raw intensity values were background corrected and normalized using RMA, MCF-7aro parental cells were used for baseline correction, and one-way ANOVA was performed with a 3.5-fold up-regulated or down-regulated gene expression cutoff and a 0.0001 \( P \) value criteria (equivalent to FDR <1%) to select 264 significant genes. Hierarchical clustering was performed using Pearson’s correlation using Partek Genomics Suite, version 6.3Beta. Biological replicates of resistant lines are shown horizontally and significant genes are vertically clustered; red, up-regulation; green, down-regulation of gene expression. LTED (LTEDaro), TAM (T+TAM R), ANA (T+ANA R), LET (T+LET R), EXE (T+EXE R), and T (T-Only) cell lines are shown.
ER phosphorylation were found in T+EXER or T+TAMR lines, with no phosphorylation in MCF-7aro. Moreover, phosphorylation at serine 167 of ERα was not observed in any of the resistant lines (data not shown). Based on these results, there is a clear difference in ERα functionality in the LTEDaro, T+LET R, and T+ANA R lines, which contain a constitutively active ER, versus the parental MCF-7aro, T+EXER, and T+TAMR lines, which depend on E2 for activation of ERα.

Based on the observation that ERα is transcriptionally active without E2 in select resistant lines, ER promoter occupancy of an estrogen-responsive gene (TFF1 or pS2) was performed to establish functional relevance of this finding. ChIP assays were done in the resistant lines, using MCF-7aro and T-only cell lines as controls. The LTEDaro, T+LET R, and T+ANA R lines showed a ligand-independent recruitment of ERα to the pS2 promoter at the location of the ERE (Fig. 4C). This recruitment of ERα was seen in the DMSO-treated cells which was comparable with E2 treatment. The control cell lines, as well as T+EXER and T+TAMR lines, showed a ligand-dependent recruitment of ERα to the pS2 promoter. These ChIP results agree with ER activity assays and phosphorylation profiles of ERα, suggesting that ER is constitutively activated only in the LTEDaro, T+LET R, and T+ANA R lines, which differ from parental MCF-7aro, T+TAMR, and T+EXER R cells.

Further characterization: cross-resistance, cell cycle, and immunofluorescence analysis. Cross-resistance studies were performed to determine whether acquired resistance to one endocrine therapy agent would confer resistance to all agents, or if response to a different inhibitor was achievable as a second-line

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**Figure 2.** Quantitative real-time PCR for select estrogen-responsive genes. Real-time PCR analysis is shown for the following estrogen-responsive genes: CTSD, PGR, GJA1, and CA2. Cells were grown in appropriate growth medium, and RNA was isolated and reverse transcribed to obtain cDNA. This cDNA was used for real-time PCR analysis in a 25 μL total reaction containing 1 μL cDNA, gene-specific primer, and iQ5 SYBR Green Supermix. All samples were run in triplicate and multiple independent biological replicates of each type of resistant line were used. Gene expression was normalized with the β-actin housekeeping gene. SD was calculated, and normalized gene expression values were averaged to obtain graphs shown.

**Figure 3.** Comparison of global gene expression profiles of resistant cell lines. One-way ANOVA analysis comparing gene expression of resistant lines to control MCF-7aro cells was performed, and Venn diagrams were produced using top regulated genes with Partek Genomics Suite. T+LET R and T+ANA R lines contained 64% gene expression overlap (A), 48% overlap was seen among the three AI-resistant lines (T+LET R, T+ANA R, and T+EXER R; B), 35% overlap was observed between the AI and TAM (T+TAM R) lines (C), and 25% overlap in gene expression among the AI and LTED (LTEDaro) lines was observed (D).
treatment. The AI-resistant lines showed statistically significant growth suppression to other AIs over TAM, whereas the growth of T+TAM R lines was almost completely inhibited by AI exposure (Fig. 5). In contrast, the LTEDaro lines do not show statistically significant inhibition of cell growth upon treatment with an AI or TAM.

Cell cycle analysis of the resistant cell lines was done and compared with the cell cycle profiles of parental MCF-7aro, an extremely robust cell line. As expected, MCF-7aro cells exhibit a testosterone-dependent increase in cell cycle, especially in G2-M, compared with DMSO control (Table 1). In contrast, the T+LET R lines have similar cell cycle profiles under all conditions, suggesting the constitutive activity of ER, which is no longer hormone dependent. Both T+LET R and T+ANA R cells show similar cell cycle profiles, whereas the T+EXE R and T+TAM R cells require the presence of testosterone to drive the cell cycle (representative data shown from T+LET R). All resistant cell lines no longer respond to the presence of the inhibitor, as these cells have acquired resistance.

