Amplified in Breast Cancer 1 in Human Epidermal Growth Factor Receptor – Positive Tumors of Tamoxifen-Treated Breast Cancer Patients

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Abstract

Purpose: Amplified in breast cancer 1 (AIB1) is a member of the p160/steroid receptor coactivator family and is involved in estrogen-dependent gene transcription by reducing the antagonistic activity of tamoxifen-bound estrogen receptor-α (ER-α). The present study was carried out to test the hypothesis that AIB1 protein expression and/or gene amplification mediates tamoxifen resistance in breast cancer.

Experimental Design: Immunohistochemistry using AIB1 antibody and fluorescence in situ hybridization using probes specific for AIB1 and chromosome 20 was done on 402 ER-α –positive tamoxifen-treated breast cancers.

Results: AIB1 overexpression was not associated with relapse during treatment with tamoxifen. In contrast, high AIB1 expression in patients with human epidermal growth factor receptor (HER) 2– and HER3-overexpressing tumors or tumors expressing one or more of HER1, HER2, or HER3 (HER1-3 positive) was associated with an increased risk of relapse on tamoxifen [hazard ratio, 2.20; 95% confidence interval, 1.07-3.52 (P = 0.0416); hazard ratio, 2.42; 95% confidence interval, 1.32-4.43 (P = 0.0030), respectively]. AIB1 gene amplification was observed in 18 of 362 (5%) patients. High AIB1 gene copy number had no effect on overall or disease-free survival.

Conclusions: Data presented here support a role for AIB1 expression on relapse during tamoxifen treatment in hormone-responsive HER-expressing clinical breast cancers and support clinical evidence, suggesting a cross-talk between ER-α and growth factor receptor pathways through changes in expression of specific coactivator proteins, such as AIB1. This study highlights the potential that tumor profiling, using multiple markers of treatment response, may improve patient selection for endocrine treatment, such as tamoxifen or aromatase inhibitors.

Tamoxifen has been the standard adjuvant endocrine treatment of postmenopausal women with primary estrogen receptor (ER)-α-positive breast cancer since the 1970s. Unfortunately, some of these patients have a poor prognosis that requires more heavy treatment and approximately one fourth of tamoxifen-treated breast cancers exhibit de novo (present before tamoxifen treatment) or acquired resistance during the course of therapy (1). Recently, alternative endocrine therapies, such as treatment with aromatase inhibitors (AI), are found to be effective after disease relapse because most patients remain ER-α positive (2). Switching therapeutic agents in the adjuvant setting is however unwarranted for many patients as ~75% of patients treated with tamoxifen remain alive and disease-free up to 15 years after treatment (3). The potential indiscriminate implementation of AIs in breast cancer underlines the need to identify the molecular mechanisms involved in development of tamoxifen resistance. The aim is ultimately to identify patients at high risk of developing tamoxifen resistance, who would derive maximum benefit from selective targeting of other drugs, such as AIs.

Estrogen-mediated tumor growth and disease progression is controlled not only by binding of estrogen to ER-α but also by posttranslational events, such as phosphorylation of ER-α and interactions between ER-α and specific coregulator proteins (4 –6). In vitro studies have shown that coregulator proteins can be either coactivators, which enhance ER-α-mediated gene transcription, or corepressors, which suppress it (7, 8). In the absence of ligands, ER-α–mediated gene transcription is repressed via recruitment of corepressors. Ligand binding to ER-α triggers the release of the corepressors and subsequently recruitment of coactivators through conformational changes that expose a coactivator-binding groove on the surface of the ligand-binding domain (9). Cell line studies suggest that crosstalk between ER-α and growth factor receptor pathways...
contribute to endocrine resistance, at least in part, through changes in expression and activation of coactivator proteins (10). Overexpression of coactivators in the presence of ligand-bound nuclear receptor significantly increases hormone-induced gene transcription (11). Tamoxifen and other antiestrogens antagonize estrogen action by competing for mone-induced gene transcription (11). Tamoxifen and other ligand-bound nuclear receptor significantly increases hormone-induced gene transcription (11). Tamoxifen and other ligand-bound nuclear receptor significantly increases hormone-induced gene transcription (11). Tamoxifen and other

