A Gene Expression Signature from Human Breast Cancer Cells with Acquired Hormone Independence Identifies MYC as a Mediator of Antiestrogen Resistance

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Abstract

Purpose: Although most patients with estrogen receptor α (ER)-positive breast cancer initially respond to endocrine therapy, many ultimately develop resistance to antiestrogens. However, mechanisms of antiestrogen resistance and biomarkers predictive of such resistance are underdeveloped.

Experimental Design: We adapted four ER+ human breast cancer cell lines to grow in an estrogen-depleted medium. A gene signature of estrogen independence was developed by comparing expression profiles of long-term estrogen-deprived (LTED) cells to their parental counterparts. We evaluated the ability of the LTED signature to predict tumor response to neoadjuvant therapy with an aromatase inhibitor and disease outcome following adjuvant tamoxifen. We utilized Gene Set Analysis (GSA) of LTED cell gene expression profiles and a loss-of-function approach to identify pathways causally associated with resistance to endocrine therapy.

Results: The LTED gene expression signature was predictive of high tumor cell proliferation following neoadjuvant therapy with anastrozole and letrozole, each in different patient cohorts. This signature was also predictive of poor recurrence-free survival in two studies of patients treated with adjuvant tamoxifen. Bioinformatic interrogation of expression profiles in LTED cells revealed a signature of MYC activation. The MYC activation signature and high MYC protein levels were both predictive of poor outcome following tamoxifen therapy. Finally, knockdown of MYC inhibited LTED cell growth.

Conclusions: A gene expression signature derived from ER+ breast cancer cells with acquired hormone independence predicted tumor response to aromatase inhibitors and associated with clinical markers of resistance to tamoxifen. Activation of the MYC pathway was associated with this resistance. Clin Cancer Res; 17(7): 1–11. ©2011 AACR.

Introduction

Estrogen receptor α (ER) and/or progesterone receptor (PR) are expressed in approximately 70% of breast cancers, which typically indicates that breast cancer cells depend upon estrogens for growth. Preferred therapies for such patients target ER signaling either by antagonizing binding of estrogens to the ER (tamoxifen), downregulating the ER (fulvestrant), or blocking estrogen biosynthesis (aromatase inhibitors, AI). Although endocrine therapies have changed the natural history of hormone-dependent breast cancer, many tumors exhibit de novo or acquired resistance (1). While clinical data link ErbB2/HER2 overexpression with resistance to endocrine therapy (2–4), less than 10% of hormone receptor–positive breast cancers overexpress HER2 (2), suggesting that mechanisms of escape from endocrine therapy remain undefined for the majority of patients.

Several studies have generated gene expression signatures using patient data sets with disease outcome to predict resistance to endocrine therapy. While this approach may yield predictive information, it does not provide a model in which to evaluate causal contributions of gene expression changes to an antiestrogen-resistant phenotype. To model breast cancers that are resistant to estrogen deprivation, we generated long-term estrogen-deprived (LTED) derivatives...
Gene expression microarray analysis of LTED cells

Raw data are available on the Gene Expression Omnibus (GEO; GSE19639). RNA harvested from cells incubated for 24 hours in 10% DCC-FBS was analyzed using Affymetrix HG_U133_Plus_2 microarrays as described previously [ref. 6; detailed in Supplementary Methods]. We compared the lists of probe sets altered across each LTED/parental comparison [≥1.5-fold, false discovery rate (FDR)-adjusted P ≤ 0.05; Fig. 1A]. Probe sets commonly deregulated in 3 or more LTED lines were included in the LTED gene signature.

Correlation of LTED signature genes with response to anastrozole

This patient cohort (SMITH69) was described previously (7). Core biopsies were collected prior to treatment (detailed in Supplementary Methods). Patients with hormone receptor–positive disease were treated with anastrozole. A repeat core biopsy was taken 14 days later. Ki67 immunohistochemical (IHC) scoring was performed on 4-μm formalin-fixed, paraffin-embedded sections from biopsies. This study was approved by the Institutional Review Board (IRB) and Research and Ethics Committees of the recruiting centers.

