Estrogen-Related Receptor α Expression and Function Is Associated with the Transcriptional Coregulator AIB1 in Breast Carcinoma

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Abstract

The significance of the estrogen-related receptor α (ERα) as prognostic marker for poor clinical outcome in breast carcinoma has recently been reported. Transcriptional activity of nuclear receptors such as ERα depends on coregulatory proteins. Thus, we compared the expression of different receptors, coregulators, and target genes on RNA and protein level in identical primary breast tumor samples (n = 48). We found a positive correlation between the transcripts of ERα and AIB1 (amplified in breast cancer-1), a coactivator overexpressed in breast cancers and associated with resistance to antihormone treatment. These data were confirmed on protein level, studying an independent patient collection (n = 237). Expression of the estrogen-regulated gene pS2 was associated with ERα only in tumors, where estrogen receptor (ERα) expression was low or absent. In ERα high expressing tumors, no correlation of ERα and pS2 was observed. AIB1 interacts directly with ERα as shown by fluorescence-resonance energy transfer, mammalian two-hybrid, and coimmunoprecipitation assays with endogenous proteins. It enhances ERα transcriptional activity in ERα-negative breast cancer cell lines as shown in functional reporter gene assays. Blocking ERα with an inverse agonist abolished interaction and coactivation by AIB1. Recruitment of both proteins to ERα target gene promoters further supports the significance of their interaction. Our findings identify AIB1 as functionally relevant co-factor for ERα in breast carcinoma. ERα/AIB1 complexes may control estradiol-regulated genes in a hormone-independent manner. Accordingly, ERα might be a rewarding target for treatment of endocrine-resistant tumors. [Cancer Res 2009;69(12):5186–93]

Introduction

Estrogen hormones regulate the growth and development of normal as well as malignant human breast epithelial cells through activation of estrogen receptor α (ERα; ref. 1). Antagonists of ERα, such as tamoxifen, therefore play an essential role in the treatment of ERα-positive mammary carcinomas (2). However, ~30% of primary breast carcinomas do not express ERα, and some patients acquire resistance to tamoxifen (3). Thus, ERα-negative and hormone treatment–resistant breast cancers are strongly associated with a poor prognosis. An effective targeted therapy for these tumors is still not available (4). Lately, the orphan nuclear receptor ERα emerges as a promising target for alternative treatment of breast carcinomas (5, 6). Expression of ERα in breast tumors correlates with an increased risk of recurrence, a poor prognosis of the patients (7), and high expression of the tyrosine kinase ErbB2 (Her2/neu; ref. 8). Recently, ERα was shown to be critical for the growth of ERα-negative breast cancer (9).

The structure and function of ERα resembles that of ERα, particularly with regard to recognition and regulation of target gene promoters (10). Like ERα, ERα binds to classic estrogen response elements (ERE) but also to binding sites consisting of an extended half of the palindromic ERE, referred to as ERR response elements (ERE; refs. 6, 10). Consequently, ERα can affect ERα transcriptional activity (11). This is reflected in the mutual regulation of common target genes, for instance the breast cancer marker gene pS2 (12) and other genes involved in mammary gland biology such as lactoferrin (6) and aromatase (13).

ERα functions as a constitutively active transcription factor. It binds neither natural estrogens nor other physiologic ligands known thus far (14). However, ERα can be activated by phosphorylation via epidermal growth factor and the ErbB2/Her2 signaling pathway (15, 16). Pharmacologic inactivation of ERα has been described for a few small molecules termed inverse agonists because they bind to the receptor but reverse its constitutive activity. Diethylstilbestrol (DES) is a weak inverse agonist for ERα (17). Remarkably, the ERα antagonist 4-hydroxytamoxifen (4-OHT), which is effective in the treatment of ERα-positive breast carcinomas, is not an inhibitor of ERα (18). More recently, the synthetic inverse agonist XCT790 was described to specifically inhibit ERα (19, 20).

Transcriptional activity of ERα is strongly dependent on interaction with coregulatory proteins. The binding of ERα to coactivators is constitutive and ligand independent. The peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) was identified as a strong coactivator of ERα in the context of energy metabolism (6, 21). Transcription through ERα is also enhanced by members of the steroid receptor cofactor (SRC) family, namely, SRC-1, GRIP1, and AIB1 (22). However, the physiologic relevance of ERα/cofactor interactions in estrogenic pathways in breast carcinoma has not been shown until now.