One of the best-characterized groups of ER-α coactivators is the p160/steroid receptor coactivator family consisting of steroid receptor coactivator-1, TIF-2 (GRIP1), amplified in breast cancer 1 (AIB1; steroid receptor coactivator-3, RAC3, ACTR) and p/CIP; (15–17). These have been identified as nuclear cofactors that activate the AF-2 function of hormone-bound ER-α to enhance gene transcription. AIB1 is located on chromosome 20q12, which is a frequently amplified chromosome region in breast cancer (16). The AIB1 gene is amplified in 5% to 10% and the protein overexpressed in >30% of primary breast carcinomas (15, 16, 18, 19). AIB1 amplification is preferentially found in ER-α- and progesterone receptor-positive breast tumors (18). High levels of tumor AIB1 expression has been associated with decreased risk of relapse in untreated patients. However, in tamoxifen-treated patients, AIB1 overexpression acts as a marker of disease relapse by reducing the antagonistic activity of tamoxifen-bound ER-α in breast cancer patients, leading ultimately to ineffective endocrine treatment (6).

The human epidermal growth factor receptor (HER) family of type-1 receptor tyrosine kinases consists of four members, epidermal growth factor receptor/HER1, HER2/ErbB-2, HER3/ErbB-3, and HER4/ErbB-4 (20), which regulate cellular proliferation, differentiation, and apoptosis in a growth factor–dependent manner (21–23). Previously, we have shown, in a cohort of 402 ER-positive breast cancer patients, that patients whose tumors overexpress HER2 or one or more of HER1, HER2, or HER3 had increased risk of relapse on tamoxifen (24). HER signaling is a frequent phenomenon in various human cancers (25). HERs are activated by ligand binding, which activates downstream signal transduction pathways, such as the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways. Signaling through the HER receptor pathways activates AIB1 by phosphorylation at six different phosphorylation sites (10, 26, 27).

Overexpression of AIB1 has been shown previously to mediate early relapse in patients with HER2-overexpressing tumors (6). However, as ER-α–positive tumors are more likely to be HER2 negative, the current study was carried out to extend this previous study by testing the hypothesis that gene amplification and/or protein overexpression of AIB1, especially in HER1-3–positive tumors, predicts for early relapse in tamoxifen-treated breast cancers.

Materials and Methods

Patients. A total of 402 ER-α–positive breast carcinomas of patients diagnosed between 1983 and 1999 at Glasgow Royal Infirmary were collected retrospectively for analysis. ER-α status was determined as described previously (24). ER-α positivity was defined as 10% or more positive tumor cells staining at any intensity, in accordance with current local pathology guidelines. Ethical approval was obtained from the local ethics committee.

Immunohistochemistry. Immunohistochemistry for AIB1 (BD Transduction Laboratories, San Diego, CA) was carried out on tissue microarrays as described previously (24, 28) using a standard immunoperoxidase procedure. In brief, antigen retrieval was done by microwaving the slides under pressure for 5 min in TE buffer [1 mmol/L EDTA, 5 mmol/L Tris base (pH 8.0)]. Endogenous peroxidase activity was quenched in 1% H2O2 for 10 min and nonspecific binding blocked by incubation in casein for 10 min at room temperature. AIB1 antibody was applied at 4°C overnight at a concentration of 5 μg/mL. EnVision (DAKO A/S, Glostrup, Denmark) was used for signal amplification, and positive staining was visualized using 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA). Nuclei were counterstained with hematoxylin before mounting. Protein expression was evaluated by two observers using a semiquantitative weighted histoscore method (28–31). The interclass correlation coefficient for nuclear AIB1 obtained when comparing scores from two observers was 0.93. The specificity of the AIB1 antibody was checked by Western blotting using a standard protocol.

Immunohistochemistry using antibodies against epidermal growth factor receptor, HER2, and HER3 was done as described previously (24) and the cutoff was previously defined (24).

