Coassociation of Estrogen Receptor and p160 Proteins Predicts Resistance to Endocrine Treatment; SRC-1 is an Independent Predictor of Breast Cancer Recurrence

Aisling M. Redmond,1 Fiona T. Bane,1 Anthony T. Stafford,1 Marie McIlroy,1 Mary F. Dillon,1,2 Thomas B. Crotty,3 Arnold D. Hill,1 and Leonie S. Young1

Abstract Purpose: This study investigates the role of the p160 coactivators AIB1 and SRC-1 independently, and their interactions with the estrogen receptor, in the development of resistance to endocrine treatments.

Experimental Design: The expression of the p160s and the estrogen receptor, and their interactions, was analyzed by immunohistochemistry and quantitative coassociation immunofluorescent microscopy, using cell lines, primary breast tumor cell cultures, and a tissue microarray with breast cancer samples from 560 patients.

Results: Coassociation of the p160s and estrogen receptor was increased in the LY2 endocrine-resistant cell line following treatment with tamoxifen in comparison with endocrine-sensitive MCF-7 cells. In primary cultures, there was an increase in association of the coactivators with estrogen receptor following estrogen treatment but dissociation was evident with tamoxifen. Immunohistochemical staining of the tissue microarray revealed that SRC-1 was a strong predictor of reduced disease-free survival (DFS), both in patients receiving adjuvant tamoxifen treatment and untreated patients (P < 0.0001 and P = 0.0111, respectively). SRC-1 was assigned a hazard ratio of 2.12 using a Cox proportional hazards model. Endocrine-treated patients who coexpressed AIB1 with human epidermal growth factor receptor 2 had a significantly shorter DFS compared with all other patients (P = 0.03). Quantitative coassociation analysis in the patient tissue microarray revealed significantly stronger colocalization of AIB1 and SRC-1 with estrogen receptor in patients who have relapsed in comparison with those patients who did not recur (P = 0.026 and P = 0.00001, respectively).

Conclusions: SRC-1 is a strong independent predictor of reduced DFS, whereas the interactions of the p160 proteins with estrogen receptor can predict the response of patients to endocrine treatment.

Adjuvant endocrine therapy offers substantial benefit in terms of reduction in risk of tumor recurrence in women with estrogen receptor–positive tumors. However, although most patients initially respond to tamoxifen, in 30% to 40% of cases these tumors recur within 5 years. This precipitates cessation of the regime and the initiation of second-line therapy. However, despite new targeted treatments for breast cancer, the vast majority of patients still depend on endocrine manipulation for management of their breast cancer.

The magnitude of estrogen receptor gene regulation is influenced not only by the ligand but also by the presence of specific coregulatory proteins, present at rate-limiting levels, which modulate transcription. Over the past 10 years a number of nuclear receptor–interacting proteins have been isolated using various screening strategies. These include the p160 family coactivator proteins – steroid receptor coactivator-1 (SRC-1/NCoA-1), SRC-2 (TIF2/GRIP1), and SRC-3 (AIB1/pCIP/RAC3/ACTR). The coactivator proteins drive nuclear receptor transcriptional activity by doing the significant reactions required for control of enhancer-dependent gene expression. These coactivator proteins modulate the entire transcriptional process, including chromatin modification, transcription initiation, chain elongation, and RNA splicing, through to termination of the transcriptional response (1).

SRC family members contain multiple structural and functional domains. The central region of SRC proteins includes several conserved LXXLL motifs. These regions are responsible for interaction with ligand-bound nuclear receptors.
Translational Relevance

Breast cancer continues to affect one in ten women in the western world and despite the phenomenal advances in recent years the mortality rate still remains at around 35%. There is, therefore, a pressing need, both in terms of health and economics, to identify patients with a poor prognosis to optimize treatment strategies. This study provides compelling evidence of a role for the coactivator SRC-1 as a prognostic indicator in breast cancer, independent of endocrine treatment. Using quantitative coassociation analysis, the relevance of colocalization of the coactivator proteins, SRC-1 and AIB1, with estrogen receptor in predicting endocrine resistance was established in a patient population for the first time. Identification of tissue markers that can accurately predict prognosis and response to endocrine therapies is a substantial advancement in the pursuit of a personalized medical program for breast cancer patients.