Immunofluorescence experiments were carried out to look at subcellular localization of ER in the resistant cell lines, compared with MCF-7aro (Fig. 6A and B). Upon treatment of MCF-7aro cells with E2, nuclear translocation of ER was apparent (Fig. 6B). This is different from LTEDaro and T+LET R lines (representative lines shown), where ER staining, after E2 treatment, is seen in both the cytoplasm and the nucleus (Fig. 6B). Immunofluorescence analysis also revealed a unique morphology to the LTEDaro lines. As shown in Fig. 6, the LTEDaro cells exhibit long axon-like extensions, or pseudopodia, as described by Santen and colleagues (32). These pseudopodia were not seen in other resistant lines, or the parental MCF-7aro.

**Discussion**

From the clinical viewpoint, acquired resistance to AIs or TAM renders patients unresponsive to current modes of endocrine treatment for hormone-responsive breast cancer. Therefore, a great deal of laboratory research has been dedicated to understanding the mechanisms of acquired resistance on the molecular level. We have established a cell culture model system for AI-resistance,
T+LET R, T+ANA R, and T+EXE R, and for comparison, we have also generated LTEDaro and T+TAM R cell lines. This study has shown that acquired resistance to AI is inherently different from LTEDaro and T+TAM R, and that the transcriptional program of ERs plays a critical role in these resistance mechanisms.

Our laboratory has previously carried out microarray analysis of MCF-7aro cells treated short-term with AIs and TAM, to identify genes that are responsive to inhibitor treatment. Genes such as TFF1, SULF1, PDZK1, MGP, EGR3, and CTSD were up-regulated by hormone treatment alone and down-regulated by AI or TAM treatment (29). In contrast, MCF-7aro AI-resistant cell lines showed an up-regulation of estrogen-responsive gene expression, despite the presence of the inhibitor. This suggests that acquired resistance to AI has indeed changed the gene expression profiles of these cells. Using hierarchical clustering and Venn diagram analysis, the nonsteroidal AI-resistant lines, T+LET R and T+ANA R, exhibit similar gene expression profiles, suggesting their mechanisms of resistance may be related. The steroidal AI, T+EXE R lines, do share some global similarity with the T+LET R and T+ANA R lines, based on hierarchical clustering, but experiments addressing ER functionality differentiate T+EXE R from T+LET R and T+ANA R. Based on work with EXE, we have observed that this steroidal AI does have weak estrogenic properties (33). EXE may bind directly to and activate ER, therefore eliminating the need for constitutive ER activation, which was seen in the T+LET R and T+ANA R lines. In addition, our laboratory has further investigated EXE-resistant lines and data suggests that amphiregulin is involved in an autocrine loop, which is ER and EGFR dependent (34).

Using hierarchical clustering and Venn diagram analysis, T+TAM R and especially LTEDaro gene expression profiles differed immensely from the AI-resistant lines. Our microarray as well as ER functional studies suggest that T+TAM R lines retain estrogen dependency and gene expression signatures between the AI- and T+TAM R lines can be effectively differentiated. As shown in Supplementary Tables S3 and S5, the top 20 up-regulated genes in the T+TAM R lines are quite unique from the AI-resistant lines, with some common estrogen-responsive genes (PDZK1, GREB1, PGR, and TFF1). Previous microarray analysis of TAM resistance has been quite variable, with some groups reporting an increase in expression of HER2 or EGFR (35) with contrasting data reporting no changes in expression levels (36). In terms of TAM resistance, our microarray data shows slight decreases in HER2 and EGFR expression levels, with little changes seen in MAPK expression levels. Schiff and Osborne (37) have presented the idea of altered ER coregulatory proteins in TAM resistance, such as AIB1, which can be phosphorylated and subsequently activated by HER2 and downstream MAPK activity. The role of coactivators in TAM resistance may serve to differentiate these cells from AI-resistant lines.

