AIB1:ERα Transcriptional Activity Is Selectively Enhanced in Aromatase Inhibitor–Resistant Breast Cancer Cells

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Abstract

**Purpose:** The use of aromatase inhibitors (AI) in the treatment of estrogen receptor (ER)-positive, postmenopausal breast cancer has proven efficacy. However, inappropriate activation of ER target genes has been implicated in the development of resistant tumors. The ER coactivator protein AIB1 has previously been associated with initiation of breast cancer and resistance to endocrine therapy.

**Experimental Design:** Here, we investigated the role of AIB1 in the deregulation of ER target genes occurring as a consequence of AI resistance using tissue microarrays of patients with breast cancer and cell line models of resistance to the AI letrozole.

**Results:** Expression of AIB1 associated with disease recurrence ($P = 0.025$) and reduced disease-free survival time ($P = 0.0471$) in patients treated with an AI as first-line therapy. In a cell line model of resistance to letrozole (LetR), we found ERα/AIB1 promoter recruitment and subsequent expression of the classic ER target genes pS2 and Myc to be constitutively upregulated in the presence of both androstenedione and letrozole. In contrast, the recruitment of the ERα/AIB1 transcriptional complex to the nonclassic ER target cyclin D1 and its subsequent expression remained sensitive to steroid treatment and could be inhibited by treatment with letrozole. Molecular studies revealed that this may be due in part to direct steroid regulation of c-jun-NH₂-kinase (JNK), signaling to Jun and Fos at the cyclin D1 promoter.

**Conclusion:** This study establishes a role for AIB1 in AI-resistant breast cancer and describes a new mechanism of ERα/AIB1 gene regulation which could contribute to the development of an aggressive tumor phenotype. *Clin Cancer Res; 18(12); 3305–15. © 2012 AACR.*

Introduction

Resistance to endocrine treatment, including both tamoxifen and aromatase inhibitors (AI), is marked by a shift from steroid dependence to growth factor dependence (1). This increase in growth factor signaling can result in ligand-independent activation of the steroid receptor, estrogen receptor (ER)α, and inappropriate recruitment of coactivator proteins, including AIB1 (2). AIB1 (SRC-3, TRAM-L, RAC3, NCaA3, ACTR, and p/CIP) is a member of the p160 nuclear receptor coactivator family and was identified as a gene frequently amplified in breast cancer (3). The coactivator protein has a central role in promoting cell proliferation, migration, invasion, and metastasis by signaling through ERα, as well activating growth factor receptors HER2, EGFR, and insulin-like growth factor receptor (IGFR; reviewed extensively in ref. 4). Reports of in vivo studies show that, knock-out of AIB1 suppresses mammary tumor initiation, growth, and metastasis (5), whereas forced expression in mouse mammary epithelial cells is sufficient to induce spontaneous mammary tumorigenesis (6).

In tamoxifen-treated patients, expression of AIB1 in conjunction with an activated HER2 cascade has been associated with treatment resistance and early disease recurrence (2). A direct role for AIB1, in ER-mediated transcriptional regulation of HER2, as well as in growth factor receptor signaling has recently been described (7, 8). These studies establish a mechanistic role for AIB1 in reversing tamoxifen responsiveness in ER-positive breast cancer.

The role of AIB1 in resistance to AI therapy, however, is less well understood. AI resistance is characterized by a shift to growth factor–dependent cell growth (9–12), where coactivator proteins can use steroid receptor–independent mechanisms to drive transcription of genes relevant to the metastatic phenotype (13). Several groups have also described an adaptive hypersensitivity of the intact ER
Translational Relevance

Aromatase inhibitors (AI) are now the treatment of choice for estrogen receptor (ER)-positive postmenopausal patients with breast cancer. AIs however do not remove all of the estrogen ligand—data from molecular and in vivo studies suggest that this can result in adaptive hypersensitivity of the intact ER via increased signaling through growth factor pathways. In this study, we provide evidence of a central role for the steroid coactivator protein AIB1 in regulating selective ER transcriptional activity and driving tumor recurrence in AI-treated patients. Tackling the emerging problem of AI resistance in a timely fashion will enable us to tailor existing therapies and improve outcome in specific patient groups before disease recurrence becomes a clinical issue.

