AIB1:ERalpha transcriptional activity is selectively enhanced in AI-resistant breast cancer cells

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**Translational relevance**

Aromatase inhibitors (AIs) are now the treatment of choice for ER positive post menopausal breast cancer patients. 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 signalling 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 tumour 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.
Abstract

Purpose: The use of aromatase inhibitors (AIs) in the treatment of ER-positive, post menopausal breast cancer has proven efficacy. However inappropriate activation of ER target genes has been implicated in the development of resistant tumours. 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 breast cancer patient tissue 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 ERalpha/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 ERalpha/AIB1 transcriptional complex to the non-classic 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 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 ERalpha/AIB1 gene regulation which could contribute to the development of an aggressive tumour phenotype.
Introduction

Resistance to endocrine treatment, including both tamoxifen and aromatase inhibitors (AIs), is marked by a shift from steroid dependence to growth factor dependence (1). This increase in growth factor signalling can result in ligand independent activation of the steroid receptor ERalpha and inappropriate recruitment of coactivator proteins, including AIB1 (2). AIB1 (SRC-3, TRAM-1, RAC3, NCoA3, 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 signalling through ERalpha, as well activating growth factor receptors HER2, EGFR and IGFR (Reviewed extensively in (4). Reports of in vivo studies show that, knockout of AIB1 suppresses mammary tumour 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 signalling 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 characterised 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 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 signalling 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 discriminating ER transcriptional activity associated with AI resistance, could provide a further mechanism for AIB1 to mediate tumour 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 breast cancer patients. 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, harbouring full or half ERE binding sites respectively, were constitutively over expressed in the resistant phenotype, whereas expression of the non-classic 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 JNK pathway retained its responsiveness to letrozole suggesting that in AI resistance, it remains dependent on steroid signaling. Differential ERalpha/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 (TMA)
Following ethical approval, two tissue microarrays (TMAs) from two separate clinical institutions were constructed. For the first TMA, breast tumour samples were obtained from archival cases at Beaumont Hospital, Dublin, Ireland over period from 2000 to 2008. Patients received either tamoxifen (n=132) 20 mgs/day for five years or Arimidex (anastrozole) 1mg/day 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 tumour 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 tumour 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: and rabbit antihuman AIB1 (3μg/mL; sc-25742, Santa Cruz, CA) (2), rabbit antihuman Myc (3μg/mL; sc-788, Santa Cruz)(19), mouse anti-human MMP2 (5μg/ml) (Thermo Scientific, Fremont, CA), mouse anti-human MMP9 (2μg/ml) (Chemicon, Temecula, CA), rabbit anti-human pSRC(Tyr 416) (3μg/mL; 2101, Cell Signaling),rabbit antihuman pERK1/2 (3μg/mL; 4370, Cell Signaling). The primary antibody was incubated for 1 hour at room temperature, followed by corresponding biotin-labelled secondary antibody (1:2000) (Vectastain Elite kit, Vector Labs, Burlingame, CA, USA) according to the manufacturer’s instructions. Staining was assessed using 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, Texas, USA) was used in the analysis. Univariate analysis was performed using Fisher’s exact test for categorical variables and Wilcoxon’s test for continuous variables. The difference in incidence of recurrence, over a five
year period, was analysed according to AIB1 status and commonly used pathological 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, tumour grade, tumour 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 (aromatase inhibitor) sensitive cells (Aro) were developed by stable transfection of the aromatase gene (CYP19) (Invitrogen, Paisley, UK). Expression and activity of aromatase was established by western blot 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, Basel, Switzerland). Cells were maintained in steroid depleted medium for 72 hours prior to treatment with hormones [estradiol (\( E_2 \); \( 10^{-8} \) M; Sigma Aldrich, Poole, UK) or androstenedione (\( 10^{-7} \) M; Sigma Aldrich, St. Louis, MO, USA)], letrozole (\( 10^{-6} \) M), epidermal growth factor (EGF; 1 ng/mL), or SP600125 (c-Jun NH2-terminal kinase inhibitor; 50 \( \mu \)M; 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\(^0\)C, 5% CO\(_2\) in a humidified incubator. Primary cell cultures were derived from patient tumours 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-ERalpha (10 \( \mu \)g/mL) and rabbit anti-AIB1 (10 \( \mu \)g/mL), followed by the corresponding fluorescent conjugated antibodies TRITC anti-rabbit or TRITC anti-mouse (Molecular Probes, Paisley, UK). Colocalisation of ERalpha with AIB1 was assessed by fluorescent confocal microscopy. Coassociations between the two proteins were quantified by Pearson’s correlation coefficient as previously described (2).