It was described that AIB1 is an oncogenic coactivator of ERα that is frequently amplified and overexpressed in human breast
carcinomas and is associated with resistance to endocrine treatment (23, 24). Similarly, expression of the coactivator SRC-1 is associated with resistance to antihormone therapy and breast tumor recurrence (25). Conversely, expression of NCOR1, a transcriptional corepressor of ERα, predicts a good prognosis in breast carcinomas (26).

To assess the contribution of coregulatory proteins to ERα activity in breast cancer, we investigated the relative expression levels of various cofactors, receptors, and target genes in primary breast carcinomas. Moreover, functional studies in breast cancer cell lines including ERα inhibitory ligands were done. As from our study AIB1 is a major coactivator of ERα in breast carcinoma, we also discuss therapeutic implications.

Materials and Methods

Tissue samples, patient characteristics, and tissue microarrays. Breast carcinoma specimens were randomly collected from 2001 to 2003 at the University Women’s Hospital, Heidelberg. Patient and tumor characteristics are summarized in Supplementary Table S1. None of the patients had received treatment for breast cancer before surgery. For mRNA studies, shock-frozen core biopsies from 48 unselected primary breast carcinomas taken at time of diagnosis were analyzed. Tissue microarrays (TMA) were custom-made at the tissue bank of the National Center for Tumor Diseases (NCT), Heidelberg. One TMA was composed of the same 48 breast tumors that were also analyzed in the mRNA expression studies (tumor set I). A second TMA consisted of 257 breast cancer samples (tumor set II) and 48 normal glandular breast samples (normal tissue). Each tumor was arranged on the array in duplicate.

Quantitative real-time PCR. Frozen tumor samples were disrupted in a dmembrator (B. Braun) and total RNA was extracted using TRIzol reagent (Invitrogen) and RNeasy (Qiagen) purification. Integrity, purity, and concentration of the RNA were determined (Agilent 2100 Bioanalyzer). The cDNA synthesis, quantitative real-time PCR analyses, and normalization to housekeeping genes (PGK1, LMNBL, and PPIA) were done as described previously (27). Primer sequences are available in Supplementary Table S2. Concentration of the RNA were determined (Agilent 2100 Bioanalyzer). The data shown are mRNA expression levels normalized to housekeeping genes (PPIA).

Immunohistochemistry. Staining of TMAs was done essentially as described before (28). Buffers, primary antibodies, and their dilutions are given in Supplementary Table S3. Each individual section on the TMA was evaluated according to the immunoreactivity score system suggested by Remmele and Stenger (29), resulting in scores ranging from 0 to 12 (for details, see Supplementary Fig. S2). Tumor samples were analyzed in duplicate and scores were averaged. The TMAs were evaluated by two independent investigators who were blinded to clinical and molecular variables.

Statistical analyses. The mRNA expression clusters (continuous variables) were obtained by unsupervised robust K-means clustering. ERα and ERα protein expressing groups were defined as immunoreactivity scores (discrete variables) above and below the 25% quartile. Associations between ERα mRNA expression levels and clinicopathologic parameters or AIB1 expression were evaluated by exact Wilcoxon rank sum test. Relationships among the expression levels of the various genes were assessed by Spearman’s rank correlation. Linear regression analysis was applied to test the relationship between pS2 and ERα expression. Differences with P < 0.05 were considered significant. For cluster analysis, we used the multiscale bootstrap resampling for hierarchical clustering (R package pvclust; ref. 30). Statistical computations were done with the software environment R, version 2.7.0.

Fluorescence–resonance energy transfer assay. For fluorescence-resonance energy transfer assay (FRET) assays, the ligand binding domain (LBD) of ERα was expressed in E. coli as glutathione S-transferase fusion protein using the vector pDEST15 (Invitrogen). A biotinylated AIB1 cofactor peptide was purchased (SynPep Corp.). Purified ERα-LBD, AIB1 peptide, and compounds were mixed and processed as described previously (31). Measurements were done in a Victor V fluorometer (Perkin-Elmer, Inc.) using 340 nm as excitation and 615 nm (donor signal) and 665 nm (acceptor signal) as emission wavelengths. Dose-response curves and IC50 values were calculated by Prism software (GraphPad Software, Inc.).

Compounds, cell lines, and plasmids. See Supplementary Materials.