occurring as a response to low levels of circulating ligand (14–17). Enhanced ER transcriptional activity either through adaptive hypersensitivity of the receptor and/or increased cross-talk with growth factor signaling mechanisms leads to increased ER target gene expression. There is evidence to suggest, however, that selective deregulation of ER target genes occurs in the AI-resistant phenotype, which has the potential for significant functional consequences (18). This inappropriate and discriminative ER transcriptional activity associated with AI resistance could provide a further mechanism for AIB1 to mediate tumor aggression independent of HER2.

In this study, we investigated the role of AIB1 in the deregulation of ER target genes occurring as a consequence of AI resistance. From immunohistochemical studies AIB1 alone was observed to be a significant independent predictor of poor disease-free survival in AI-treated patients with breast cancer. Further investigation of AIB1’s role in the transcriptional regulation of ER target genes in a letrozole-resistant breast cancer cell model showed evidence of differential gene expression. Classical ER target genes, pS2 and Myc, harboring full or half estrogen response element (ERE)-binding sites respectively, were constitutively overexpressed in the resistant phenotype, whereas expression of the nonclassic ER target, cyclin D1, remained sensitive to steroid and AI treatment. Recruitment of the transcription factor c-jun to the cyclin D1 promoter via an activated c-jun-NH2-kinase (JNK) pathway retained its responsiveness to letrozole suggesting that in AI resistance, it remains dependent on steroid signaling. Differential ERBe/AIB1 gene regulation may, therefore, play a role in disease progression in AI-treated patients.

Materials and Methods

Patient information and construction of tissue microarray

Following ethical approval, 2 tissue microarrays (TMA) from 2 separate clinical institutions were constructed. For the first TMA, breast tumor samples were obtained from archival cases at Beaumont Hospital, Dublin, Ireland over period from 2000 to 2008. Patients received either tamoxifen (n = 132) 20 mg/d for 5 years or Arimidex (anastrozole) 1 mg/d for 5 years (n = 67), which was discontinued only in those patients who suffered a relapse while on endocrine therapy. Detailed follow-up data (median, 57 months) was collected on the patients to determine disease-free survival. For the second TMA, breast tumor samples were obtained from archival cases at St. Vincent’s University Hospital, Dublin, Ireland over period from 1987 to 1999 (n = 560). Data on the patients for both TMAs included tumor size, grade, lymph node status, HER2 status, and ER status. TMAs were constructed as previously described (2). Excluded from the analysis were patients who did not have breast surgery, those who had neoadjuvant therapy, or those whose tissue specimens were irretrievable.

Immunohistochemistry

Sections were incubated with primary antibodies as follows: rabbit anti-human AIB1 (3 μg/mL, sc-25742, Santa Cruz; ref. 2), rabbit anti-human Myc (3 μg/mL, sc-788, Santa Cruz; ref. 19), mouse anti-human matrix metalloproteinase (MMP)2 (5 μg/mL, Thermo Scientific), mouse anti-human MMP9 (2 μg/mL; Chemicon), rabbit anti-human p-SRC (Tyr 416; 3 μg/mL, 2101; Cell Signaling), rabbit anti-human pERK1/2 (3 μg/mL, 4370; Cell Signaling). The primary antibody was incubated for 1 hour at room temperature, followed by corresponding biotin-labeled secondary antibody (1:2,000; VectastainElite kit, Vector Labs) according to the manufacturer’s instructions. Staining was assessed with a modified Allred scoring system as previously described (20). Independent observers, without knowledge of prognostic factors, scored slides.