Fluorescence in situ hybridizations. AIB1 fluorescence in situ hybridization was done on the tissue microarrays using a probe mix consisting of Texas red–labeled DNA cosmId clones covering the AIB1 gene and FITC-labeled peptide nucleic acid probes for chromosome 20 (DAKO A/S). The mix was tested for specificity on metaphase spreads of normal cells and showed hybridization to 20q12. Fluorescence in situ hybridization was done using buffers from DAKO Histology FISH Accessory kit (DAKO A/S). In brief, dewaxed and rehydrated tissue was incubated in pretreatment solution for 10 min at 96°C, treated with pepsin for 18 min at 25°C, washed, dehydrated, and air dried. Ten

![Table 1. Patient clinical and pathologic variables](https://www.aacrjournals.org/clinican/2007/3103/1031406_T1.jpg)
microliters of probe mix were applied and denatured at 82°C for 22 min, and tissue was incubated overnight at 45°C in a humidified hybridization chamber. Tissue was washed in stringent wash buffer (DAKO A/S) for 10 min at 65°C, dehydrated, and air dried, and the slides were mounted in 0.5 Ag/mL 4',6-diamidino-2-phenylindole in Vectashield antifade (Vector Laboratories). Gene copy number status was determined as the ratio of red signals (AIB1) over the number of green signals (chromosome) in 20 cancer cell nuclei for each tissue microarray core.

Statistical analysis. All statistical analysis was done using the SPSS statistical package (version 9.0 for Windows). Kaplan-Meier life tables with log-rank testing were plotted to assess overall survival (OS), disease-free survival (DFS), and DFS during treatment with tamoxifen. Spearman rank tests were conducted to test the associations between AIB1 and molecular or clinical markers. Cox’s multivariate analysis (Cox’s regression) was done with inclusion of biological markers alongside known predictive factors, such as size, nodal status, and grade. A value of P < 0.05 was considered statistically significant.

Results

Patient information. All patients were treated with tamoxifen for a median of 5 years (range, 0.6-18 years) and followed up for a median of 6.45 years (range, 0.64-18.42 years). In addition to tamoxifen, 99 of 399 (24.8%) patients had chemotherapy (3 unknown) and 110 of 399 (27.57%) had radiotherapy (3 unknown). At the end of the study, there were 74 breast cancer – specific deaths and 100 breast cancer relapses, 78 of which occurred during tamoxifen treatment. Clinical and pathologic data are shown in Table 1. Data on HER1-3 expression has been published previously (24).

AIB1 protein expression. AIB1 expression was evaluated in 377 of 402 (93.8%) breast carcinomas. The remaining 25 cases were excluded from the study because of insufficient material, either due to loss of core or insufficient tumor material in the cores. AIB1 protein expression was confined to invasive breast carcinoma cells with no staining in normal breast epithelial cells. Due to weak cytoplasmic staining, only nuclear staining was analyzed (Fig. 1A and B). High levels of AIB1 expression was weakly associated with high tumor levels of ER-α (correlation coefficient, 0.142; P = 0.006) and HER2 (correlation coefficient, 0.186; P = 0.001), whereas no association was noted between AIB1 expression and other molecular or clinical markers, such as tumor size, progesterone receptor expression, and time to relapse (data not shown). The median AIB1 histoscore was 56 (interquartile range, 30-100; Fig. 1C). For subsequent analysis, AIB1 expression was categorized as high (above upper quartile, histoscore of 100 and above) or low (below upper quartile; Fig. 1C) as described previously (6).

AIB1 protein expression and patient response to tamoxifen. Although there was a trend for patients whose tumors had high AIB1 expression to exhibit reduced OS compared with those
whose tumor had low AIB1 expression ($P = 0.059$, log-rank test; data not shown), statistical significance was not reached. No significant association between tumor AIB1 expression and DFS or DFS on tamoxifen was observed (data not shown).

