Expression Levels of Estrogen Receptor-α, Estrogen Receptor-β, Coactivators, and Corepressors in Breast Cancer

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ABSTRACT
Recent studies have indicated that a complex machinery of transactivation of target genes by estrogen or androstren through estrogen receptor (ER) exists. However, the substantial roles of ER-β, coactivators, and corepressors in the development and progression of breast cancer remain to be elucidated. To obtain some clue to these roles, we screened the expression levels of ER-α, ER-β, coactivators (SRC-1, TIF2, AIB1, CBP, and P/CAF) and corepressors (N-CoR and SMRT) in 6 normal mammary glands, 6 intraductal carcinomas, 22 invasive ductal carcinomas, and 7 breast cancer cell lines using a multiplex reverse transcription-PCR. ER-α mRNA expression levels significantly correlated with ER-α protein levels measured by enzyme immunoassay in the breast cancer tissues and cell lines. A significant correlation of expression levels was observed between ER-α and TIF2, AIB1, P/CAF, and N-CoR, and between ER-β and AIB1 and CBP in the tissue samples. A significant correlation was also observed between ER-α and ER-β and between ER-β and CBP in the cell lines. The expression levels of ER-α, TIF2, and CBP were significantly higher in the intraductal carcinomas than those in the normal mammary glands. In addition, the expression levels of ER-α and N-CoR were significantly higher in the intraductal carcinomas than those in the invasive ductal carcinomas. These findings suggest a positive correlation of expression levels among ER-α and cofactors and among ER-β and cofactors, an up-regulation of expression levels of ER-α and cofactors during the development of intraductal carcinomas from normal mammary glands, and a decrease in their expression levels during the progression of breast cancer.

INTRODUCTION
ER<sup>1</sup> belongs to the steroid/thyroid nuclear receptor family and is an estrogen-dependent transcriptional factor that regulates growth, development, differentiation, and homeostasis by binding to estrogen response elements in DNA to modulate transcription of target genes, including progesterone receptors and transforming growth factors, in target organs, such as the breast and uterus. Recent studies have disclosed a complex machinery of transactivation mediated by ER. There are several cofactors, termed coactivators and corepressors, which interact with ER and basal transcriptional machinery and activate or repress ER-mediated transcription (1–3). In addition, a new subtype of ER, ER-β, also participates in the transcriptional machinery as homodimers or heterodimers with ER-α (4–6).

The definitive roles of ER in the development and progression of breast cancer have been elucidated (7–9), but the substantial roles of ER-β and cofactors in breast cancer have not been clarified. Recently, it has been suggested that the relative expression levels of ER-β versus those of ER-α decrease during human breast tumorigenesis (10). There have been some reports suggesting a possible relationship between the expression levels of a coactivator, SRC-1, and the clinical responses to an antiestrogen, tamoxifen (11), between the expression levels of another coactivator, AIB1 and ER-α expression levels (12, 13), and between the development of tamoxifen resistance and the expression levels of a corepressor, N-CoR, in breast cancer (14). Taken together with the fundamental knowledge of ER-mediated transcriptional machinery, these recent findings suggest ER-β and cofactors may play certain roles in the development and progression of breast cancer.

To obtain some clue to the roles of ER-β and cofactors in breast cancer, we decided to use a semiquantitative multiplex RT-PCR method to measure the relative expression levels of ER-α, ER-β, and cofactors. We screened their expression levels in 7 human breast cancer cell lines, and then 6 normal mammary glands, 6 intraductal carcinomas, and 22 invasive ductal carcinomas were studied in the same manner.