RNA harvested from pretreatment and post–2-week treatment frozen tumor samples was analyzed using Illumina HumanWG-6 v2 Expression BeadChips (detailed in Supplementary Methods). To examine the association between the 99-gene LTED signature and Ki67 score among patient tumors, 104 available probe sets mapping to 79 or 99 genes were extracted from the tumor data set. Microarray data were filtered to extract the most variable probe set for each gene (in R software using package: genefilter). Next, all resulting signal intensities (log2-transformed) were multiplied by (−1) for genes upregulated in LTED cells and (+1) for genes downregulated in LTED cells. The sum value across all genes was then calculated for each patient tumor, generating an unscaled LTED "score" that recapitulates the degree of similarity to the LTED cells. Thus, a high tumor LTED score corresponds to a signature highly concordant with resistance to estrogen deprivation. LTED scores were standardized to Z-scores by subtracting the mean score for the cohort and then dividing by the standard deviation of the scores for the cohort (Supplementary Table S3). The Z-scores for any given cohort have a mean of 0 with a standard deviation of 1. Z-scores were evaluated using a linear model in comparison to post–2-week treatment Ki67 scores. Gene expression and Ki67 data were available for 69 patients.

Correlation of LTED signature genes with response to letrozole

This patient data set (MILLER48) was described previously (8). This study was approved by the local ethics committee (LREC; 2001/8/80 and 2001/8/81). Postmenopausal women with ER+ breast cancer were treated with neoadjuvant letrozole. Tumor gene expression data (.CEL files) were downloaded from GEO (GSE5462). Ki67

Translational Relevance

Breast cancers which express estrogen receptor α (ER) and/or progesterone receptor typically respond to endocrine therapies (i.e., tamoxifen, aromatase inhibitors). However, a significant fraction of such cancers exhibit or develop therapeutic resistance, and metastatic recurrences are uniformly incurable. For the majority of antiestrogen-resistant breast cancers, mechanisms and predictive biomarkers of resistance await discovery. We generated a gene expression signature of estrogen independence using ER+ breast cancer cell lines which acquired hormone-independent growth. In patients, this signature was predictive of (i) high tumor cell proliferation following neoadjuvant therapy with an aromatase inhibitor and (ii) shorter time to recurrence following adjuvant tamoxifen therapy. In addition, we identified a signature of MYC transcription factor activation associated with estrogen-independent growth. An MYC activation signature and high MYC protein levels were predictive of shorter time to recurrence following adjuvant tamoxifen, thus offering MYC as a therapeutic target in breast cancers resistant to endocrine therapy.

Materials and Methods

Cell lines

MCF-7, ZR75-1, MDA-361, and HCC-1428 cells (American Type Culture Collection, ATCC) were maintained in Improved modified Eagle’s medium (IMEM)/10% FBS (Gibco). LTED cell lines were generated by culturing cells under hormone-depleted conditions [phenol red-free IMEM/10% dextran–charcoal-treated FBS (DCC-FBS, Hyclone; contains <0.0367 pmol/L 17β-estradiol)] as described previously (6).

Of 4 ER+ human breast cancer cell lines by maintaining them in conditions that mimic the low estrogen levels in patients treated with AIs (≤3 pmol/L plasma 17β-estradiol; ref. 5). We compared LTED cells with respective parental controls to evaluate global changes in gene expression and defined a signature of 99 genes commonly deregulated across LTED lines. This LTED signature was predictive of a lower tumor response to neoadjuvant therapy with an AI and poor outcome following adjuvant tamoxifen therapy in patients with hormone receptor-positive breast cancer. Furthermore, we bioinformatically derived a gene expression signature of MYC transcription factor activation in LTED cells that was predictive of poor outcome following tamoxifen therapy. High tumor MYC protein level was also predictive of shorter time to recurrence following adjuvant tamoxifen. Finally, small interfering RNA (siRNA)-mediated depletion of MYC reduced the hormone-independent growth of LTED cells, suggesting that activated MYC could be a useful target for the treatment of antiestrogen-resistant breast cancer.
IHC scoring was performed as above. Gene expression and Ki67 data were available for 48 patients. Principal components analysis revealed a batch effect in the microarray data. LTED scores were calculated as above (using 78/99 available genes) and evaluated in a linear model (including the batch term) compared with posttreatment Ki67 values.