Transient transfections and luciferase reporter assays. For the mammalian two-hybrid assay, HEK293 cells in 96-well plates were cotransfected with the reporter gene pFR-Luc (100 ng) and expression plasmids pCMVBD-AIB1 (5 ng) and pCMV-AD-ERα-LBD (25 ng) using polyethyleneimine (Sigma). Direct luciferase reporter gene assays were done with MDA-MB 231 and EVSA-T cells in 96-well plates cotransfected with 50 ng 2xERE-TK-Luciferase and 100 ng of either pTREX-ERαs or pTREX-AIB1 expression plasmid (or 50 ng of each for coexpression) using Effectene (Qiagen). Transfection efficiency was controlled by pRL-TK Renilla luciferase reporter (Promega). Compounds were added 4 h after transfection and luciferase activities were measured after 16 h according to Dyer and colleagues (32). Firefly relative light units were normalized to the Renilla control.

Chromatin immunoprecipitation and coimmunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were done with the ChIP assay kit (Millipore) according to the manufacturer’s instructions with minor modifications. MCF-7 cells were formaldehyde cross-linked, collected, and resuspended in SDS lysis buffer. After sonification (Bioruptor, Diagenode), chromatin was diluted 10-fold in ChIP dilution buffer (1% Triton X-100, 2 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L Tris-HCl pH 8.1) and immunoprecipitated overnight using the respective antibodies (Supplementary Table S3). After addition of salmon sperm DNA/protein A-agarose for 3 h at 4°C, immune-protein-DNA complexes were washed and eluted with 1% SDS in 0.1 mol/L NaHCO3 for 4 h at 65°C. Enriched fragments were isolated and subjected to quantitative real-time PCR using primers depicted in Supplementary Table S2. For coimmunoprecipitations, EVSA-T cells were treated with phorbol-12-myristate-13-acetate (PMA); 80 ng/mL; Sigma) for

### Table 1. Pairwise relationships between relative mRNA expression levels of ERα and selected receptors, cofactors, and target genes in breast cancer tissues (tumor set I, n = 48)

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Cofactors</th>
<th>Target genes</th>
</tr>
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<tbody>
<tr>
<td>ERα</td>
<td>PR</td>
<td>ErbB2</td>
</tr>
<tr>
<td>ERα</td>
<td>PGC-1α</td>
<td>AIB1</td>
</tr>
<tr>
<td>ERα</td>
<td>SRC-1</td>
<td>NCOR1</td>
</tr>
<tr>
<td>ERα</td>
<td>pS2</td>
<td>Aromatase</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>PR</th>
<th>ErbB2</th>
<th>PGC-1α</th>
<th>AIB1</th>
<th>SRC-1</th>
<th>NCOR1</th>
<th>pS2</th>
<th>Aromatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>(r) 0.028</td>
<td>(r) –0.032</td>
<td>(r) –0.086</td>
<td>(r) 0.411</td>
<td>(r) 0.183</td>
<td>(r) 0.221</td>
<td>(r) 0.006</td>
<td>(r) 0.231</td>
<td></td>
</tr>
<tr>
<td>(p) 0.851</td>
<td>(p) 0.877</td>
<td>(p) 0.558</td>
<td>(p) 0.004</td>
<td>(p) 0.214</td>
<td>(p) 0.131</td>
<td>(p) 0.224</td>
<td>(p) 0.965</td>
<td>(p) 0.114</td>
<td></td>
</tr>
<tr>
<td>ERα</td>
<td>(r) 0.326</td>
<td>(r) 0.464</td>
<td>(r) –0.014</td>
<td>(r) 0.645</td>
<td>(r) 0.410</td>
<td>(r) 0.444</td>
<td>(r) 0.447</td>
<td>(r) 0.224</td>
<td></td>
</tr>
<tr>
<td>(p) 0.024</td>
<td>(p) 0.001</td>
<td>(p) 0.927</td>
<td>(p) &lt;0.001</td>
<td>(p) 0.004</td>
<td>(p) 0.002</td>
<td>(p) 0.002</td>
<td>(p) 0.125</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: (r), Spearman’s rank correlation coefficient. (p), P values; P < 0.05 was considered significant (boldface).
Results

ERRα mRNA expression correlates with expression of the coactivator AIB1 in breast carcinoma. The expression of ERRα together with that of ERRα and progesterone receptor (PR); ErbB2; the transcriptional coregulators PGC-1α, AIB1, SRC-1, and NCOR1; and the ERRα target genes pS2 and aromatase was determined in 48 unselected primary breast carcinomas by quantitative real-time PCR (tumor set I, Supplementary Table S1). About 67% of the carcinomas were classified as tumors with elevated ERRα expression by cluster analysis. ERRα expression increases with tumor grade, but only in the low ERRα expressing group (Supplementary Fig. S1).