Two transcriptional activation domains (AD) are located in the COOH-terminal region of the protein. The AD1 contains multiple LXXLL motifs that are responsible for interaction with general transcription coactivators CBP and p300, which can facilitate transcription through histone acetylation (2). In models of mammary gland tumorigenesis, induced either by the expression of the mouse tumor virus-Ha-ras transgene in mammary epithelial cells or by the chemical carcinogen DMBA, tumor progression is significantly suppressed in AIB1 knockout mice compared with the wild type (3, 4). SRC-1−/− knockout models also provide indirect evidence regarding the role of SRC-1 in steroid resistance. SRC-1−/− deficient mice exhibit an altered hypothalamic-pituitary-adrenal axis function (5) and alteration of uterine growth, mammary gland ductal side branching, and alveolar formation in response to estrogen compared with wild-type mice (6).

In human breast cancer, a role for SRC proteins in tumor progression is rapidly being established. Recent studies suggest that disruption of estrogen receptor interactions with coactivator proteins may inhibit cell growth of endocrine-resistant breast cancer (7). Depletion of AIB1 can significantly reduce estrogen-induced cell proliferation and inhibition of apoptosis (8, 9). In patient populations, variable amplification frequencies of the AIB1 gene have been reported (10). In a large patient population, Osborne et al. have described an association between high expression of AIB1 and reduced disease-free survival (DFS), which was compounded by coexpression with the growth factor receptor human epidermal growth factor receptor 2 (HER2; ref. 11). In line with these observations we have reported that AIB1 protein expression associates with breast tumor recurrence in a HER2-positive population (12). The function of the coactivator SRC-1 in breast tumor development is perhaps less well described than that of AIB1. We have previously reported that estrogen can induce SRC-1 recruitment to the estrogen receptor response element in breast cancer cell lines and in primary cell cultures derived from patient tumors (13). In patients undergoing endocrine treatment, SRC-1 protein expression strongly associates with reduced DFS on both univariate and multivariate analysis (14). These data are supported by prostate cancer studies, in which SRC-1 expression is associated with both clinical and pathologic variables of increased tumor aggression (15).

In this study we examined the role of SRC-1 and AIB1 as independent prognostic markers of DFS in breast cancer, and assessed their ability to act as predictors of response to endocrine treatment. The significance of p160 coassociation with the steroid receptor estrogen receptor was investigated in vitro using established endocrine-sensitive and -resistant cell lines and ex vivo using both primary cell cultures derived from patient tumors and in breast cancer patient tissue.

Materials and Methods

Cell culture and cell treatments. Breast cancer cell lines, endocrine-sensitive MCF-7 (American Type Culture Collection, LGC Promochem) and endocrine-resistant MCF-7–derived LY2 (ref. 16; kind gifts from Robert Clarke, Georgetown University, Washington DC) were maintained in MEM (Eagle) with 2 mmol/L L-glutamine and supplemented with 10% fetal bovine serum (Invitrogen). All cell lines were incubated at 37°C, under 5% CO2. Cells were steroid depleted for 72 h prior to treatment with 17β-estradiol (10−8 mol/L), 4-hydroxytamoxifen (10−8 mol/L), and ICI 182780 (10−8 mol/L). For growth assays, hormone treatment was added for 24 h before analyzing cell proliferation by cell counts using trypan blue exclusion. For immunofluorescent microscopy experiments, cells were treated for 1 h, cytocyntrifuged onto slides at 700 rpm for 2 min using a Shandon Cytospin 3, and fixed in 100% methanol for 5 min.