Statistical analysis

STATA 10 Data analysis statistical software (Stata Corp LP) was used in the analysis. Univariate analysis was conducted using Fisher exact test for categorical variables and Wilcoxon test for continuous variables. The difference in incidence of recurrence, over a 5-year period, was analyzed according to AIB1 status and commonly used pathologic variables including HER2 status. A P value of less than 0.05 was considered to be significant. Survival times between groups were compared using the Wilcoxon test adjusted for censored values. Difference in rate of incidence over a 5-year survival period was assessed according to AIB1, HER2, tumor grade, tumor size, and nodal status.

Cell lines, primary cell cultures, and treatments

Endocrine-sensitive MCF-7 breast cancer cells were obtained from American Type Culture Collection (ATCC). Letrozole (AI)-sensitive cells (Aro) were developed by stable transfection of the aromatase gene (CYP19; Invitrogen). Expression and activity of aromatase was established by Western blotting and enzyme activity assay, respectively (Supplementary Fig. S1). Letrozole-resistant cells (LetR) were created by long-term treatment (>3 months) of Aro...
cells with the letrozole (Novartis). Cells were maintained in steroid-depleted medium for 72 hours before treatment with hormones estradiol (E2; 10^{-8} \text{ mol/L; Sigma-Aldrich}) or androstenedione (10^{-7} \text{ mol/L; Sigma-Aldrich}), letrozole (10^{-6} \text{ mol/L}), EGF (1 \text{ ng/mL}), or SP600125 (INKinhibitor; 50 \text{ \mu mol/L; Calbiochem}). Resistance to AI was confirmed regularly by cell proliferation assay, where cell growth in the LetR cells was found to be independent of steroid or AI treatment (Supplementary Fig. S2). All cells were maintained at 37°C, 5% CO₂ in a humidified incubator. Primary cell cultures were derived from patient tumors and cultured for 72 hours as previously described (2).

**Immunostaining and coassociation analysis**

Breast cancer cell lines and primary cultures were immunostained with mouse anti-ERα (10 \text{ \mu g/mL}) and rabbit anti-AIB1 (10 \text{ \mu g/mL}), followed by the corresponding fluorescent-conjugated antibodies TRITC anti-rabbit or TRITC anti-mouse (Molecular Probes). Colocalization of ERα with AIB1 was assessed by fluorescent confocal microscopy. Coassociations between the 2 proteins were quantified by Pearson correlation coefficient as previously described (2).

**Co-immunoprecipitation and Western blotting**

Protein lysate (500 \text{ μ g}) was immunoprecipitated with rabbit anti-AIB1 antibody (sc-25742; Santa Cruz) and subsequently immunoblotted with either rabbit anti-AIB1 (as before) or mouse anti-ERα (sc-8002; Santa Cruz). Protein from breast cancer cells was lysed, electrophoresed, and immunoblotted with antibodies against the following proteins: Myc (rabbit, sc-788; Santa Cruz), cyclin D1 (mouse, sc-246; Santa Cruz), c-jun (rabbit, sc-44; Santa Cruz), c-fos (rabbit, sc-52; Santa Cruz), aromatase (rabbit, ab-18995; Abcam), ERα (rabbit, sc-453; Santa Cruz), and AIB1 (rabbit, sc-25742; Santa Cruz).

**PCR**

Aro and LetR cells were seeded into 6-well plates at a density of 2 × 10^5 cells per mL and steroid-depleted in phenol red-free media containing 10% CDS-FCS for 72 hours. Cells were treated with vehicle (ethanol; 0.01%), E2 phenol red–free media containing 10% CDS-FCS for 72 hours. Cells were treated with vehicle (ethanol; 0.01%), E2 phenol red–free media containing 10% CDS-FCS for 72 hours. Total RNA was isolated by an RNA extraction kit (Invitrogen). One microliter of 20 \text{ ng/cDNA} was amplified by PCR or quantitative PCR. Levels of pS2 and cyclin D1 mRNA were assessed in Aro and LetR cells with PCR. For each set of PCR samples, 2 controls were included: an RT- and a PCR-negative control containing no cDNA. The samples were heated in a thermocycler. Annealing temperatures were as follows: pS2, 65°C; cyclin D1, 59°C; β-actin, 60°C. Samples were electrophoresed on a gel. pS2 and cyclin D1 mRNA levels were compared with β-actin mRNA. Primers for pS2, forward: GTGCCCTGG-TGGTCCATGCTC; myc, forward: TTTGGAGTGGAAGCAGGAGT; reverse: CAGCAAGCTCAATGCTTCC; and for cyclin D1, forward: CTACACGGCAGAATCTCC; and reverse: TGTCTCTCTTGCGCCTCG.