Co-immunoprecipitation and Western blot

Protein lysate (500 \( \mu \)g) was immunoprecipitated with rabbit anti-AIB1 antibody (sc-25742; Santa Cruz, CA) and subsequently immuno-blotted with either rabbit anti-AIB1 (as before) or mouse
anti-ERalpha (sc-8002; Santa Cruz Biotechnology, CA). Protein from breast cancer cells was lysed, electrophoresed and immunoblotted with antibodies against the following proteins: Myc (rabbit, sc-788; Santa Cruz), CyclinD1 (mouse, sc-246; Santa Cruz), c-jun (rabbit, sc-44; Santa Cruz), c-fos (rabbit, sc-52; Santa Cruz), aromatase (rabbit, ab-18995; Abcam, UK), ERalpha (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 2x10^5 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%), E\(_2\) (10nM), androstenedione (andro; 100nM), letrozole (letro; 1µM), or a combination of andro and letro, for 6 hours. Total RNA was isolated using a RNA extraction kit (Qiagen). RNA (1µg) was reverse-transcribed using an RT-PCR kit (Invitrogen). 1µl of the 20µl total RT product (cDNA) was amplified by PCR or qPCR. Levels of pS2 and CyclinD1 mRNA were assessed in Aro and LetR cells with PCR. For each set of PCR samples, two controls were included; an RT-minus and a PCR negative control containing no cDNA. The samples were heated in a thermocycler. Annealing temperatures were as follows: pS2: 65°C, cyclinD1: 59°C, β-actin: 60°C. Samples were electrophoresed on a gel. pS2 and cyclinD1 mRNA levels were compared with β-actin mRNA. Primers for pS2, forward: GTCCCCTGGTGCTTCTATCC and reverse: GAGTAGTCAAAGCTAAGGAGTCATCT, myc forward: TTCGGGTAGTGAAACCAG and reverse: CAGCAGCTCGAATTTCTCC, and for CyclinD1, forward: CTACACGGACAACTCCATCC and reverse: TGTTCTCCTCCGCTCTTG. Semi-quantitative qPCR was carried out using a Roche Light Cycler (Roche Applied Science, Indianapolis, IN, USA) with specific real-time PCR primers for pS2, myc and β-actin.

**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}M) or letrozole (10^{-6}M) for a further 23 hours. The concentration of estrone was measured using an estrone enzyme immunoassay kit (Cosmo Bio Co. Ltd, Tokyo, Japan). The absorbance was measured at 492nm, and the estrone concentration was calculated based on a logarithmic standard curve.
**Proliferation assay**

Aro and LetR cells were plated at equal confluence. Both Aro and LetR cells were steroid depleted for 72 hours. Both cell lines were then treated with either E2 (10^{-8}M), androstenedione (10^{-7}M) or letrozole (10^{-6}M) for a further 12 days, changing medium and treatments every fourth day. Total cells were stained with neat crystal violet stain (20 min). Cells were washed in water, air dried and the stain was dissolved with 33% glacial acetic acid. Absorbance was read at 620nm.