Correlation of LTED signature genes with response to tamoxifen

Tumor gene expression data (.CEL files) and patient characteristics from 3 cohorts (LOI164, LOI181, MDACC298) of patients with ER⁺ breast cancer treated with adjuvant tamoxifen for 5 years (9, 10) were downloaded from GEO. Patients from sites GUIJ (n = 87, GSE6532) and GUIJ² (n = 77, GSE9195) comprised LOI164, which were analyzed on Affymetrix U133 plus 2.0 microarrays (9). Patients from sites KIT and OXFT (n = 181, GSE6532) comprised LOI181, which were analyzed on Affymetrix U133A and U133B microarrays. MDACC298 analysis was performed on Affymetrix U133A microarrays (GSE17705). Accession numbers of the patients which comprised LOI181 and LOI164 are annotated in Supplementary Table S10 and analyses are described in Supplementary Methods. LTED scores were calculated as above (using 99/99, 97/99, and 78/99 available genes in LOI164,
LOI181, and MDACC298, respectively). Patients were dichotomized based on median LTED score and Kaplan-Meier survival curves were compared by log-rank test. The association of LTED score with relapse-free survival (RFS) was analyzed using Cox proportional hazards (PH) regression.

Molecular subtyping of clinical microarray data sets using the PAM50 classifier

Molecular subtyping of clinical microarray was performed using R package: genefu (11). Gene expression data from 230 breast cancers of mixed subtypes (GEO GSE20194; ref. 12, POPOVICI230 cohort) were used to determine the association of LTED score with molecular subtype using robust scaling. Molecular subtyping was similarly performed on the tamoxifen-treated cohorts described above using no scaling, as all tumors were ER+.

Gene Set Analysis

Gene Set Analysis (GSA) is a variation on the Gene Set Enrichment Analysis algorithm and is described elsewhere (13, 14). Curated (c2) gene sets were downloaded from Molecular Signatures Database (14). GSA was performed using the 2-class paired algorithm comparing each LTED cell line to its respective parental control in R (package: GSA; 1,000 permutations). Positively regulated gene sets (i.e., upregulated in LTED cells) were noted. Two gene sets specific to MYC activation were identified, which were combined to form a union set (231 genes), and filtered to identify genes scoring greater than 1.0 by GSA; this yielded 46 genes. To test associations with clinical parameters, the expression values (log2-transformed) were summed across all 46 genes for each tumor and the resulting MYC score was standardized to a Z-score within each cohort as above.

Reverse-phase protein array analysis

Forty-seven early-stage hormone receptor–positive primary breast tumor samples (MDACC47) were obtained from the Breast Tissue Frozen Tumor Bank at MD Anderson Cancer Center. These were from deidentified patients that received adjuvant tamoxifen therapy for 5 years. Specimens were collected under an IRB-approved protocol. Protein was extracted from tumors and analyzed by reverse-phase protein array (RPPA) using a MYC antibody (Cell Signaling) as described previously (refs. 6, 15, 16; detailed in Supplementary Methods).

siRNA targeting of MYC

Cells transfected with siRNA targeting MYC or nonsilencing control (detailed in Supplementary Methods) were reseeded for cell proliferation and immunoblotting assays in 10% DCC-FBS. In proliferation assays, cells were trypsinized and counted after 6 days using a Coulter counter. Significant differences were determined by 2-sided t test (P < 0.05). Protein lysates collected at 2 days posttransfection were analyzed by immunoblotting using antibodies against c-Myc (Santa Cruz) and actin (Sigma).

Results and Discussion

Derivation of a gene expression signature of estrogen independence

We first compared gene expression profiles from 4 parental and their respective LTED cell lines. Venn diagrams were generated depicting microarray probe sets commonly altered across matching LTED/parental comparisons (Fig. 1A). We found 110 and 16 probe sets that were commonly down- or upregulated across 3 or more LTED lines (≥1.5-fold, FDR-adjusted P ≤ 0.05), respectively. These probe sets mapped to 86 downregulated and 13 upregulated genes, which we defined as the 99-gene LTED signature of estrogen independence (Supplementary Table S4). A heatmap depicting the levels of expression of these 99 genes (118 probe sets) in LTED cells relative to respective parental controls is shown in Figure 1B. Eight probe sets which mapped to multiple genes or were not annotated were excluded from further analysis.