Similarly, LTEDaro showed the least overlap with AI-resistant lines in clustering and Venn diagram analysis, and many genes up-regulated in the AI-resistant lines show minimal or negative regulation in LTEDaro. Also, significantly up-regulated genes in the LTEDaro lines are unique from AI-resistant lines, and according to GO function and IPA, some of these genes are involved in membrane/cytoskeletal organization (Supplementary Tables S6 and S7), a finding that is supported by morphologic changes seen in immunofluorescence experiments (Fig. 6). Similar to our findings, Mackay and colleagues (38) have reported changes in expression profiles of genes involved in extracellular matrix remodeling in breast cancer patient samples treated short term with LET or ANA. Interestingly, progesterone receptor (PR), a known estrogen-responsive gene, was up-regulated in all lines except LTEDaro, suggesting that LTEDaro may be a model for ER+/PR− breast cancers. It has been reported that loss of PR expression may be due to growth factor activity, nongenomic ER activity, and/or altered ER coregulator levels (37, 39, 40). The LTEDaro lines, never being exposed to estrogen, but having a constitutively active ER, may have undergone a survival mechanism to drive breast cancer proliferation. Although ER is functional in LTEDaro, the transactivation potential of ER is altered, as seen in gene expression profiles that greatly differ from the AI-resistant lines. Based on the importance of ER in breast cancer progression as well as our own microarray data of estrogen-responsive gene profiles, we also focused on the functionality of this nuclear receptor in our resistant cell lines. We observed that the LTEDaro, T+LET R, and T+ANA R lines contained a constitutively active ERα, which did not depend on the presence of E2 for activation. Our results of ERα phosphorylation at serine 118 (ligand-independent activation) are supported by previous findings in LTED lines (23), with different results from LET-resistance studies that implicate serine 167 ER phosphorylation (26). In terms of TAM resistance, there are contradictory reports regarding ERα phosphorylation, an increase in serine 118 phosphorylation (22, 41) with contrasting results indicating a decrease in ERα phosphorylation at serine 118 (42) and phosphorylation of ERα at serine 167 (43). Because our T+TAM R lines do seem to be E2 dependent, the very low levels of serine 118 phosphorylation of ER does not correlate with the T+TAM R lines being E2 independent.

| Table 1. Cell cycle analysis of MCF-7aro and T+LET R cell lines |
|------------------|----------|--------|--------|
| Cell line       | Treatment | G₁     | S      | G₂-M   |
| MCF-7aro        | DMSO     | 87.3 ± 1.2 | 6.3 ± 1.1 | 5.6 ± 0.8 |
| MCF-7aro        | T        | 68.0 ± 1.7 | 20.9 ± 1.4 | 10.5 ± 1.3 |
| MCF-7aro        | T+LET    | 78.5 ± 5.5 | 12.7 ± 3.5 | 8.1 ± 1.4 |
| T+LET R         | DMSO     | 74.1 ± 2.9 | 13.9 ± 1.5 | 11.1 ± 1.2 |
| T+LET R         | T        | 70.0 ± 2.5 | 17.1 ± 1.9 | 12.0 ± 1.3 |
| T+LET R         | T+LET    | 71.9 ± 1.5 | 14.5 ± 1.3 | 12.8 ± 0.4 |

NOTE: Data are shown as percent of total cell number for G₁, S, and G₂-M phases and is representative of three independent experiments, with ± SDs shown.
with this observation. Yet, this phosphorylation of ER in the LTEDaro as well as nonsteroidal AI-resistant lines does suggest the activation of the MAPK pathway, which has been previously shown at serine 118 of ER (23, 44, 45). This potential involvement of MAPK, as well as the reported role of EGFR in EXE resistance (34), support the use of signal transduction inhibitors or even pan kinase inhibitors as combination treatment to combat endocrine therapy resistance.

Using a well-defined model system, we believe our results establish a fundamental difference among acquired resistance mechanisms of AIs, TAM, and LTEDaro. Based on expression profiles, nonsteroidal AI-resistant lines are very similar, but steroidal and nonsteroidal AI-resistant lines do differ in ER functionality, potentially due to agonist qualities of EXE. In addition, previous reports as well as our data suggest that TAM resistance is inherently different from AI resistance, with ER coregulators potentially playing an important role. Lastly, although ER functionality seems to be similar in LTEDaro versus AI-resistant lines, estrogen-responsive gene profiles greatly differ and suggest changes in ER function. Therefore, we believe that ER

Figure 6. Immunofluorescence of resistant cell lines. Immunofluorescence was carried out to look at subcellular localization of ER in the resistant cell lines. Cells were seeded onto 4-well chamber slides, serum starved for 24 h, and treated with DMSO (A) or 1 nmol/L E2 (B) for 10 min. Cells were stained with ERα primary antibody and fluorescein-conjugated secondary antibody. Slides were washed with PBS, mounted with vectashield hardset mounting medium with DAPI, and visualized with an upright 2-photon confocal microscope. ER staining, DAPI visualization, and a merge of both images are shown.
is a master regulator in differentiating between LTEDaro, TAM, and AI resistance mechanisms. This report provides valuable information regarding acquired resistance to AIs, which may be effective in treatment strategies to circumvent breast cancer relapse.

Disclosure of Potential Conflicts of Interest

L. Wagman: Medwaves, Inc. consultant. The other authors disclosed no potential conflicts of interest.

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