Western blotting. Western blotting for AIB1 and SRC-1 was conducted as previously described (17).

Primary cultures. After ethical approval, breast tumor specimens were obtained from patients undergoing surgery for removal of a histologically confirmed breast tumor. The breast cancer cells were dissociated using a digestion mixture (DMEM-F12, 10% fetal bovine serum, 10 μg/mL insulin, 5 μg/mL fungizone, 500 U/mL penicillin, 500 μg/mL streptomycin, 1 mg/mL neomycin, 100 U/mL hyalurondases, 200 U/mL collagenases) and then transferred to culture medium (DMEM-F12, L-Glutamine, 10 μg/mL basic fibroblast growth factor, 20 μg/mL epidermal growth factor, 5 μg/mL insulin, 0.4% bovine serum albumin). Cells were treated for 1 h and cytocyntrifuged onto slides at 700 rpm for 2 min using a Shandon Cytospin 3, and fixed in 100% methanol for 5 min.

Knockdown of SRC-1 and AIB1 using siRNA. Predesigned small interfering RNA (Ambion) directed against exon 5 of the SRC-1 gene was used to knock down SRC-1 expression. Small interfering RNA–AIB1 was constructed and both genes were silenced as previously described (18). Twenty-four hours posttransfection, hormone treatments were added to the cells for a further 24 h. Cell proliferation was monitored by cell counts done using trypan blue exclusion.

Immunofluorescent microscopy and quantitative colocalization. Breast cancer cells and sections were incubated in goat anti-rabbit antibody (1/200; Molecular Probes) for 60 min. Sections were blocked again with goat serum for 60 min. Mouse anti-human estrogen receptor α (10 μg/mL in 10% human serum; Santa Cruz) was placed on each slide for 90 min. The sections were incubated with Alexa 594 conjugated goat antirabbit antibody (1/200; Molecular Probes) for 60 min. Sections were blocked again with goat serum for 60 min. Mouse anti-human estrogen receptor α (10 μg/mL in 10% human serum; Santa Cruz) was placed on each slide for 90 min. The sections were incubated with Alexa 488 conjugated goat antismouse antibody (1/200; Molecular Probes) for 60 min. Sections were mounted using fluorescent mounting media (DAKO). Slides were examined under a Zeiss LSM 510 META confocal fluorescent microscope. Cells were
imaged using the 63× objective lens (1.40 NA). Quantitative colocalization analyses were done with Zeiss 510 META Software using the Pearson’s correlation coefficient \( R(r) \); ref. 19. The \( R(r) \) coefficient was calculated for nine images per treatment and tissue specimen, and the average calculated values for each condition were plotted on the histogram (error bars represent SE).

**Patient information and construction of tissue microarray.** Following ethical approval, breast tumor samples were obtained from archival cases at St Vincent’s University Hospital, Dublin, Ireland over period from 1987 to 1999. Excluded from the analysis were patients who did not have breast surgery, those who had neoadjuvant therapy, or those whose tissue specimens were irretrievable. A total 560 patients were used in this study; this sample was ample for all subgroup analysis of biomarkers. Patients received either no endocrine treatment (\( n = 200; \) estrogen receptor–positive 68%) or tamoxifen (\( n = 360; \) estrogen receptor–positive 74%), 20 mg/day for 5 y, which was discontinued only in those patients who suffered a relapse while on endocrine therapy. Data on the patients included pathologic characteristics (tumor size, grade, lymph node status, estrogen receptor status) as well as treatment with radiotherapy, chemotherapy, or tamoxifen. Follow-up data, median 7.72 y, was collected on the patients to determine DFS and overall survival.

Archival paraffin-embedded breast cancer tissue from the Department of Pathology, St Vincent’s University Hospital, was attained for the purposes of tissue microarray construction. A H&E slide was used on all specimens to mark the site of carcinoma. Three 0.6-mm punch biopsies were taken from each specimen, and transplanted into a recipient block. The recipient block was cut into 5-μm sections, mounted on Superfrost Plus slides (BDH), and baked in an oven for 1 h at 60°C.