**AIB1 and HER2 expression as a combined biological marker of patient outcome.** Patients with HER2-positive tumors that expressed high levels of AIB1 (HER2/AIB1, $n = 20$, 5.5%) exhibited decreased DFS on tamoxifen ($P = 0.042$, log-rank test; Fig. 2) with a 2.20-fold increased risk of relapse [95% confidence interval (95% CI), 1.07-3.52; $P = 0.0472$; Table 2]. This group of patients also showed decreased OS ($P = 0.036$, log-rank test; data not shown) with a 2.26-fold (95% CI, 1.03-4.95; $P = 0.041$; Table 2) increased risk of dying from disease relative to patients whose tumors did not overexpress both HER2 and AIB1. No significant association was observed between patients with HER2/AIB1–overexpressing tumors and overall DFS (data not shown). Multivariate analysis did not suggest that tumor HER2/AIB1 overexpression was an independent marker of patient outcome either for DFS, DFS on tamoxifen, or OS when analyzed alongside known prognostic markers, such as tumor size, grade, and nodal status.

**AIB1 and HER3 expression as a combined biological marker of patient outcome.** Patients with HER3-positive tumors that expressed high levels of AIB1 (HER3/AIB1, $n = 19$, 5.6%) exhibited decreased DFS on tamoxifen ($P < 0.001$, log-rank test; Fig. 3) with a 3.67-fold increased risk of relapse (95% CI, 1.81-7.41; $P < 0.001$; Table 2). This group of patients also showed decreased OS ($P < 0.001$, log-rank test; data not shown) with a 3.79-fold (95% CI, 1.79-8.03; $P < 0.001$; Table 2) increased risk of dying from disease and increased risk of recurrence in general ($P < 0.001$, log-rank test) with a 3.35 risk (95% CI, 1.72-6.49; $P < 0.001$; Table 2) of recurrence relative to patients whose tumors did not overexpress both HER3 and AIB1. Multivariate analysis did, however, not suggest that tumor HER3/AIB1 overexpression was an independent marker of patient outcome either for DFS, DFS on tamoxifen, or OS when analyzed alongside known prognostic markers, such as tumor size, grade, and nodal status.

**AIB1 and HER1-3 expression as combined biological marker of patient outcome.** Overexpression of AIB1 in HER1-3–positive tumors (HER1-3/AIB1, $n = 34$, 10%) was significantly associated with a decreased DFS during the patient’s treatment with tamoxifen ($P = 0.003$, log-rank test, Fig. 4A) with a 2.42-fold increased risk of relapse (95% CI, 1.32-4.43; $P = 0.004$; Table 2). When patients were stratified according to their AIB1 expression and HER1-3 positivity (HER1-3/high AIB1, HER1-3/low AIB1, no HER1-3/high AIB1, and no HER1-3/low AIB1), patients with AIB1-overexpressing HER1-3–positive tumors had a significantly decreased DFS during the treatment with tamoxifen ($P = 0.011$, log-rank test, Fig. 4B) compared with the other three patient groups. These patients (HER1-3/AIB1) also showed significantly reduced overall DFS ($P = 0.0371$, log-rank test, Fig. 4C) and OS ($P = 0.005$, log-rank test, Fig. 4D) with a 1.79-fold (95% CI, 1.03-3.13; $P = 0.039$; Table 2) increased risk of relapse and a 2.40-fold (95% CI, 1.27-4.50; $P = 0.007$; Table 2) increased risk of dying of the disease. Multivariate analysis suggested that overexpression of AIB1 in HER1-3–positive tumors was an independent predictor of decreased DFS on tamoxifen ($P = 0.023$, Cox’s regression) and OS ($P = 0.044$, Cox’s regression) when analyzed alongside known prognostic markers, such as tumor size ($P = 0.009$ and 0.010 for DFS on tamoxifen and OS, respectively), grade ($P = 0.013$ and 0.001 for DFS on tamoxifen and OS, respectively).