Pairwise comparison of the mRNA expression levels of the different factors revealed various significant correlations in the breast tumor tissues (Table 1; Supplementary Table S4). Already known positive interrelations between the classic ERα and its target genes PR (P = 0.001) and pS2 (P = 0.002), as well as with the coactivators AIB1 (P < 0.001), SRC-1 (P = 0.004), and the corepressor NCOR1 (P = 0.002), were found, confirming the validity of this study. We also observed a significant positive association of ERα and ERRα expression (P = 0.024).

In contrast to ERα, a highly significant correlation of ERRα expression in the breast tumors was only detected with AIB1 (P = 0.004), not with the other coregulators and not with PR or ErbB2 or the target genes pS2 and aromatase (Table 1). The median level of ERRα mRNA was ~2-fold higher in high AIB1 expressing tumors compared with low AIB1 expressing tumors (P = 0.01, Fig. 1A). Likewise, AIB1 mRNA was more than 2-fold more abundant in tumors with high ERRα expression (P = 0.004), showing the coordinate expression of both factors. To find the genes that are expressed in a similar manner across the set of breast tumors, we performed cluster analyses (Fig. 1B). In the resulting dendrogram, ERRα and AIB1 built a single cluster with a probability similar to the cluster consisting of ERα and PR, the main markers for response to hormonal breast cancer therapy (probability of 81% and 84%, respectively). Interestingly, the two ERRα target genes, pS2 and aromatase, built another cluster (probability of 90%), just as ErbB2 and NCOR1 do (probability of 81%).

ERRα and AIB1 expression also correlate on the protein level. To ascertain whether the correlation between ERRα and AIB1 mRNA expression also translates to protein levels, we used a custom-made TMA consisting of sections from the same tumor specimens that were used for the mRNA expression studies (46 informative cases of tumor set I). Immunohistochemistry was done to detect ERRα, AIB1, and pS2 protein in the tumor tissues (Supplementary Fig. S2). Comparisons of the immunoreactivity scores of the three factors showed a significant correlation of ERRα with AIB1 protein expression in the tumor tissues (Table 2; P = 0.012) but not with pS2 protein.

To further validate this result, we screened a larger set of 257 unselected primary breast carcinomas (tumor set II, Supplementary Table S1), as well as 48 normal glandular breast samples for expression of ERRα, AIB1, and pS2 protein. Again a significant positive correlation of ERRα with AIB1 protein expression was detected in this independent patient cohort (Table 2; P < 0.001), but not in the group of normal mammary tissue (P = 0.412). Cluster analysis done with the immunoreactivity data from this larger tumor set displayed a dendrogram similar to the RNA data. Again, ERRα and AIB1 built one cluster, which occurs with high probability (Supplementary Fig. S3). These findings clearly show that the interrelation between ERRα and AIB1 does not only occur at

![Figure 1](image-url)
significant positive correlation between pS2 and ERRα in tumors with either high or low ERα observed in our expression studies, we investigated their relation in the same tumors.

The mRNA level but is also reflected in similar protein levels within the same tumors.

**Association between ERRα and the estrogen-responsive breast cancer marker gene pS2 depends on the status of ERα.** Because no significant association of ERRα with pS2 was observed in our expression studies, we investigated their relation in tumors with either high or low ERα mRNA expression. A significant positive correlation between pS2 and ERRα was found in those carcinomas, where ERα was low or absent (Table 3, tumor set I; \( P = 0.035 \)). In cases with high ERα mRNA expression, no significant association between pS2 and ERRα was observed (\( P = 0.856 \)).

The same relationship was found on the protein level in an independent patient collection. Protein expression of pS2 correlated with the expression of ERRα in the ERα low expressing group, but not in tumors with elevated ERα expression (Table 3, tumor set II; \( P = 0.024 \) and \( P = 0.981 \), respectively). These data suggest that the regulation of estrogen-responsive genes in breast carcinoma may be controlled by ERRα depending on the presence of ERα.