**Immunohistochemistry.** Sections were incubated with primary antibodies as follows: rabbit antihuman SRC-1 (2 μg/mL; Santa Cruz) and mouse antihuman AIB1 (3 μg/mL; Cayman). The primary antibodies were incubated for 1 h at room temperature. Sections are subsequently incubated with the corresponding biotin-labeled secondary (0.5% in PBS; Vector Laboratories) for 30 min, followed by peroxidase-labeled avidin biotin complex (Vector Laboratories) for 30 min. Sections were developed in 3,3-diaminobenzidine tetrahydrochloride for 8 min and counterstained with hematoxylin for 3 min, then passed through increasing concentrations of Industrial Methylated Spirits (70% and 100%) and then xylene. Immunostained slides were scored using the Allred scoring system (20). A nuclear score of \( =3 \) was defined as positive staining. Independent observers, without knowledge of prognostic factors, scored slides.

**Fig. 1.** Quantitative coassociation analysis of SRC-1 with estrogen receptor α in MCF-7 and LY2 cell lines. Cells were steroid-depleted for 72 h prior to treatment with 17β-estradiol, 4-hydroxytamoxifen, or a combination of both (10⁻⁸ mol/L) for 1 h, then cytocentrifuged and fixed onto slides. Cells were analyzed under a Zeiss LSM 510 META confocal fluorescent microscope. A. representative immunofluorescent confocal images (630×) of MCF-7 are shown with insets illustrating a magnification of a selected subnuclear region (white square). B. histogram represents normalized Pearson’s correlation coefficient, shown as mean ± SE, in MCF-7 (blue) and LY2 (red) cells. C. Quantitative coassociation analysis of SRC-1 with estrogen receptor α in primary breast cancer cultures. D. representative immunofluorescent confocal images (630×) of LY2 cells with insets illustrating a magnification of a selected subnuclear region (white square).
Statistical analysis. Univariate analysis was done using Fisher’s exact test for categorical variables and Wilcoxon’s test for continuous variables. Multivariate analysis was carried out using Cox’s proportional hazard model. Two-sided $P \leq 0.05$ was considered to be statistically significant. Kaplan-Meier estimates of survival functions were computed, and the Wilcoxon test was used to compare survival curves.

Results

Interaction of SRC-1 and AIB1 with estrogen receptor $\alpha$ in MCF-7 and LY2 cell lines and in primary cell cultures. Co-activator levels and their role in cell proliferation were examined in the endocrine-sensitive MCF-7 and endocrine-resistant LY2 cell lines. SRC-1 and AIB1 protein levels were higher in the LY2 cell line in comparison with the MCF-7 cell line. In both cell lines, treatment with estrogen (17$\beta$-estradiol) for 24 hours resulted in an increase in SRC-1 and AIB1 protein levels (Supplementary Fig. S1A). Treatment of the MCF-7 and LY2 cell lines with 17$\beta$-estradiol resulted in increased cell proliferation. Exposure to the antiestrogen tamoxifen (4-hydroxytamoxifen) caused a decrease in MCF-7 cell number, whereas in the endocrine-resistant LY2 cell line, 4-hydroxytamoxifen resulted in an increase in cell proliferation (Supplementary Fig. S1B). Knockdown of the p160 coactivators SRC-1 and AIB1 resensitized the LY2 cells to 4-hydroxytamoxifen treatment (Supplementary Figs. S1C and D).