**Chromatin immunoprecipitation analysis**

Aro and LetR cells were treated with either E2 (10^{-8}M), androstenedione (10^{-7}M) or letrozole (10^{-6}M). ChIP was performed as previously described (21). Goat anti-AIB1 (6µg; Santa Cruz, sc-7216) mouse anti-ERalpha (6µg; Santa Cruz, sc-8002), mouse anti-c-jun (6 µg; Santa Cruz, sc-44), mouse anti-c-fos (6 µg; Santa Cruz, sc-52) or H4 antibody (7µg; Millipore; positive control) were added to the supernatant fraction and incubated overnight at 4°C with rotation. Proteins were uncrosslinked using Chelex-100 resin (Bio-Rad, Hercules, CA) and primers were used to amplify the DNA upstream of the transcriptional start sites of the promoters of the pS2, Myc and CyclinD1 genes. pS2 promoter specific primers, forward: GCCCACTCTCTATGAATCTGATCTGCA and reverse: GGCAGGTCTGTGTGC TTAAGAGCGTGGATA . Myc promoter specific primers, forward: CCGCCTGCGATGATTTATAC and reverse: CGGAGATTAGCGAGAGAGGA. CyclinD1 promoter specific primers, forward: AACAAAACCCTTATACCTT and reverse: ATTTCTTTCATCTTTGCTCCTTCT.

**Bioinformatics**

Global ER and AIB1 binding sites were taken from published data (22-24). Peaks located within 1kbp from each other were considered to be co-localized and assessed by the ChippeakAnno package in R/Bioconductor (25). ER peaks on promoters (UCSC hg18, upstream 5kbps from TSS) were assessed with BEDTools (26). 1493 (15%) out of 10191 ER peaks were found to overlap with promoters, 537 of which co-localized with AIB1 peaks. DNA sequences under peak regions were retrieved using the BSgenome package within R/Bioconductor. The ERalpha/ESR1 DNA-binding motif (ERE) was identified from the TRANSFAC database. The first 5bps of this motif were deemed...
to have low-information content and removed from further consideration, resulting in a full ERE motif of 15bps (AGGTCANNTGACCT) being employed for binding site discovery. *TFfinder* (27) was used to search within all 1493 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 two 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’s 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**

*AIB1 associates with poor disease-free survival in AI treated breast cancer*

Strong nuclear expression of AIB1 was observed in 63% of breast cancer patients following immunohistochemical staining of a tissue microarray (n=447) (Fig 1A). AIB1 associated significantly with a positive ER status and inversely with lower histological grade (*p* =0.007, *p*=0.009, respectively, Supplementary table 1). With regards to disease recurrence, AIB1 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, AIB1 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 five year period according to AIB1 (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, tumour size or tumour grade (Supplementary table 2).

*Interactions between ERalpha and AIB1 are unrestricted in AI resistance*

Interactions between ERalpha and AIB1 in response to drug treatments were examined in the primary cell cultures derived from ER positive breast cancer patients using quantitative
colocalisation. Cells were stained for ERalpha and AIB1 and coassociations between the two proteins were measured by Pearson’s correlation coefficient. Treatment with androstenedione significantly increased coassociations between the two proteins, compared with vehicle, (p=0.007, Fig. 2A). Exposure to letrozole significantly decreased coassociations of ERalpha with AIB1 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 tumours, 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). Coimmunoprecipitation studies confirmed the dysregulation of ERalpha/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, ERalpha and ERalpha p-Ser118 was assessed in LetR cells. In addition to elevated levels of AIB1 these cells displayed significantly higher levels of phosphorylated ERalpha than either the aromatase overexpressing Aro or parental cell line MCF7 (Figure 2D). Similar increases in basal expression of ERalpha 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 if 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 harbours an imperfect ERE, Myc, with a ½ ERE and Cyclin D1 with an AP-1 binding site (Fig. 3A). In endocrine sensitive Aro cells, recruitment of ER and AIB1 (Fig. 3B, Supplementary Fig S6) to all three 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 ERalpha/AIB1 transcriptional complex may differentially regulate target genes depending on the promoter context.

To determine whether the increased interactions between ERalpha 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, while 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 ERalpha/AIB1 was maintained despite treatment with letrozole for pS2 and Myc, however, resistance was not observed in the case of the non-classical target gene, Cyclin D1.