**ERRα and AIB1 interact but can be dissociated by ERRα inverse agonists.** As our analyses revealed a close relationship of ERRα with AIB1 in breast carcinomas, we further investigated their physical interaction by performing a FRET assay with the ERRα LBD and an AIB1 peptide. A constitutive binding of ERRα and AIB1 was observed. Addition of the ERRα inverse agonists DES or XCT790 at increasing concentrations resulted in inhibition of this interaction with IC_{50} values of 9.8 μmol/L for DES and 0.9 μmol/L for XCT790 (Fig. 2A).

In a second step, we verified the interaction between ERRα and AIB1 in a mammalian two-hybrid assay by transient transfection of a Gal4-luciferase reporter gene with Gal4-AIB1 and ERRα-AD fusion proteins into HEK293 cells. Expression of ERRα together with AIB1 resulted in constitutive transcriptional activity of the reporter gene that was not observed in absence of ERRα (Fig. 2B). Again, this interaction could be blocked by treating the cells with increasing amounts of DES or XCT790. The calculated IC_{50} values in this cellular assay were 7 μmol/L for DES and 3.5 μmol/L for XCT790. These data show that AIB1 interacts constitutively with ERRα and that this interaction can be disrupted by ERRα inverse agonists.

To study whether ERRα and AIB1 also interact in vivo, nuclear extracts from the ERα-negative breast cancer cell line EVSA-T were immunoprecipitated with an anti-ERRα or anti-AIB1 antibody and subjected to Western blot analysis to detect either ERRα or AIB1 protein. As expected, ERRα was found in the precipitate from its own antibody but also in that from the AIB1 antisera, whereas no precipitate was present in the IgG control (Fig. 2C, top). Vice versa, in the extract precipitated with an ERRα antibody, we also coprecipitated AIB1 (Fig. 2C, bottom), clearly showing in vivo interaction of the endogenous proteins.

**ERRα transcriptional activity is enhanced by AIB1 and both proteins are recruited to ERRα target gene promoters.** To investigate the functional relevance of an ERRα/AIB1 interaction in absence of ERα, we cotransfected an ERRα-responsive luciferase reporter gene with expression vectors for ERRα, AIB1, or both into the ERα-negative breast cancer cell line MDA-MB

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### Table 2. Pairwise relationships between protein expression levels in breast cancer tissues from independent patient cohorts

<table>
<thead>
<tr>
<th></th>
<th>AIB1 (r)</th>
<th>pS2 (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERRα (n = 46)</td>
<td>0.360</td>
<td>0.105</td>
</tr>
<tr>
<td>Tumor set I</td>
<td>0.012</td>
<td>0.480</td>
</tr>
<tr>
<td>ERRα (n = 257)</td>
<td>0.393</td>
<td>0.049</td>
</tr>
<tr>
<td>Tumor set II</td>
<td>&lt;0.001</td>
<td>0.432</td>
</tr>
<tr>
<td>ERRα (n = 48)</td>
<td>0.122</td>
<td>-0.040</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>0.412</td>
<td>0.788</td>
</tr>
</tbody>
</table>

NOTE: (r), Spearman’s rank correlation coefficient. (p), \( P \) values; \( P < 0.05 \) was considered significant (boldface).

*Same breast tumor set (I) as for RNA expression.

†Independent breast tumor set (II).

‡Normal glandular breast tissue set.

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### Table 3. Relationship between the estrogen-responsive target gene pS2 and ERRα according to ERα status in breast tumor samples

<table>
<thead>
<tr>
<th>ERα expression</th>
<th>RNA (n = 48)</th>
<th>Protein (n = 195)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor set I</td>
<td>Tumor set II</td>
</tr>
<tr>
<td></td>
<td>No. of ERRα high expressing tumors</td>
<td>Correlation pS2 - ERRα</td>
</tr>
<tr>
<td>Low</td>
<td>23</td>
<td>( P = 0.035 )</td>
</tr>
<tr>
<td>High</td>
<td>9</td>
<td>( P = 0.856 )</td>
</tr>
</tbody>
</table>

NOTE: (p), \( P \) values; \( P < 0.05 \) was considered significant (boldface).

*Independent breast tumor set (II); due to the quality of the arrayed spots on the TMA, the number of informative cases of tumor set II on the TMA was reduced to 195.

†For reasons of comparability of the protein data, we performed additional immunohistochemical staining of ERα protein independent from routine diagnostics.

‡Multivariate linear regression analysis after clustering the ERRα-expressing tumors into two clusters according to ERα status.