Breast cancer is composed of several molecular subtypes with different clinical outcomes (17). ER+ breast cancer can generally be segregated into luminal A and luminal B subtypes, with luminal B exhibiting reduced sensitivity to endocrine therapy and a worse prognosis compared with luminal A tumors (18). We hypothesized that the gene expression changes reflected in the LTED signature are associated with luminal B breast cancer. We performed molecular subtyping of 230 unselected breast tumors (POPOVICI230 cohort; ref. 12) using the 50-gene PAM50 classifier (11). PAM50 subtypes displayed patterns consistent with clinical parameters (ER, PR, and HER2).

Using the 99 LTED signature genes (Fig. 1A), we generated an LTED score for each patient tumor (detailed in the Materials and Methods section). The tumor LTED score, which according to our analysis should reflect the degree of resistance to estrogen deprivation, significantly varied among subtypes (ANOVA, P < 0.001, Fig. 1C). A post hoc analysis demonstrated that LTED score was significantly higher in luminal B than in luminal A tumors (t test, P < 0.001), suggesting that the LTED signature associates with clinically relevant molecular subtypes of breast cancer.

LTED signature predicts clinical response to an aromatase inhibitor

We next examined whether the expression profiles in LTED cells correlate with response to neoadjuvant letrozole. In a microarray data set of primary tumor samples from 48 patients with ER+ breast cancer (MILLER48 cohort; ref. 8), we calculated pretreatment and posttreatment (2 weeks) LTED scores for each tumor. To our knowledge, long-term follow-up in patients treated with an AI with tumor microarray data is currently unavailable. However, higher posttreatment Ki67 scores, a marker of high tumor cell proliferation (19), in primary cancers from patients treated with an AI for 2 weeks have been shown to predict poor long-term disease outcome (1). Thus, we performed Ki67 IHC on tumor biopsies obtained after 2 weeks of therapy with letrozole (Fig. 2A). Tumors with high
pretreatment and posttreatment LTED scores showed significantly higher posttreatment (2 weeks) Ki67 scores (Fig. 2B and C; pretreatment LTED score $r = 0.27$, batch-adjusted $P = 0.047$; posttreatment LTED score $r = 0.32$, batch-adjusted $P = 0.014$). Therefore, the genes up- or downregulated in LTED cells are expressed at higher or lower levels, respectively, in tumors where cell proliferation was not suppressed by letrozole. Notably, only 4 of 99 genes were classified as “cell growth/proliferation” genes by Gene Ontology analysis (20), suggesting that the ability of the LTED signature to predict early response to the AI (as determined by Ki67) is not only based on quantification of cell proliferation genes. Therefore, the LTED score likely reflects the underlying biology of a tumor cell population refractory to estrogen deprivation rather than the proliferative state of the cancer. Since our previous analysis suggested that the LTED score is higher in luminal B than luminal A tumors (Fig. 1C), these results further corroborate our contention that the LTED score is capturing expression patterns indicative of reduced proliferative dependency on the ER pathway (i.e., luminal B tumors).

To confirm these results, we assessed whether the LTED signature can predict response to anastrozole in another cohort of 69 patients with ER$^+$ breast cancer treated with neoadjuvant anastrozole for 14 days (cohort SMITH69). Ki67 scoring was performed on pre- and posttreatment tumor biopsies. E and F, pretreatment (E) and posttreatment (F) LTED scores were calculated for tumors from (D) and compared with the 2-week Ki67 scores as in (B and C).

LTED scores demonstrated a weaker correlation with Ki67 (Fig. 2F; $r = 0.19$, $P = 0.11$), suggesting that the pretherapy LTED score may be a stronger predictor of response to estrogen deprivation.

**LTED signature predicts disease outcome following adjuvant tamoxifen**

To determine whether the LTED signature is predictive of long-term outcome following adjuvant endocrine therapy, we assessed the correlation between pretreatment tumor LTED scores and RFS in 2 cohorts of patients with ER$^+$ breast cancer treated with adjuvant tamoxifen for 5 years ($n = 164$ in cohort LOI164, $n = 181$ in cohort LOI181, with median follow-ups of 8.94 and 5.11 years, respectively; ref. 9).