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

Elevated production of selective ER target genes, including the oncogene Myc may promote tumour aggression in AI resistant breast cancer. In a cohort of breast cancer patients, expression of AIB1 in the primary tumour was found to positively associate with the steroid receptor ERalpha (Table 1). Reflecting increased growth factor activity reported in AI resistance, AIB1 expression associated with the growth factor second messenger signalling proteins, pSrc and pERK1/2, but not the receptor HER2 (Table 1). Supporting molecular observations of ERalpha/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 ERalpha/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. ERalpha 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 signalling 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 employed to investigate this signalling 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 ERalpha/AIB1 target genes**

Data reported here suggest that the promoter context could enable the ERalpha/AIB1 transcriptional complex to differentially regulate specific gene sets. To examine this we undertook global analysis of ERalpha and AIB1 binding sites from published data sets. ERalpha- AIB1 co-localised peaks on promoters were examined and target genes were analysed relative to their ER binding site (i.e. full ERE, partial ERE or non-ERE binding) (Supplementary table 3A, B and C). Pathway enrichment analysis revealed that ER-AIB1 genes which contained either a full or partial ERE contributed to pathways promoting tumour progression, including prostate cancer, mTOR and cell adhesion pathways, relative to genes which did not contain an ER response element (Figure 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 tumour 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 ERalpha may contribute to tumour 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 co-expressed 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 neo-adjuvant AI treatment in breast cancer patients, which may reflect a highly steroid dependent tumour, 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 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 provides valuable information regarding the role of transcription factor complexes in disease progression (2). In primary cultures derived from endocrine sensitive patient tumours and in cell line models, steroid induced interactions between ERalpha and AIB1 were successfully interrupted by letrozole treatment. In cell models of resistance to the AI letrozole however, basal interactions between ERalpha 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 (UMB-1Ca), ligand independent activation of ER as well as increased sensitivity to growth factor signaling has been reported (8, 32). Moreover, activation of ERalpha 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 ERalpha/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 ERalpha/AIB1 in AI resistant cells and were insensitive to treatment with androstenedione or letrozole. In contrast, Cyclin D1 whose promoter harbours an AP-1 site remained sensitive to steroid treatment and was down-regulated 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 ERalpha/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 Fos to interact with the AP-1 site in the promoter of Cyclin D1, with subsequent recruitment of ERalpha 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 demonstrate preferential deregulation of ER alpha target genes. Our data supports the hypothesis that ligand independent transactivation of target genes can contribute to tumour progression in AI resistance. Estrogen independent activation of ERalpha/AIB1 leads to substantial increases in pS2 and Myc, ER target genes involved in tumour cell proliferation. These molecular observations were consistent with our ex vivo clinical studies where AIB1 was found to associate with strong Myc expression in breast cancer patients. Moreover AIB1 expression associated with growth factor signalling proteins, pSrc 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 TKI inhibitor lapatinib was shown to reduce transcript levels of pS2 and Myc.
Cyclin D1 however remained endocrine sensitive. A role for Cyclin D1 in tumour progression and, in particular endocrine resistance, has been well described (reviewed extensively in; (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 tumour progression. Specifically, loss of Cyclin D1 in AI resistance described here may contribute to the loss of estrogen mediated cellular differentiation and increased tumour adaptability which are associated with the development of a metastatic phenotype. Globally from bioinformatic analysis of ERalpha/AIB1 binding sites from published data sets, genes containing full or partial EREs may activate more aggressive pathways relative to genes containing no ER response elements. These data serve to broaden the debate on how ERalpha and AIB1 contribute to tumour 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 tumour recurrence is the differential regulation of ERalpha/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 tumour to progress. This study establishes a role for AIB1 in AI resistant breast cancer and describes a new mechanism of ERalpha/AIB1 mediated gene regulation which could contribute to the development of a more aggressive tumour phenotype.
References


Figure Legends

Figure 1. AIB1 associates with poor disease-free survival in AI treated breast cancer. A, Immunohistochemical localization of AIB1 (100X magnification, inset 200X) on a tissue microarray (TMA) constructed with archival tissue from 447 breast cancer patients, examples of positively (AIB1+) and negatively (AIB1-) stained cores are shown. B, Kaplan-Meier estimates of disease-free survival of breast cancer patients according to AIB1 status in tamoxifen (n=132, p=0.9189) and AI treated patients (n=67, p=0.0471).