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Overexpression of ERRα resulted in a 4.5-fold increase of reporter gene transcription, and increasing concentrations of the ERRα-specific inverse agonist XCT790 inhibited this activation (Fig. 3A, left). Further, overexpression of AI1 increased the reporter gene activity ∼3-fold whereas addition of XCT790 almost completely blocked this AI1-mediated induction (Fig. 3A, middle). Thus, endogenous ERRα was responsible for this effect, showing that AI1 can function as coactivator for ERα in ERα-negative breast cancer cell lines. Finally, coexpression of ERRα and AI1 together led to a synergistic activation of reporter gene transcription, ∼2-fold higher than with each of the factors alone (Fig. 3A, right). XCT790 completely blocked this activation, confirming that indeed ERRα is coactivated by AI1 in absence of ERα. Identical results were obtained with another ERα-negative breast cancer cell line (EVSA-T; Supplementary Fig. S4). Noteworthy, treatment of the cells with 4-OHT had no effect on ERRα- and AI1-induced transcription (data not shown), indicating that the observed activations were not due to other receptors binding to this reporter gene that could be inhibited by 4-OHT (e.g., ERβ, ERRαγ, and ERRγ).

As we detected in vivo interaction of ERα and AI1 in nuclear cell extracts (Fig. 2C), we investigated whether both proteins are recruited simultaneously to ERRα-containing promoter regions by ChIPs. To study a known target gene of ERRα, we investigated the pS2 promoter. Despite the presence of ERα, we detected recruitment of both ERRα and AI1 to this promoter in MCF-7 cells, with ERα being recruited at a lower level compared with AI1 (Fig. 3B, left). We also found strong recruitment of ERα (data not shown). To check whether other breast cancer relevant genes are targeted by ERRα/AI1 as well, we examined recruitment to the lactoferrin gene, containing another ERRα-regulated promoter. We found the ERRα region of this promoter to be also occupied by both proteins, albeit with higher levels of enrichment for ERRα compared with AI1 (Fig. 3B, right). Altogether these data suggest a concerted role of ERRα and AI1 in regulation of transcription in breast cancer cells.

Discussion

Although ERRα has been identified as a negative prognostic marker in various cancer types, its role in progression of breast carcinoma is far from being understood. In the present study, we measured elevated levels of ERα in two thirds of breast carcinomas, which is in good agreement with previous work (7). Comparison of ERα expression with histopathologic parameters revealed a correlation between ERα and tumor grade only in ERα low expressing carcinomas. In the report of Suzuki and colleagues (7), no significant association was detected between ERα immunoreactivity and clinicopathologic parameters. However, ERα expression turned out to be an independent prognostic factor for clinical outcome of the patients. In the current study, the follow-up time of patients was too short (42 months in average) to perform appropriate survival analyses.

In our expression analysis, we found a positive association between ERRα and ERα expression, which was observed by Suzuki and colleagues (7) as well, whereas another study reports on an inverse correlation between ERRα and the ERα and PR status of breast tumors (8). Nevertheless, ERRα gene expression itself is induced by ERα (33), which rather argues for a positive relationship between ERRα expression and estrogen signaling. Because ERRα is a modulator of ERα-mediated responses (11), these findings suggest a potential feedback loop on ERα action through up-regulation of ERRα.

Remarkably, ERα expression was positively correlated with the coactivators AI1 and SRC-1 as well as with the corepressor
NCOR1. It was already reported that both AIB1 and NCOR1 expression are positively associated with ERα in breast tumors (34), which is analogous with our findings. Coactivators and corepressors are jointly required to fully ensure ERα transcriptional activity as parts of the same regulatory pathway. Consequently, we would assume that both kinds of modulators are present in tumors with elevated ERα levels.

As a constitutive active orphan nuclear receptor, ERα is strongly dependent on interaction with coregulatory proteins. This study is the first to investigate and compare the expression of ERα together with various cofactors in primary breast carcinoma. We found a strong correlation of ERα expression with that of the coactivator AIB1, but not with other cofactors tested (SRC-1, PGC-1α, and NCOR1). Thus far, PGC-1α is the best characterized coactivator of ERα (6, 21). Surprisingly, we did not detect an association between ERα and PGC-1α expression in breast carcinomas. Moreover, the mRNA expression level of PGC-1α was very low in the tumor tissues. This is in agreement with the finding that PGC-1α expression is reduced in human breast cancer compared with normal mammary epithelium (35). Hence, we conclude that PGC-1/ERα complexes do not play a major role in breast tumorigenesis.