**Aromatase activity (estrone concentration) assay**

Aro and LetR cells were plated at equal confluence. Cells were steroid depleted for 72 hours and then treated with either androstenedione (10^{-7} \text{ mol/L}), or letrozole (10^{-6} \text{ mol/L}) for a further 12 days, changing medium and treatments every 4 days. Total cells were stained with real-time PCR primers for pS2, myc, and β-actin.

**Chromatin immunoprecipitation analysis**

Aro and LetR cells were treated with either E2 (10^{-8} \text{ mol/L}), androstenedione (10^{-7} \text{ mol/L}), or letrozole (10^{-6} \text{ mol/L}). Chromatin immunoprecipitation (ChIP) was carried out as previously described (21). Goat anti-AIB1 (6 \mu g; Santa Cruz, sc-7216) mouse anti-ERα (6 \mu g; Santa Cruz, sc-8002), mouse anti-c-jun (6 \mu g; Santa Cruz, sc-44), mouse anti-c-fos (6 \mu g; Santa Cruz, sc-52), or H4 antibody (7 \mu g; Millipore; positive control) were added to the supernatant fraction and incubated overnight at 4°C with rotation. Proteins were uncross-linked with Chelex-100 resin (Bio-Rad) and primers were used to amplify the DNA upstream of the transcriptional start sites of the promoters of the pS2, Myc, and cyclin D1 genes. pS2 promoter-specific primers, forward: GGGGCACTCTTCTAGATGCACCTGGA and reverse: GGGGCACTCTTCTAGATGCACCTGGA; and for pS2 promoter-specific primers, forward: AAAAAAACCAATGGAACCCCT and reverse: ATTTCCCTCTACTTGCCTCTTCT.

**Bioinformatics**

Global ER- and AIB1-binding sites were taken from published data (22–24). Peaks located within 1 kbp from each other were considered to be colocalized and assessed using the transcriptional start sites of the promoters of the pS2, Myc, and cyclin D1 genes. pS2 promoter-specific primers, forward: GGGGCACTCTTCTAGATGCACCTGGA and reverse: GGGGCACTCTTCTAGATGCACCTGGA; and for pS2 promoter-specific primers, forward: AAAAAAACCAATGGAACCCCT and reverse: ATTTCCCTCTACTTGCCTCTTCT.
sequences under peak regions were retrieved using the BSgenome package within R/Bioconductor. The ERα/ESR1 DNA-binding motif (ERE) was identified from the TRANSFAC database. The first 5 bps of this motif were deemed to have low information content and removed from further consideration, resulting in a full ERE motif of 15 bps (AGGTCANNNTGACCT) being used for binding site discovery. Tffinder (27) was used to search within all 1,493 peaks for instances of the ERE motif, allowing up to 2 mismatches. Peaks without an ERE hit were further searched for instances of half-ERE motifs, allowing up to 1 mismatch. Half-ERE motifs were defined as 2 subsets of the full motif—left half–ERE (AGGTCA) and right half–ERE (TGACCT). A \( P \) value was calculated in each case based on the Fisher exact test statistic. Pathway enrichment analysis was conducted using the hypergeometric test available in the R/Bioconductor package GOstats (28). The nearest downstream genes corresponding to 1,493 peaks were retrieved with PeakAnalyzer (3). A total number of 1,565 genes, either overlapping with peak(s) or with peak(s) in their promoter regions, were included during this process.