In order to investigate the interactions of SRC-1 and AIB1 with estrogen receptor $\alpha$ upon treatment with 17$\beta$-estradiol and 4-hydroxytamoxifen, these proteins were examined within individual cells using quantitative colocalization. In the MCF-7 cells, SRC-1 (Fig. 1A and B) exhibited increased coassociation with estrogen receptor $\alpha$ upon treatment with 17$\beta$-estradiol, in comparison with the control cells. In 4-hydroxytamoxifen–treated MCF-7 cells, SRC-1 and estrogen receptor $\alpha$ appeared in a more diffuse distribution in the nucleus (Fig. 1A and B). This is visible in the insets of Fig. 1A, with distinct red and green foci in the inset of the 4-hydroxytamoxifen–treated cells in contrast to the orange color of the nucleus in the cells treated with 17$\beta$-estradiol alone. Treatment of the MCF-7 cells with a combination of 17$\beta$-estradiol and 4-hydroxytamoxifen resulted
in a similar colocalization to that seen in the control cells (Fig. 1A). In primary tumor cell cultures derived from estrogen receptor–positive patients prior to adjuvant endocrine treatment, exposure to 4-hydroxytamoxifen decreased the colocalization of SRC-1 and estrogen receptor α, indicating disruption of the interactions between these proteins in an ex vivo setting (Fig. 1C). In contrast, in the endocrine-resistant LY2 cell line, treatment with 17β-estradiol, 4-hydroxytamoxifen, and a combination of both, caused increased nuclear localization of the p160s and estrogen receptor α and also increased their coassociation, as illustrated by the vivid yellow-orange of the nucleus (inset; Fig. 1B and D). These results show the association of SRC-1 with estrogen receptor α in the development of endocrine resistance.

In a similar manner the interactions between AIB1 and estrogen receptor α were driven by 17β-estradiol and disrupted by 4-hydroxytamoxifen in an endocrine-sensitive setting (Fig. 2A and B). Indeed in primary cell cultures 17β-estradiol drove AIB1 and estrogen receptor α interactions to a greater extent than was observed between SRC-1 and estrogen receptor α.

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**Fig. 3.** A, immunohistochemical localization of SRC-1 and AIB1 (100×, inset: 400×) on tissue microarray constructed from archival tissue from 560 breast cancer patients, showing positively (+) and negatively (−) stained cores and IgG controls. B, Kaplan-Meier estimates of DFS of breast cancer patients who were treated with tamoxifen (left panels) or untreated (middle panels) according to SRC-1 or AIB1. DFS according to SRC-1 and HER2 coexpression (top, right hand panel) and AIB1 and HER2 coexpression (bottom, right hand panel). Statistical analysis was conducted using the Wilcoxon’s test. P values and patient populations within the individual groups are noted in the bottom left-hand and bottom right-hand corner of the graphs, respectively.
We applied a Cox proportional hazards model, with time to recurrence as the end point and tumor size, grade, nodal status, age, estrogen receptor, HER2, and SRC-1 or AIB1 as the predictor variables. A stepwise evaluation of variables determined that SRC-1 was the best predictor tested. Significant predictors were SRC-1, nodal status, and tumor size, with SRC-1 being the strongest predictor of disease recurrence (hazard ratio, 2.12; \( P < 0.001 \)). AIB1 was found to have a hazard ratio of 1.09 when analyzed with the clinicopathologic variables.