According to the PAM50 classifier, LOI164 was composed of 77 (47%) luminal A, 74 (45%) luminal B, and 13 (8%) HER2-positive tumors. LOI181 was composed of 156 (86%) luminal A, 21 (12%) luminal B, 3 (2%) HER2-positive, and 2 (1%) normal-like tumors. In univariate Cox PH models, the LTED score was associated with RFS in LOI164 ($P = 0.0065$) and showed a trend in LOI181 ($P = 0.085$). The LTED gene signature was a better predictor of RFS than 95.9% and 92.6% of 10,000 randomly generated 99-gene and 97-gene signatures by univariate analyses in the 2 data sets, respectively. In multivariate Cox PH models adjusting for age, tumor grade, tumor size, lymph node status, and molecular subtype in patients for which covariate data were available, the LTED score was independently predictive of RFS in LOI164 ($P = 0.0018$, $n = 128$,
Supplementary Table S5) but not in LOI181 (P = 0.175, n = 149, Supplementary Table S6). Notably, the LTED score was independently predictive of RFS in the presence of molecular subtype in LOI164. We underscore that molecular subtype is linked with clinical outcome (18) but is routinely excluded from the majority of multivariate models predicting outcome in breast cancer. To visualize differences in RFS between patients with a high versus low LTED score, we dichotomized patients on the basis of median LTED score and generated Kaplan–Meier survival curves. Analysis of LOI164 showed that patients bearing tumors with high LTED scores (those demonstrating high concordance with the signature of LTED cells) had significantly worse outcome following adjuvant tamoxifen (Fig. 3A;
A high LTED score was significantly predictive of early recurrence during adjuvant tamoxifen and tumor cell proliferation (i.e., Ki67) after short-term therapy with an AI. Although each element is potentially useful to inform clinical outcome, neither can be directly used to determine therapeutically targetable mechanisms causally associated with resistance to endocrine therapy. The current clinical standard is to add chemotherapy to the treatment regimen when disease progresses and/or antiestrogen therapies are exhausted. However, rational therapies targeted against molecules or pathways causal to such resistance would be preferable. Thus, we next assessed whether the LTED gene expression pattern could be used to identify distinct molecular alterations or pathway activation signatures that inform novel drug targets.

For this purpose, we utilized GSA. A paired design was invoked where each LTED cell line was compared with its parental control using the GSA algorithm. This analysis may be more informative for identifying coordinate regulated and biologically related gene sets. Among the gene sets enriched in LTED lines (Supplementary Table S7), we found 2 signatures of MYC activation [COLLER_MYC_oncoGENIC_SIGNATURE; refs. 22, 23]. The MYC oncogene is amplified in 15% of breast cancers (24). Further, breast cancer cells may overexpress MYC as a result of posttranslational mechanisms (25). Given that prior reports have identified a large overlap in estrogen- and MYC-responsive genes (26, 27), we hypothesized that MYC-coordinated gene expression promotes resistance to endocrine therapy by overriding the inhibitory effects of estrogen deprivation on genomic ER signaling. We created a union set of the genes activated by MYC from the 2 identified gene sets above, which yielded 231 genes (Supplementary Table S8). We then determined which of these 231 genes were specifically upregulated in LTED cells versus the parental lines and contributed most strongly to the correlation with the activated MYC signatures [gene score (s) > 1]. We identified 46 such genes (Supplementary Table S9), only one of which overlapped with the 99-gene LITED signature, suggesting that the 46-gene activated-MYC signature provides distinct biological information. The log2 signal intensities for these 46 genes were summed for each patient in cohort LOI164 to create an activated MYC score. A high MYC score was associated with poor RFS (univariate Cox PH model, \(P = 0.004\)). When the data were stratified into "high," "intermediate," and "low" MYC tertiles, it was apparent that the highest tertile represented a distinct fraction of the patients with a poor time to recurrence (not shown). Thus, we divided the patients into "high" and "low/intermediate" MYC score groups and compared RFS curves by log-rank test (Fig. 4A; \(P = 0.0068\)). After removing the contribution of the MYC gene itself, the MYC score was linearly associated with MYC mRNA levels (Fig. 4B; \(P = 5 \times 10^{-16}\)). Interestingly, MYC mRNA levels alone were not significantly predictive of RFS (not shown). This was confirmed in the LOI181 and MDACC298 data sets (below), suggesting that a signature of MYC activation, which better reflects MYC
transcriptional output, may be more informative than MYC gene expression alone.