**Table 2. Hazard ratio**

<table>
<thead>
<tr>
<th>AIB1</th>
<th>DFS on tamoxifen, HR (95% CI)</th>
<th>$P$</th>
<th>DFS, HR (95% CI)</th>
<th>$P$</th>
<th>OS, HR (95% CI)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIB1</td>
<td>1.32 (0.80-2.17)</td>
<td>NS</td>
<td>1.23 (0.80-1.90)</td>
<td>NS</td>
<td>1.62 (0.97-2.69)</td>
<td>NS</td>
</tr>
<tr>
<td>HER2</td>
<td>1.94 (1.07-3.52)</td>
<td>0.029</td>
<td>1.35 (0.76-2.42)</td>
<td>NS</td>
<td>1.83 (0.96-3.48)</td>
<td>NS</td>
</tr>
<tr>
<td>HER3</td>
<td>1.18 (0.74-1.88)</td>
<td>NS</td>
<td>0.99 (0.66-1.49)</td>
<td>NS</td>
<td>1.524 (0.93-2.49)</td>
<td>NS</td>
</tr>
<tr>
<td>HER1-3</td>
<td>1.93 (1.20-3.00)</td>
<td>0.006</td>
<td>1.38 (0.90-2.10)</td>
<td>NS</td>
<td>1.62 (0.98-2.72)</td>
<td>NS</td>
</tr>
<tr>
<td>HER2/AIB1</td>
<td>2.20 (1.07-3.52)</td>
<td>0.047</td>
<td>1.32 (0.61-2.87)</td>
<td>NS</td>
<td>2.26 (1.03-4.95)</td>
<td>0.041</td>
</tr>
<tr>
<td>HER3/AIB1</td>
<td>3.67 (1.81-7.41)</td>
<td>&lt;0.001</td>
<td>3.35 (1.72-6.49)</td>
<td>&lt;0.001</td>
<td>3.79 (1.79-8.03)</td>
<td>0.001</td>
</tr>
<tr>
<td>HER1-3/AIB1</td>
<td>2.42 (1.32-4.43)</td>
<td>0.004</td>
<td>1.79 (1.03-3.13)</td>
<td>0.040</td>
<td>2.40 (1.27-4.50)</td>
<td>0.007</td>
</tr>
</tbody>
</table>

NOTE: HR, relative increased hazard, with 95% CIs, from Cox’s regression analysis showing relative OS, DFS, and DFS on tamoxifen of patients with high expression of AIB1, HER1, HER2, and HER1-3 alone or high HER2/AIB1 expression, high HER3/AIB1 expression, and high HER1-3/AIB1 expression. $P$ values are derived from Cox regression.

Abbreviation: NS, not significant.
and nodal status \((P = 0.0001\) and 0.0001 for DFS on tamoxifen and OS, respectively).

**AIB1 gene amplification.** AIB1 gene amplification status was evaluated in 362 of 402 (90\%) breast carcinomas. The remaining 40 cases were excluded from the study because of insufficient tumor material and loss of cores or because it was not possible to successfully fluorescence in situ hybridized the samples. AIB1 gene amplification was seen in 18 (5\%) carcinomas \((\text{gene/chromosome ratio, } 2.09-6.84)\). There was no consistent association between AIB1 gene amplification and AIB1 protein overexpression among the patients (data not shown).

**Discussion**

Data presented here show the significant role of AIB1 in promoting early relapse and death following tamoxifen treatment in hormone-responsive HER-expressing clinical breast cancers. The study provides clinical evidence that suggests a cross-talk between ER-\(\alpha\) and growth factor receptor pathways through changes in expression of specific coactivator proteins, such as AIB1 (6, 13).

In line with the study by Osborne et al (6), AIB1 expression alone was not a predictor of early relapse and death in this patient cohort. However, we confirmed that high AIB1 expression was a marker of tamoxifen resistance in HER2-overexpressing tumors (6) and also showed that high AIB1 expression was a highly significant marker of relapse and death of tamoxifen-treated patients with HER3-overexpressing tumors (Figs. 2 and 3). Patients with HER2/AIB1–overexpressing tumors exhibited 45\% 10-year DFS on tamoxifen compared with 75\% 10-year DFS on tamoxifen for all other patients (Fig. 2), whereas patients with HER3/AIB1–overexpressing tumors exhibited only 30\% 10-year DFS on tamoxifen compared with 70\% 10-year DFS on tamoxifen for all other patients (Fig. 3). The same patient groups also exhibited...
significantly 10-year reduced OS compared with the rest of the patients. AIB1 overexpression in HER2- or HER3-overexpressing tumors was, however, not an independent predictive marker of outcome in this patient cohort either for DFS during treatment with tamoxifen or OS when analyzed alongside known prognostic markers, such as tumor size, grade, and nodal status.