Quantitative coassociation of SRC-1 and AIB1 with estrogen receptor α in breast cancer patient tissue microarray. In order to investigate the associations of the p160 coactivators with estrogen receptor α in an ex vivo setting, we used quantitative immunofluorescent microscopy to look at the colocalization of SRC-1 and AIB1 with estrogen receptor α in relation to recurrence of disease in our patient tissue microarray. Twenty patients were selected for this study, 10 patients who did not recur and 10 patients who suffered recurrence of disease. All patients studied were estrogen receptor α–positive and had received tamoxifen treatment. In the “recurrent group” DFS was <3 years. “Non recurrent” patients had no tumor relapse within the study period. Staining of the tissue microarray samples revealed SRC-1 and estrogen receptor α to have significantly increased colocalization in the patients who recurred in comparison with the patients with nonrecurrence disease (\( n = 10; \quad P = 0.00001; \quad \text{Fig. 4A} \)). Coassociation of SRC-1 and estrogen receptor α was noted in the merged immunofluorescence images and the inset from a recurrent patient, with yellow nuclei visible (Fig. 4B, lower panel). In contrast, there were distinct green nuclei and red cytoplasmic staining in tissue from patients who did not relapse (Fig. 4B, top panel). The p160 protein AIB1 was localized to the cytoplasm with estrogen receptor α present in the nucleus in the nonrecurrent patient (inset, Fig. 4C, top panel). In the patients who had recurrent disease, there was a significant increase in AIB1 and estrogen receptor α colocalization (\( n = 10; \quad P = 0.026; \quad \text{Fig. 4A and B, lower panel} \)). These results indicate the coassociation of the p160 coactivators with estrogen receptor α can predict the response of patients to endocrine treatment.

### Table 1. Associations of markers SRC-1 and AIB1 expression with each other using the Fisher’s exact test

<table>
<thead>
<tr>
<th>Total %</th>
<th>SRC-1 +</th>
<th>SRC-1 -</th>
<th>AIB1 +</th>
<th>AIB1 -</th>
<th>P</th>
<th>( \text{Total N} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRC-1 +</td>
<td>34.42%</td>
<td>40.51%</td>
<td>36.58%</td>
<td>34.42%</td>
<td>0.001</td>
<td>34.42%</td>
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<tr>
<td>SRC-1 -</td>
<td>65.58%</td>
<td>59.49%</td>
<td>63.42%</td>
<td>65.58%</td>
<td></td>
<td>65.58%</td>
</tr>
<tr>
<td>AIB1 +</td>
<td>73.58%</td>
<td>36.58%</td>
<td>40.51%</td>
<td>73.58%</td>
<td>0.001</td>
<td>73.58%</td>
</tr>
<tr>
<td>AIB1 -</td>
<td>26.42%</td>
<td>63.42%</td>
<td>59.49%</td>
<td>26.42%</td>
<td></td>
<td>26.42%</td>
</tr>
<tr>
<td>ER +</td>
<td>67.91%</td>
<td>35.26%</td>
<td>77.14%</td>
<td>67.91%</td>
<td>0.044</td>
<td>67.91%</td>
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<tr>
<td>ER -</td>
<td>32.09%</td>
<td>64.74%</td>
<td>22.86%</td>
<td>32.09%</td>
<td></td>
<td>32.09%</td>
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<tr>
<td>Her2m +</td>
<td>19.55%</td>
<td>47.37%</td>
<td>77.53%</td>
<td>19.55%</td>
<td>0.419</td>
<td>19.55%</td>
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<tr>
<td>Her2m -</td>
<td>80.45%</td>
<td>52.63%</td>
<td>22.47%</td>
<td>80.45%</td>
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<tr>
<td>Her2n+</td>
<td>11.97%</td>
<td>33.14%</td>
<td>72.73%</td>
<td>11.97%</td>
<td>0.870</td>
<td>11.97%</td>
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<tr>
<td>Her2n-</td>
<td>88.11%</td>
<td>66.86%</td>
<td>27.27%</td>
<td>88.11%</td>
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<td>88.11%</td>
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<tr>
<td>Cox-2+</td>
<td>47.15%</td>
<td>43.09%</td>
<td>79.31%</td>
<td>47.15%</td>
<td>0.017</td>
<td>47.15%</td>
</tr>
<tr>
<td>Cox-2-</td>
<td>52.85%</td>
<td>56.94%</td>
<td>20.69%</td>
<td>52.85%</td>
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<td>52.85%</td>
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<tr>
<td>pERK1/2+</td>
<td>29.84%</td>
<td>45.18%</td>
<td>70.16%</td>
<td>29.84%</td>
<td>0.039</td>
<td>29.84%</td>
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<tr>
<td>pERK1/2-</td>
<td>70.16%</td>
<td>54.82%</td>
<td>29.84%</td>
<td>70.16%</td>
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<td>70.16%</td>
</tr>
</tbody>
</table>

NOTE: Expression of variables (top row) is expressed as a percentage of patients in individual clinical classifications (1st column). Abbreviations: ER, estrogen receptor; cox-2, cyclooxygenase-2.