Results

**AI\(^1\)B1 associates with poor disease-free survival in AI-treated breast cancer**

Strong nuclear expression of AI\(^1\)B1 was observed in 63% of patients with breast cancer following immunohistochemical staining of a TMA (\( n = 447 \); Fig. 1A). AI\(^1\)B1 associated significantly with a positive ER status and inversely with lower histologic grade (\( P = 0.007, P = 0.009, \) respectively, Supplementary Table S1). With regards to disease recurrence, AI\(^1\)B1 was not found to associate with disease recurrence in either the entire ER-positive population or in tamoxifen-treated patients, however, it was found to associate significantly with disease recurrence in patients treated with AIs as first-line therapy (\( P = 0.025 \)). Similarly, AI\(^1\)B1 significantly associated with reduced time to disease recurrence in AI-treated patients, (\( P = 0.0471 \)), but not in patients treated with tamoxifen or the entire ER-positive population (Fig. 1B, Supplementary Fig. S3). In the AI-treated patients, there was a significant difference in the incidence over a 5-year period according to AI\(^1\)B1 (21.8%) and nodal status (17.35%, \( P = 0.022 \) and 0.047, respectively). No significant difference in the incidence of recurrence was observed for HER2, tumor size, or tumor grade (Supplementary Table S2).

**Interactions between ER\(^a\) and AI\(^1\)B1 are unrestricted in AI resistance**

Interactions between ER\(^a\) and AI\(^1\)B1 in response to drug treatments were examined in the primary cell cultures derived from ER-positive patients with breast cancer using quantitative colocalization. Cells were stained for ER\(^a\) and AI\(^1\)B1 and coassociations between the 2 proteins were measured by Pearson correlation coefficient. Treatment with androstenedione significantly increased coassociations between the 2 proteins, compared with vehicle, (\( P = 0.007, \) Fig. 2A). Exposure to letrozole significantly decreased coassociations of ER\(^a\) with AI\(^1\)B1 compared with androstenedione alone (\( P = 0.018 \)), indicating that interactions...
between these proteins could be disrupted by the AI in an ex vivo setting (Fig. 2A). Consistent with our observations in primary cell cultures derived from patient tumors, analysis of a cell model system sensitive to AI (Aro) revealed that AIB1/ER interactions could be steroid regulated. This was not reflected in alterations of coactivator protein expression (Supplementary Fig. S4). Interactions between the coactivator and the transcription factor were diminished in the presence of the AI letrozole. In contrast, in cells resistant to letrozole (LetR), basal associations between AIB1 and ER were significantly stronger than the sensitive cells (Fig. 2B) and these interactions remained unchanged in the presence of androstenedione and letrozole, alone or in combination (Fig. 2B). Co-immunoprecipitation studies confirmed the dysregulation of ERα/AIB1 interactions in letrozole-resistant cells, where steroid treatment had no influence on strong basal interactions between the coactivator and the transcription factor (Fig. 2C).
Basal protein expression of AIB1, ERα, and ERα p-Ser118 was assessed in LetR cells. In addition to elevated levels of AIB1, these cells displayed significantly higher levels of phosphorylated ERα than either the aromatase-overexpressing Aro or parental cell line MCF-7 (Fig. 2D). Similar increases in basal expression of ERα p-Ser118 and AIB1 were observed in cells resistant to the AI anastrozole in comparison to the endocrine-sensitive parent cells (Supplementary Fig. S5).