MATERIALS AND METHODS
Breast Cancer Cell Lines and Tissue Samples. The KPL-1, KPL-3C, and KPL-4 cell lines were established in our laboratory, and their characterization has been published elsewhere (15–17). All these cell lines were derived, respec-

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3 The abbreviations used are: ER, estrogen receptor; RT-PCR, reverse transcription-PCR; HAT, histone acetyltransferase; TIF2, transcription intermediary factor 2; AIB1, amplified in breast cancer 1; CBP, CREB-binding protein; N-CoR, nuclear receptor corepressor; SMRT, silencing mediator of retinoid and thyroid receptors; P/CAF, p300/CBP-associated factor.
tively, from the malignant pleural effusion of three different Japanese patients with recurrent breast cancer. The KPL-1 and KPL-3C cell lines are ER positive, but the KPL-4 cell line is ER negative. Four other human breast cancer cell lines, the MCF-7 (ER-positive), T-47D (ER-positive), MDA-MB-231 (ER-negative), and SkBr-3 (ER-negative) cell lines were kindly provided by Dr. Robert B. Dickson (at the Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC). A total of 28 breast cancer specimens and 6 normal mammary glands, which were located more than 5 cm away from the breast cancer in mastectomy specimens, were selected from the tumor bank of our department. Each breast cancer specimen was confirmed to include tumor tissues by pathologists of our institute. Six tumors were derived from patients with intraductal carcinomas, 11 tumors from patients with node-negative invasive ductal carcinomas, and the other 11 tumors were from patients with node-positive invasive ductal carcinomas. In breast cancer specimens, ER in 26 of 28 samples were measured by an enzyme immunoassay (Dinabot, Tokyo, Japan). Tumor specimens, were selected from the tumor bank of our department, Washington, DC). A total of 28 breast cancer specimens and 6 normal mammary glands, which were located more than 5 cm away from the breast cancer in mastectomy specimens, were selected from the tumor bank of our department. Each breast cancer specimen was confirmed to include tumor tissues by pathologists of our institute. Six tumors were derived from patients with intraductal carcinomas, 11 tumors from patients with node-negative invasive ductal carcinomas, and the other 11 tumors were from patients with node-positive invasive ductal carcinomas. In breast cancer specimens, ER in 26 of 28 samples were measured by an enzyme immunoassay (Dinabot, Tokyo, Japan). Tumor samples with >10 fmol/mg protein of ER were defined as ER positive.

**Multiplex RT-PCR Method.** Total cellular RNA from the human breast cancer cell lines and tissue samples was extracted with a TRIzol RNA extraction kit (Life Technologies, Inc., Gaithersburg, MD), according to the manufacturer’s recommendations. One μg of total RNA and 1μl Oligo(dT)-18 primer in 12.5 μl of diethyl pyrocarbonate-treated water were heated to 70°C for 2 min, followed by cooling on ice for 1 min. cDNA synthesis was initiated with 200 units of recombinant Moloney murine leukemia virus reverse transcriptase (Clontech Laboratories, Inc., Palo Alto, CA) under conditions recommended by the manufacturer, and the reaction was allowed to proceed at 42°C for 1 h. The reaction was terminated by heating at 94°C for 5 min. cDNA was dissolved to a final volume of 100 μl by adding 80 μl of diethyl pyrocarbonate-treated water and then was frozen at −20°C until use. Oligonucleotide primers for the RT-PCR were designed using a published sequence of each target gene and synthesized by the solid-phase triester method. The primers and conditions used in this study and the expected sizes from the reported cDNA sequence are shown in Table 1. To amplify both β-actin, the housekeeping control gene, and the target gene in a single reaction, the multiplex RT-PCR was carried out. The ratios of primer sets between the target gene and β-actin are also shown in Table 1. These ratios and PCR cycles were determined to amplify both products logarithmically and in relatively similar amounts. Each RT-PCR reaction contained 1/100 of cDNA, the indicated concentrations of primers of each target gene and β-actin, 200 μM deoxyxynucleotide triphosphates, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, 50 mM KCl, 0.08% NP40, and 1 unit of recombinant *Thermus aquaticus* DNA polymerase (MBI Fermentas, Vilnius, Lithuania) in a final volume of 20 μl. After an initial denaturation at 94°C for 4 min, various cycles of denaturation (at 94°C for 15 s), annealing (at various temperatures, as shown in Table 1 for 15 s), and extension (at 72°C for 30 s) for the respective target genes were performed on a DNA Thermal Cycler 2400 (PC-960G Microplate Gradient Ther-
Fig. 1  Multiplex RT-PCR analysis of ERs and cofactors in seven human breast cancer cell lines (A) and in representative tissue samples (B). The gel images are shown in inverted presentation. Primers and PCR conditions are shown in Table 1. The upper band and lower band in each lane show PCR products of β-actin and each target gene, respectively.