We also tested the ability of the activated-MYC signature to predict outcome in the LOI181 and MDACC298 cohorts. Although MYC score was not linearly associated with RFS in LOI181 or MDACC298 in univariate Cox PH models, Kaplan–Meier analy{}ses comparing high versus intermediate/low tertiles revealed an association with outcome (Fig. 4C and D; log-rank $P = 0.0029$ and $P = 0.0017$, respectively). Thus, an alternative explanation for the lack of significance in the Cox PH model could be that MYC-mediated endocrine resistance may behave as a binary “on/off” characteristic, rather than a continuous variable where the degree of MYC activation is linearly related to the degree of antiestrogen resistance. To test if MYC expression is predictive of response to endocrine therapy in a large multi-study data set, we utilized the Kaplan–Meier Plotter tool (28). In a population of 399 patients with ER$^+$ breast cancer, MYC expression was associated with RFS in patients treated with endocrine therapy (Supplementary Fig. S1, log-rank $P = 0.00069$). In contrast, no difference in RFS curves was noted when the analysis was restricted to 609 patients not
treated with adjuvant therapy. This analysis strongly suggests that MYC expression is predictive of response to endocrine therapy and not simply a prognostic indicator. Correlations have been found between MYC and HER2 gene coamplification and poor prognosis in breast cancer (29, 30), but other studies reported only rare coamplification (31, 32). Similarly, we found a correlation between MYC score and HER2 mRNA levels (from microarray data) in the MDACC298 data set ($P = 0.01$, $r = 0.14$) but not in LOI164 or LOI181. Therefore, the association between MYC and HER2 overexpression requires further study.

Next, we used RPPA to quantify MYC protein levels in 47 patients with early-stage ER$^+$ breast cancer treated with adjuvant tamoxifen for 5 years (cohort MDACC47, median follow-up of 7.75 years). MYC levels were significantly associated with RFS following adjuvant tamoxifen (Cox PH model, $P = 0.019$). Stratification of the patients by high versus low/intermediate MYC expression demonstrated a similar association (Fig. 4E; log-rank $P = 0.017$).

Finally, since the 2 gene signatures ("LTED" and "MYC activation") derived from the LTED cells did not significantly overlap, we hypothesized that each signature may provide unique biological information. Thus, we asked whether stratification according to both factors (high LTED score and/or high MYC score) would serve as a better predictor of outcome. Indeed, patients with a high score for both signatures (MYC$^+$ LTED) demonstrated the shortest RFS in all 3 cohorts, whereas patients with low scores for both signatures (NONE) had the best outcome (Fig. 5). Patients with either a high MYC score or a high LTED score (MYC/LTED) had intermediate RFS rates.

siRNA-mediated depletion of MYC inhibits estrogen-independent growth

Since both MYC protein overexpression and a signature of MYC activation were predictive of antiestrogen resistance, we hypothesized that MYC inhibition should suppress estrogen-independent breast cancer cell growth. Thus, we utilized siRNA-mediated depletion of MYC in LTED cells to determine whether MYC contributes to hormone-independent growth. MYC knockdown suppressed the growth of MCF-7/LTED and ZR75-1/LTED cells (Fig. 6A). MYC knockdown was confirmed by immunoblotting in Figure 6B. Similarly, MYC overexpression has been shown to confer resistance to tamoxifen in vitro (33), and siRNA-mediated downregulation of MYC impairs estrogen-induced growth (26). We were unable to effectively knockdown MYC expression in HCC-1428/LTED cells; MDA-361/LTED cells express very low MYC levels (not shown).
These data suggest that MYC signaling may substitute for estrogen-induced ER signaling in a subset of breast cancers which are resistant to estrogen deprivation.

In summary, data generated from these cellular models of ER\(^+\) breast cancer have revealed biomarkers that upon further development may prospectively identify patients at risk of early recurrence during or following endocrine therapy. These findings also support the combined use of antiestrogens with MYC-targeted therapies to abrogate or delay the emergence of endocrine resistance in patients with hormone receptor–positive breast cancer.

**Disclosure of Potential Conflicts of Interest**

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