On the other hand, we detected an association between ERα and AIB1 both on mRNA and protein levels and confirmed that finding in an independent breast tumor collection. Noteworthy, ERαs and AIB1 expression did not correlate in normal mammary tissue, suggesting a functional significance of their interaction for breast tumor development or progression.

The data on ERα and AIB1 association are intriguing, considering that AIB1 functions as an oncogene (36) that is amplified and/or overexpressed in malignant breast tissues (37, 38). AIB1 can initiate the development of malignant tumors and is necessary for estrogen-mediated cell proliferation (23, 24). Here we show that AIB1 is also a physiologically relevant coactivator of ERα in mammary tissue and breast cancer cells. We proved that endogenous ERα and AIB1 interact in breast cancer cells by coimmunoprecipitation. In functional reporter gene assays in ERα-negative breast cancer cell lines, AIB1 increases ERα transcriptional activity. Thus, overexpression of AIB1 most likely enhances ERα transcriptional activity in tumor tissues as well. The data further suggest that in absence of ERα, ERα is an activator of ERα target genes. Two possible scenarios can be envisaged: In carcinomas where both receptors are expressed, ERα possibly competes with ERα for binding to AIB1. Hence, it prevents ERα to be activated by AIB1, resulting in the repression of ERα target gene expression. This model is supported by a previous study showing that ERα actively antagonizes estradiol-induced activity of ERα in MCF-7 cells (11). In ERα-negative tumors, however, endogenous ERαs together with AIB1 may substitute ERα, resulting in hormone-independent regulation of estrogen target genes. In line with this hypothesis, we observed a positive correlation of ERαs with the expression of the estradiol-regulated gene pS2 in carcinomas with low or no expression of ERαs, but not in ERα-high expressing tumors. In a similar study, Suzuki and colleagues (7) did not find a significant association between ERαs expression and pS2 regardless of ERα status. Nevertheless, in agreement with our findings, they observed a loss of correlation between ERα and pS2 in ERα-positive breast carcinomas.
As recruitment of ERRα to the pS2 promoter has been described in MCF-7 cells previously (15), we investigated ERRα/AIB1 recruitment to this promoter in these cells by ChIP and found it to be occupied by both proteins. The rather low level of ERRα basal recruitment to this promoter is likely due to the role of EREs as major regulator of pS2 in MCF-7 cells. In addition, we found enhanced binding of ERRs at the promoter of lactoferrin, another breast cancer relevant target gene of ERRα. These findings provide evidence for an influence of ERRα/AIB1 complexes on ERR activity at target genes and confirm the significance of their interaction for transcriptional regulation in mammary cancer cells.

Altered cofactor expression or availability to ERT is discussed as a mechanism to explain resistance to antiestrogen therapies (39). In patients receiving tamoxifen treatment, high AIB1 expression is associated with tamoxifen resistance and a shorter disease-free survival (40). Interestingly, AIB1 overexpression was observed also in ERα-negative tumors (41), suggesting that AIB1 may affect breast tumorigenesis through other factors as well. Given that tamoxifen is not a ligand for ERRα, elevated levels of ERRα/AIB1 complexes in breast tumors might be one of the mediators of hormone resistance. Consequently, targeting ERRα with inhibitory ligands represents a very promising approach to overcome this resistance. Here we show that the functional enhancement of ERRα activity by AIB1 is inhibited by the ERRα inverse agonist XCT790 in ERα-negative breast cancer cell lines. Moreover, we observe inhibition of proliferation of these cell lines by XCT790 in a dose-dependent manner.5 However, the potency and selectivity of the currently available ERRα inverse agonists such as XCT790 and DES need substantial improvement. Rational design of ligands that specifically disrupt ERRα/AIB1 complexes based on the crystal structure of inverse agonist–bound ERRα (42) may provide new drugs that feature the desired properties.

Finally, ERRα expression had been assessed in carcinomas other than breast, namely, ovarian, endometrial, prostate, and colorectal carcinomas (6, 21). These reports strengthen the notion that ERRα may be involved in the pathology of these cancers as well. Intriguingly, AIB1 is found to be implicated in the same tumor entities and also in various types of hormone-insensitive tumors such as pancreatic, gastric, colorectal, and hepatocellular carcinomas (23). Thus, functional ERRα/AIB1 complexes may also be associated with the etiology of other cancer types, making them even more attractive as targets for cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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