Table 2  Relationships of mRNA expression levels among ER-α, ER-β, and cofactors in breast cancer cell lines and breast tissues

<table>
<thead>
<tr>
<th></th>
<th>ER-β</th>
<th>SRC-1</th>
<th>TIF2</th>
<th>AIB1</th>
<th>CBP</th>
<th>P/CAF</th>
<th>N-CoR</th>
<th>SMRT</th>
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<tr>
<td><strong>Breast cancer cell lines (n = 7)</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>r</strong></td>
<td>0.860</td>
<td>0.162</td>
<td>0.144</td>
<td>0.595</td>
<td>0.631</td>
<td>0.180</td>
<td>0.487</td>
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<tr>
<td><strong>P</strong></td>
<td>0.035&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.397</td>
<td>0.724</td>
<td>0.115</td>
<td>0.122</td>
<td>0.659</td>
<td>0.233</td>
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<tr>
<td><strong>r</strong></td>
<td>0</td>
<td>0.148</td>
<td>0.259</td>
<td>0.815</td>
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<td><strong>P</strong></td>
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<td>0.635</td>
<td>0.046&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.856</td>
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<td><strong>Tissue samples</strong></td>
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<td>ER-α</td>
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<td><strong>r</strong></td>
<td>0.012</td>
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<td>0.279</td>
<td>0.351</td>
<td>0.558</td>
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<tr>
<td><strong>P</strong></td>
<td>0.823</td>
<td>0.253</td>
<td>0.006&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.038&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.109</td>
<td>0.041&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.001&lt;sup&gt;c&lt;/sup&gt;</td>
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<td><strong>n</strong></td>
<td>32</td>
<td>32</td>
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<td>32</td>
<td>31</td>
<td>32</td>
<td>32</td>
<td>31</td>
</tr>
<tr>
<td>ER-β</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>r</strong></td>
<td>0.252</td>
<td>0.143</td>
<td>0.346</td>
<td>0.530</td>
<td>0.304</td>
<td>0.277</td>
<td>0.157</td>
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<td><strong>P</strong></td>
<td>0.141</td>
<td>0.405</td>
<td>0.044&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.002&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.076</td>
<td>0.106</td>
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<tr>
<td><strong>n</strong></td>
<td>32</td>
<td>32</td>
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<td>32</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>31</td>
</tr>
</tbody>
</table>

a Spearman’s rank correlation was used.
b Regression coefficient
c Statistically significant (P < 0.05).
Relationships among Expression Levels of ER-α and ER-β, Cofactors, and Clinicopathological Factors.

As shown in Table 2, significant positive correlations of expression levels were observed between ER-α and ER-β and between CBP and ER-β and between CBP and the breast cancer cell lines. A trend was also observed between ER-α and AIB1 or CBP. In contrast, significant positive correlations were observed between ER-α and TIF2, AIB1, P/CAF, or N-CoR and between ER-β and AIB1 or CBP in tissue samples. A trend was also observed between ER-α and CBP or SMRT and between ER-β and P/CAF or N-CoR. No significant correlation was observed between SRC-1 and ER-α or ER-β. Representative plots of the relative expression levels are shown in Fig. 2.