Figure 2. Ex vivo and in vitro interactions between ERalpha and AIB1. A, ERalpha (green) and AIB1 (red) immunostaining of primary breast cancer tumor cells which were treated ex vivo for 50 minutes with vehicle (V), androstenedione (A) or letrozole (L). Representative confocal images are shown (n=3, a single nucleus per image; Scale bars, 2µm). Coassociations were quantified in the histogram using Pearson’s correlation coefficient (mean ± SEM). ERalpha-AIB1 coassociations were significantly enhanced by androstenedione treatment (p=0.007, 2 tailed t-test) and inhibited by the addition of letrozole (p=0.018). B, ERalpha (green) and AIB1 (red) immunostaining of Aro and LetR cells. Representative confocal images are shown (n=3) with Pearson’s correlation coefficient displayed in the histogram (mean ± SEM). In Aro cells ERalpha-AIB1 coassociations were significantly increased with androstenedione treatment (p=0.004) and inhibited by letrozole (p=0.018). By contrast, ERalpha-AIB1 coassociations were significantly higher in LetR cells than in Aro cells (p<0.01) and were not significantly affected by steroid or letrozole treatment (n.s.). C, Co-immunoprecipitation of AIB1 and subsequent immunoblotting of ERalpha identified a strong interaction between the receptor and its coactivator. The ERalpha-AIB1 interaction was induced by steroids in Aro cells but constitutively present in LetR cells, even in the absence of steroid treatment. Data shown is representative of three independent experiments. D, Protein expression of AIB1, ERalpha and ERalpha phosphorylated at Ser118 were assessed in MCF-7, Aro and LetR cells by western blot analysis. β-actin served as a loading control. Graphs show normalised relative expression of each protein by densitometry (n=3).

Figure 3. Regulation of ERalpha and AIB1 recruitment to target genes is promoter-specific. A, Diagrammatic representation of potential ERalpha-AIB1 binding sites in the promoter regions of 3 target genes. pS2 promoter contains an imperfect estrogen response element (ERE), Myc
contains a partial ERE and Cyclin D1 contains an AP1 binding site. B, C, Chromatin immunoprecipitation (ChIP) was employed to confirm recruitment of ERalpha and AIB1 to the above mentioned binding sites. B, In Aro cells, ERalpha and AIB1 were recruited to the promoter regions of pS2, Myc and Cyclin D1. This recruitment was stimulated by steroids, and inhibited by letrozole treatment. C, In LetR cells, ERalpha and AIB1 recruitment to pS2 and Myc promoters was insensitive to letrozole treatment, however, recruitment to the Cyclin D1 promoter was sensitive to both steroids and letrozole. Treatments were Vehicle(V), Oestrogen(E2), Androstendione(A), Letrozole(L). Histone(H4) served as positive ChIP control and IgG as a negative control. Results are representative of three separate experiments and densitometry is used to illustrate the key responses. D, Expression of pS2 (mRNA) and Myc (protein) in Aro and LetR cells was measured in response to treatments. In Aro cells, expression of pS2 and Myc was increased in response to steroids and inhibited by letrozole. In LetR cells, strong expression of pS2 and Myc was observed independent of treatments. E, Expression of Cyclin D1 (mRNA and protein), was enhanced by steroid treatment and inhibited by letrozole treatment in both Aro and LetR cells. B-actin was used as a loading control. All gel images are representative of three independent experiments.