### Table 2. Comparisons of SRC-1 and AIB1 expression with clinicopathologic parameters using the Fisher’s exact test

<table>
<thead>
<tr>
<th>Total N</th>
<th>SRC-1</th>
<th>P</th>
<th>AIB1</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>% positive patients</td>
<td>560</td>
<td>34.42%</td>
<td>73.58%</td>
<td></td>
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<tr>
<td>Axilla</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>279</td>
<td>35.93</td>
<td>0.625</td>
<td>73.49%</td>
</tr>
<tr>
<td>Negative</td>
<td>265</td>
<td>33.62</td>
<td>73.68%</td>
<td></td>
</tr>
<tr>
<td>Size (cm)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;2.5</td>
<td>336</td>
<td>35.54%</td>
<td>0.921</td>
<td>69.96%</td>
</tr>
<tr>
<td>&lt;2.5</td>
<td>219</td>
<td>34.62%</td>
<td>79.88%</td>
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<tr>
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<td>III</td>
<td>200</td>
<td>35.63%</td>
<td>0.434</td>
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<tr>
<td>&lt;III</td>
<td>235</td>
<td>35.63%</td>
<td>75.68%</td>
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<td>Distant metastasis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>165</td>
<td>46.72%</td>
<td>0.001</td>
<td>70.40%</td>
</tr>
<tr>
<td>Negative</td>
<td>382</td>
<td>29.43%</td>
<td>75.85%</td>
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Discussion

Translational studies from our group and others have provided substantial evidence of a significant role for steroid receptor coactivator proteins in the development of resistance to endocrine therapy. It has been suggested that inappropriate levels of nuclear receptor coactivator proteins may be responsible for the development of agonist-like properties of estrogen receptor modulators. In patients who display resistance to endocrine treatment, both SRC-1 and AIB1 have been implicated in the conversion of the selective estrogen receptor modulator tamoxifen to its agonist profile (11, 14).

To date direct evidence of the mechanism of resistance has been more abundant for AIB1 with a paucity of information as to the exact role of SRC-1. Distinct biological differences between the two proteins have been described that help define their involvement in breast cancer recurrence on tamoxifen treatment (18, 21).

Previously we reported that silencing of AIB1 in tamoxifen-resistant breast cancer cells resulted in resensitization to the growth-arresting effects of the drug and also inhibited estrogen-stimulated cell growth (18). Indeed recent studies suggest that removal of coactivator proteins may release estrogen receptor to act as a transcriptional repressor of genes relevant to tumor progression (22). Here a functional role for SRC-1 in endocrine resistance was also confirmed using the LY2 cell line model. Pearson's quantitative coassociation has been applied previously to examine the subcellular colocalization of the p160s with estrogen receptor α (19). Using this powerful new application Amazit et al. quantified the colocalization pattern between p160 coactivators, (AIB1 and SRC-1) and estrogen receptor, and showed increased estrogen-induced...
interactions between the steroid receptor and its coactivator proteins (19). In this study we investigated if colocalization of AIB1 and SRC-1 with estrogen receptor α in the nucleus of breast cancer epithelial cells played a role in the development of endocrine resistance. In endocrine-sensitive MCF-7 breast cancer cells and in primary cell cultures derived from breast cancer patients prior to endocrine treatment, increased endogenous estrogen receptor α–p160 associations were observed in the presence of estrogen, compared with vehicle control. In line with its function as a steroid receptor modulator, treatment with tamoxifen successfully disrupted these associations. Conversely in the endocrine-resistant L2Y cells, both estrogen and tamoxifen increased nuclear colocalization of estrogen receptor α with AIB1 and SRC-1 compared with untreated controls. These data implicate inappropriate interactions between estrogen receptor and its p160 coactivator proteins in the development of the resistant phenotype.