**AIB1 ER transcriptional activity is dysregulated in AI resistance**

Unregulated ER target gene expression is a feature of AI resistance. To examine whether selective dysregulation of AIB1/ER target genes occurs in AI resistance we examined recruitment of the coactivator and the steroid receptor to ER target genes whose promoters contain different ER-binding sites: pS2, which harbors an imperfect ERE, Myc, with a 1/2 ERE and cyclin D1 with an activator protein (AP-1)-binding site (Fig. 3A). In endocrine-sensitive Aro cells, recruitment of ER and AIB1 (Fig. 3B, Supplementary Fig. S6) to all 3 target gene promoters was increased by treatment with estrogen or androstenedione. This ligand-induced recruitment was decreased in the presence of letrozole. In AI-resistant LetR cells, however, recruitment of ER and AIB1 to the pS2 promoter was constant and unchanged by treatments (Fig. 3C). Though marginal increases in recruitment...
to the Myc promoter in LetR cells were observed in the presence of androstenedione compared with control, this androstenedione-mediated recruitment was not inhibited by letrozole (Fig. 3C). Of interest, recruitment of ER and AIB1 to the cyclin D1 promoter in AI-resistant cells was enhanced by androstenedione treatment, however, unlike the other target genes containing a full or partial ERE in their promoters, letrozole treatment did suppress recruitment of both ER and AIB1 to the cyclin D1 promoter (Fig. 3C). These data suggest that the ER/AIB1 transcriptional complex may differentially regulate target genes depending on the promoter context.

To determine whether the increased interactions between ERα and AIB1 and subsequent recruitment to ER target gene promoters resulted in increased gene expression, mRNA and protein levels of the ER target genes were assessed in letrozole-sensitive and letrozole-resistant cells. In Aro cells, treatment with estrogen and androstenedione increased levels of pS2 mRNA and Myc protein, whereas letrozole treatment decreased expression levels compared with androstenedione treatment alone (Fig. 3D). In LetR cells, basal levels of pS2 mRNA and Myc protein were high and remained high independent of treatment with androstenedione and/or letrozole (Fig. 3D, Supplementary Fig. S7). Of interest, the tyrosine kinase inhibitor lapatinib successfully reduced both pS2 and Myc mRNA levels alone and in combination with letrozole (Supplementary Fig. S8).

In contrast, greater basal levels of cyclin D1 were expressed in the AI-sensitive cells in comparison to the AI-resistant cells (Supplementary Fig. S7). In Aro cells, treatment with letrozole suppressed the androstenedione-induced increase in expression, in a similar pattern to pS2 and Myc expression. In LetR cells, however, cyclin D1 levels were upregulated by steroid treatment, which were inhibited by letrozole. This result suggests that in AI-resistant cells transcriptional activity of ERα/AIB1 was maintained despite treatment with letrozole for pS2 and Myc, however, resistance was not observed in the case of the nonclassical target gene, cyclin D1.

AIB1 associates with growth factor signaling cascades and Myc in breast cancer patients

Elevated production of selective ER target genes, including the oncogene Myc may promote tumor aggression in AI-resistant breast cancer. In a cohort of patients with breast cancer, expression of AIB1 in the primary tumor was found to positively associate with the steroid receptor ERα (Table 1). Reflecting increased growth factor activity reported in AI resistance, AIB1 expression associated with the growth factor second messenger signaling proteins, p-Src and pERK1/2, but not the receptor HER2 (Table 1). Supporting molecular observations of ERα/AIB1 regulation of Myc, AIB1 associated with high expression levels of the oncogene (Table 1). These ex vivo clinical observations provide further evidence of a potential steroid independent role for the ERα/AIB1 transcriptional complex in the regulation of target gene expression.

Steroid-sensitive regulation of cyclin D1 is through JNK in AI resistance

To understand how cyclin D1 remains endocrine sensitive in AI-resistant breast cancer cells, the mechanism of steroid regulation of cyclin D1 was investigated. ERα mediates cyclin D1 expression through binding to the AP transcription factors c-jun and c-fos. In this study, protein levels of JNK and c-jun were found to be higher in our letrozole-resistant cell line LetR than in the Aro cells, with no significant change in the levels of c-fos observed (Fig. 4A). Recruitment of both c-jun and c-fos to the cyclin D1 promoter was enhanced in the presence of androstenedione in AI-sensitive and -resistant cells (Fig. 4B). Moreover, in the AI-resistant cells, steroid driven recruitment of c-jun to the cyclin D1 promoter remained sensitive to the AI letrozole (Fig. 4C). As c-jun and c-fos are regulated by signaling through JNK we hypothesised that in AI resistance, activation of JNK could be steroid-dependent. Therefore, regulation of cyclin D1 through this pathway could result in steroid-related expression of cyclin D1. A JNK inhibitor was used to investigate this signaling mechanism. Treatment with the JNK inhibitor, SP600125, decreased androstenedione-induced cyclin D1 expression in LetR cells (Fig. 4D). Of interest, SP600125 had no significant effect on Myc protein levels, suggesting that this steroid-regulated pathway is selective. Steroid signaling through JNK could therefore provide a mechanism by which regulation of cyclin D1 can remain steroid sensitive in AI resistance.
Global analysis of ERα/AIB1 target genes