Figure 4. ERalpha mediates Cyclin D1 expression through binding to the AP transcription factors c-jun and c-fos. A, Increased protein expression of JNK and c-jun are detected in LetR cells compared to Aro cells. Strong expression of c-fos was detected in both cell lines. B-actin was used as a loading control. Representative images are shown (n=3). B, ChIP analysis identified increased recruitment of c-jun and c-fos to the cyclin D1 promoter in response to androstenedione treatment in both Aro and LetR cells. C, In LetR cells, ChIP analysis revealed that the androstenedione-induced recruitment of c-Jun to the cyclin D1 promoter was sensitive to letrozole treatment. +, positive PCR control (genomic DNA); -, water negative control. Densitometry was used to quantify relative recruitment. D, Protein expression of Cyclin D1 was increased in LetR cells treated with androstenedione and this increase was inhibited in the presence of the JNK inhibitor SP600125 (SP). By contrast, protein expression of Myc was not reduced in the presence of SP600125. B-actin is used as a loading control. Densitometry illustrates relative normalised expression. Gel images are representative of three independent experiments.
Figure 5. Pathway analysis of ERalpha-AIB1 target genes. Common ERalpha-AIB1 target genes were identified by analysis of publicly available ChIPsequencing data sets (n=707 genes). The pie chart represents these genes divided into subgroups: those with full EREs in their promoters (n=417 genes), partial EREs (n=201) or no EREs (n=89). Kegg pathways which were significantly overrepresented in each of the subgroups are listed in the boxes (p<0.05).
Figure 1: AIB-1 associates with poor disease-free survival in AI treated breast cancer

A

AIB1 +

AIB1 -

B

Tamoxifen Treated Population

A.I. Treated Population

p=0.0471

p=0.9189

AIB-1 Negative  AIB-1 Positive

AIB-1 Negative  AIB-1 Positive

Time (months)

Time (months)
**Figure 2:** *Ex vivo* and *in vitro* interactions between ERα and AIB1

**A**

Vehicle, Androstenedione, Letrozole, andro + letro

**B**

Vehicle, Androstenedione, Letrozole, andro + letro

**C**

Aro cells and LetR cells

**D**

MCF7, Aro, LetR

AIB1, ERα Ser118, ERα, B-actin
Figure 3: Regulation of ERalpha and AIB1 recruitment to target genes is promoter-specific.
Table 1: Associations between expression of AIB1 and HER2, pERK1/2 and Myc in a cohort of primary breast cancer patients.

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<th>AIB1 +ve</th>
<th>AIB1 -ve</th>
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<td>ER +ve</td>
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<td>169</td>
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<td>ER -ve</td>
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Figure 4: ERalpha mediates CyclinD1 expression through binding to the AP transcription factors c-jun and c-fos

A

B

C

D

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Figure 5: Pathway analysis of ERalpha-AIB1 target genes

- Cysteine, methionine metabolism
- Antigen processing, presentation
- mTOR signaling pathway
- Prostate cancer
- Type I diabetes mellitus
- Chronic myeloid leukemia
- Arginine and proline metabolism
- Sulfur relay system
- Regulation of autophagy
- Staphylococcus aureus infection
- Graft-versus-host disease
- Allograft rejection
- Asthma

- Hematopoietic cell lineage
- Viral myocarditis
- Pentose phosphate pathway
- Complement, coagulation cascades
- Staphylococcus aureus infection
- Asthma
- Allograft rejection
- Graft-versus-host disease
- Rheumatoid arthritis
- Autoimmune thyroid disease
- Cell adhesion molecules (CAMs)
- Leukocyte transendothelial migration
- Type I diabetes mellitus

- EREs 417 genes
- ½ EREs 201 genes
- Non-EREs 89 genes

- Adipocytokine signaling pathway
- PPAR signaling pathway
- Homologous recombination

- Cysteine, methionine metabolism
- Antigen processing, presentation
- mTOR signaling pathway
- Prostate cancer
- Type I diabetes mellitus
- Chronic myeloid leukemia
- Arginine and proline metabolism
- Sulfur relay system
- Regulation of autophagy
- Staphylococcus aureus infection
- Graft-versus-host disease
- Allograft rejection
- Asthma
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AIB1:ERalpha transcriptional activity is selectively enhanced in Al-resistant breast cancer cells

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