ER-α – positive tumors are more likely to be HER1 and HER2 negative as shown in this cohort of 402 ER-α – positive breast cancer patients, of which only tumors from six (1.5%) patients were HER1 positive and 51 (12.8%) tumors were HER2 positive. Subsequently, only tumors from 20 of 364 (5.5%) patients overexpressed both AIB1 and HER2. We have shown previously that patients from this cohort whose tumors were positive for one or more of HER1, HER2, or HER3 (HER1-3 positive) were significantly more likely to relapse on tamoxifen (24), and we therefore extended the study by Osborne et al. (6) and showed that HER1-3 – positive tumors overexpressing AIB1 exhibited a significantly decreased DFS on tamoxifen (45%) compared with 75% for the rest of the patients (Fig. 4A and B; Table 1). In contrast to when AIB1 overexpression was analyzed in HER2- and HER3-overexpressing tumors, AIB1 overexpression in HER1-3 – positive tumors was an independent marker of both decreased DFS on tamoxifen \( P = 0.023 \), Cox’s regression) and decreased OS \( P = 0.044 \), Cox’s regression) when analyzed alongside known prognostic markers. The putative value of AIB1 overexpression in HER1-3 – positive tamoxifen-treated tumors could be of particular interest for the Tamoxifen Exemestane Adjuvant Multicenter (TEAM) Trial and Intergroup Exemestane Study (IES; ref. 2), in which patients are randomized to continued treatment with either an AI or tamoxifen following completion of 2 to 3 years of tamoxifen. To further investigate the potential role of AIB1 overexpression in HER1-3 – positive tumors as an independent marker of early relapse and death in tamoxifen-treated breast cancers or as a marker for a switch to AI therapy after 2 to 3 years tamoxifen treatment, further work in a larger cohort of endocrine-treated breast cancers [TEAM; Arimidex Tamoxifen, Alone, or in Combination (ATAC); or IES] is required. This would also determine whether HER1-3 – positive tumors with AIB1 overexpression are more responsive to endocrine therapies that work by mechanisms different from that of tamoxifen. ER-α coactivator levels and HER-mediated activation of ER-α are expected to be less important for therapies, such as AI treatment, which is designed to reduce the levels of estrogens rather than preventing the binding of estrogens to ER-α.

AIB1 activity is regulated by posttranslational modifications, including phosphorylation (10, 26). Six phosphorylation sites have been identified, and phosphorylation at all sites are necessary for activation (26, 27). A wide range of kinases, including p42/44 mitogen-activated protein kinase, p38 mitogen-activated protein kinase, and b-kinases, mediate AIB1 phosphorylation and subsequently its binding to ER-α (10, 26). Currently, we are aiming to use immunohistochemistry to validate activated AIB1 as a marker of early relapse and death of tamoxifen-treated breast cancer patients on tumors from this patient cohort as well as tumors from the TEAM study.

We observed only 5% (18 cases) of patients form this cohort with AIB1 gene amplification (15, 16, 18, 19). The lower amplification rate could be due to the fact that ER-α – positive breast carcinomas are normally less aggressive tumors. The small number of AIB1 amplified cases prevented robust analysis of AIB1 gene amplification as a marker of tamoxifen resistance; however, to further investigate the predictive and/or prognostic role of amplification of AIB1, a larger cohort of both endocrine-treated and untreated breast cancer patients needs to be investigated.

Tamoxifen has been the standard first-line adjuvant endocrine treatment of postmenopausal metastatic breast cancers since the 1970s; however, alternative endocrine therapy, such as treatment with AIs, is often effective after disease relapse because most patients remain ER-α positive. The present study confirms that AIB1 is amplified or overexpressed in a subgroup of primary breast carcinomas. Our data also extend previous data by showing that AIB1 expression in HER1-3 – positive tumors might be an independent marker for early relapse and death on tamoxifen-treated breast cancers. The predictive or prognostic value of these combined markers should be further investigated along with other established putative markers in similar data sets, such as TEAM or ATAC, to verify their value in selection of tamoxifen or AI-based endocrine treatment.


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