Data reported here suggest that the promoter context could enable the ERα/AIB1 transcriptional complex to differentially regulate specific gene sets. To examine this we undertook global analysis of ERα- and AIB1-binding sites from published data sets. ERα/AIB1 colocalized peaks on promoters were examined and target genes were analyzed relative to their ER-binding site (i.e., full ERE, partial ERE, or non-ERE binding; Supplementary Tables S3A–C). Pathway enrichment analysis revealed that ER/AIB1 genes which contained either a full or partial ERE contributed to pathways promoting tumor progression, including prostate cancer, mTOR and cell adhesion pathways, relative to genes which did not contain an ER response element (Fig. 5). These findings were supported in a second data set (24) which also showed ER/AIB1 genes containing a full or partial ERE contributed to pathways promoting tumor progression (Supplementary Fig. S9). This bioinformatic analysis of global ER/AIB1 gene regulation is consistent with our molecular and clinical data suggesting that AIB1 and ERα may contribute to tumor progression in AI resistance through selective gene regulation.

Discussion

Treatment with AI therapy does not remove all circulating estrogen, leading to acquired drug resistance. Published molecular and in vivo studies suggest that this results in adaptive hypersensitivity of ER via increased signaling through growth factor pathways (9–11, 14). The ER coactivator protein AIB1 has previously been associated with poor response to tamoxifen treatment, in particular when coexpressed with the growth factor receptor HER2 (2, 29). Indeed ex vivo studies suggest that inappropriate ER/AIB1 transcriptional activity is dependent on amplified HER2 in tamoxifen-treated patients (30). In contrast, AIB1 has been associated with good response to short-term neoadjuvant AI treatment in patients with breast cancer, which may reflect a highly steroid dependent tumor, initially sensitive to endocrine treatment (31). The role of AIB1 in acquired resistance and disease-free survival in AI-treated breast cancer patients however, has yet to be elucidated. In this study, we found AIB1 alone significantly associated with reduced time to disease recurrence in patients treated with single first-line AI therapy. This relationship was not observed in either the ER-positive population or the
interactions between ER patient tumors and in cell line models, steroid-induced role of transcription factor complexes in disease progression. The treatment of patients.

mediated resistance has a particular importance in AI-tamoxifen-treated population, suggesting that AIB1-mediated resistance has a particular importance in AI-treated patients.

Ex vivo interactions between steroid receptors and coactivator proteins provide valuable information about the role of transcription factor complexes in disease progression (2). In primary cultures derived from endocrine-sensitive patient tumors and in cell line models, steroid-induced interactions between ERs and AIB1 were successfully interrupted by letrozole treatment. In cell models of resistance to the AI letrozole, however, basal interactions between ERs and AIB1 were found to be elevated in comparison to the sensitive phenotype and these interactions were stable following treatment with either androstenedione or letrozole. In other models of resistance, including the long-term estrogen-deprived (LTED) models, as well as the estrogen withdrawal cell lines overexpressing aromatase (UMB1Ca), ligand-independent activation of ER as well as increased sensitivity to growth factor signaling has been reported (8, 32). Moreover, activation of ERs in models of acquired AI resistance by cross-talk mechanisms has recently been described (7). These data are compatible with elevated levels of both AIB1 and ER-Ser118 in the letrozole-resistant cells reported here.