To examine the significance of the p160 proteins in a large patient population, a tissue microarray of primary breast cancers was constructed. Molecular and clinical evidence from our group and others support a role for AIB1 in breast tumor progression (10, 11, 12, 18). The status of AIB1 as an independent tissue marker of disease recurrence, however, is somewhat contentious. Although there are recent reports that AIB1 can predict poor DFS independently of receptor status (23), other investigators have found that coexpression of AIB1 with the tyrosine kinase receptor HER2 is a stronger predictor of disease recurrence (11, 24). In support of these reports, in this study we observed that patients who were positive for both AIB1 and HER2 had a poor response to tamoxifen compared with all other patients. Furthermore, in line with recent studies which have reported estrogen receptor α–AIB1 interactions to be dependent on activated protein kinase B/mitogen-activated protein kinase pathways (18, 25), AIB1 expression was found to associate with phospho-ERK1/2.

The function of the coactivator SRC-1 in breast tumor development is perhaps less well described than that of AIB1. We have previously reported that estrogen can induce SRC-1 recruitment to the estrogen receptor response element in breast cancer cell lines and in primary cell cultures derived from patient tumors (13). Although early reports looking at SRC-1 mRNA levels suggest that expression of this coactivator indicates a favorable response to tamoxifen treatment (26), later studies from our group found that SRC-1 protein expression, in full-face immunohistochemical specimens, strongly associates with reduced DFS on both univariate and multivariate analysis (14). These data are supported by prostate cancer studies, in which SRC-1 protein expression associated with both clinical and pathologic variables of increased tumor aggression (15). From Kaplan-Meier estimates of DFS in this tissue microarray study, SRC-1 was found to significantly predict disease recurrence in both untreated patients and in patients treated with endocrine therapy. Although the significance was greater in the tamoxifen-treated group compared with patients who received no endocrine treatment, no statistical significance was observed between these two groups. These data suggest that SRC-1 can predict DFS independently of endocrine treatment. When we applied a Cox proportional hazards model, with time to recurrence as the end point and tumor size, grade, nodal status, adjuvant treatment, estrogen receptor, HER2, and SRC-1 status as the predictor variables, it was found that expression of SRC-1 was the strongest predictor of breast cancer recurrence compared with classic markers of poor DFS including HER2.

Although expression levels provide valuable information regarding disease progression, p160 coactivator proteins function in conjunction with their steroid receptor, estrogen receptor α. In order to evaluate this in our patient population we undertook, for the first time, quantitative coassociation studies in breast cancer patient tumors. In selected patients who had an early tumor recurrence on endocrine treatment, a significantly greater coassociation was observed between both coactivators and estrogen receptor α, compared with patients who had no disease recurrence within the study period. Here clinical ex vivo associations support both molecular and clinical studies which have reported roles for the p160s in tumor progression on endocrine treatment.

Data from this study verify the coactivator protein SRC-1 as a prognostic indicator in breast cancer independent of endocrine treatment. Quantitative coassociation analysis elucidated the relevance of colocalization of the coactivator proteins with estrogen receptor in predicting endocrine resistance. Taken together, these data firmly establish a clinical role for the SRC proteins in breast cancer disease recurrence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We gratefully acknowledge Dr. Gabrielle Kelly (Department of Statistics, University College Dublin), and Prof. Ronan Conroy (Department of Epidemiology and Public Health Medicine, Royal College of Surgeons in Ireland) for guidance with statistical analysis.

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Aisling M. Redmond, Fiona T. Bane, Anthony T. Stafford, et al.


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