Increased ER transcriptional activity, either through adaptive hypersensitivity of the receptor and/or increased cross-talk with growth factor signaling mechanisms leads to elevated ER target gene expression. Studies by Jeng and colleagues suggest that long-term estrogen deprivation, as manifest in AI resistance, results in a selective increase in ER-regulated genes (18). As ER can bind DNA in the promoter of its target genes at EREs, partial EREs and at non-ER response element sites we queried if the promoter context of the target gene could be relevant to ERs/AIB1 regulation of expression in the resistant phenotype. Gene expression of pS2, which contains an imperfect ERE within its promoter and Myc, which has a partial ERE in its promoter, were both upregulated by ERs/AIB1 in AI-resistant cells and were insensitive to treatment with androstenedione or letrozole.

In contrast, cyclin D1 whose promoter harbors an AP-1 site remained sensitive to steroid treatment and was downregulated in the presence of letrozole in the resistant cell model. These data suggest that in the AI-resistant phenotype, ER can activate selective target gene expression independently of steroid signaling and therefore treatment with an AI such as letrozole fails to influence target gene expression. Cyclin D1, however, which is regulated by ERs/AIB1 via AP-1 sites remained endocrine sensitive. Here, we present data which suggest that estrogen-dependent activation of JNK is required for Jun and Fox to interact with the AP-1 site in the promoter of cyclin D1, with subsequent recruitment of ERs and AIB1. Similar direct steroid activation of JNK has been described previously (33). This complex secondary messenger signaling pathway provides a mechanism for cyclin D1 to remain androstenedione sensitive in AI resistance.

In this study of AI resistance we show preferential deregulation of ERs target genes. Our data support the hypothesis that ligand-independent transactivation of target genes can contribute to tumor progression in AI resistance. Estrogen-independent activation of ERs/AIB1 leads to substantial increases in pS2 and Myc, ER target genes involved in tumor cell proliferation. These molecular observations were consistent with our ex vivo clinical studies where AIB1 was found to associate with strong Myc expression in patients with breast cancer. Moreover AIB1 expression associated with growth factor signaling proteins, p-Src and p-ERK1/2 which is in line with published studies documenting a role for AIB1 in elevated cross-talk between growth factor and steroid pathways. Furthermore, treatment with the tyrosine kinase inhibitor lapatinib was shown to reduce transcript levels of pS2 and Myc.

Cyclin D1, however, remained endocrine sensitive. A role for cyclin D1 in tumor progression and, in particular endocrine resistance, has been well described (reviewed extensively in ref. 34). Recent studies, however, provide compelling evidence that cyclin D1 has a pivotal role in the regulation of steroid receptors, in particular the
progesterone receptor (35, 36). Diminished steroid regulation due to the loss of nuclear receptor regulating genes such as cyclin D1 may participate in tumor progression. Specifically, loss of cyclin D1 in AI resistance described here may contribute to the loss of estrogen-mediated cellular differentiation and increased tumor adaptability which are associated with the development of a metastatic phenotype. Globally from bioinformatic analysis of ERα/AIB1-binding sites from published data sets, genes containing full or partial ERαs may activate more aggressive pathways relative to genes containing no ER response elements. These data serve to broaden the debate on how ERα and AIB1 contribute to tumor progression on AI therapy and may provide a source of gene predictors of response to therapy going forward.

In summary, the coactivator AIB1 significantly associated with disease recurrence and reduced disease-free survival in AI-resistant breast cancer. One of the mechanisms which may contribute to AI-mediated tumor recurrence is the differential regulation of ERα/AIB1 target genes. Selective expression of these target genes could allow the steroid receptor coactivator complex to discriminate between its proliferative and differentiation functions to enable the tumor to progress. This study establishes a role for AIB1 in AI-resistant breast cancer and describes a new mechanism of ERα/AIB1-mediated gene regulation which could contribute to the development of a more aggressive tumor phenotype.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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