Statistical Analysis. The relative mRNA expression levels of ERs and cofactors were expressed as means ± SD and analyzed using StatView computer software (ATMS Co., Tokyo, Japan). These values were compared among different groups by the Mann-Whitney U test. To analyze the correlation of expression levels between two different genes, Spearman’s rank correlation was used. Two-sided P < 0.05 was considered as statistically significant.

RESULTS

Relationships of mRNA Expression Levels among ER-α, ER-β, and Cofactors. As shown in Table 2, significant positive correlations of expression levels were observed between ER-α and ER-β and between ER-β and CBP in the breast cancer cell lines. A trend was also observed between ER-α and AIB1 or CBP. In contrast, significant positive correlations were observed between ER-α and TIF2, AIB1, P/CAF, or N-CoR and between ER-β and AIB1 or CBP in tissue samples. A trend was also observed between ER-α and CBP or SMRT and between ER-β and P/CAF or N-CoR. No significant correlation was observed between SRC-1 and ER-α or ER-β. Representative plots of the relative expression levels are shown in Fig. 2.

Relationships among ER Protein Levels and mRNA Expression Levels of ER-α, ER-β, and Cofactors. As shown in Table 3, the mRNA expression levels of ER-α and AIB1 were significantly higher in the ER-positive breast cancer cell lines than those in the ER-negative cell lines. The mRNA expression levels of ER-α were also significantly higher in the ER-positive breast cancer tissues than those in the ER-negative breast cancer tissues.

Relationships among Expression Levels of ER-α, ER-β, or Cofactors and Clinicopathological Factors. Age (>50 years or not), tumor size (>3 cm in size or not), histology (intraductal carcinomas or invasive ductal carcinomas), and recurrence (yes or no) were divided into two categories, and the expression levels of ER-α, ER-β, and cofactors between two categories were compared. As shown in Fig. 3, the expression levels of ER-α, TIF2, and CBP were significantly higher in the intraductal carcinomas than those in the normal mammary glands (3.432 ± 2.477 and 1.304 ± 1.004 for ER-α, P = 0.025; 1.213 ± 0.849 and 0.279 ± 0.102 for TIF2, P = 0.025; 1.235 ± 0.698 and 0.384 ± 0.297 for CBP, P = 0.037). A trend was observed in the expression levels of AIB1 and N-CoR (1.109 ± 0.636 and 0.520 ± 0.269 for AIB1, P = 0.078; 1.363 ± 0.535 and 0.973 ± 0.071 for N-CoR, P = 0.055). The expression levels of ER-α and N-CoR were significantly higher in the intraductal carcinomas than those in the invasive ductal carcinomas (3.432 ± 2.477 and 1.641 ± 2.026 for ER-α, P = 0.038; 1.363 ± 0.535 and 0.926 ± 0.259 for N-CoR, P = 0.016). The expression levels of TIF2, CBP, and P/CAF were significantly higher in the node-positive tumors than those in the node-negative tumors (1.447 ± 0.772 and 0.656 ± 0.556 for TIF2, P = 0.009; 1.472 ± 0.636 and 0.909 ± 0.591 for CBP, P = 0.030; 0.899 ± 0.516 and 0.486 ± 0.360 for P/CAF, P = 0.041). N-CoR expression levels were higher in the tumors from patients without recurrence than those in tumors from the patients with recurrence (1.132 ± 0.357 and 0.926 ± 0.248, P = 0.004). No such difference in expression levels of any factors was observed between the two categories of age and tumor size.
ERα, ERβ, and Cofactors in Breast Cancer

Table 3

<table>
<thead>
<tr>
<th>Cofactors</th>
<th>Cell lines</th>
<th>Tumor samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ER-positive</td>
<td>ER-negative</td>
</tr>
<tr>
<td>ER-α</td>
<td>2.019 ± 0.824</td>
<td>0.232 ± 0.402</td>
</tr>
<tr>
<td>ER-β</td>
<td>0.256 ± 0.304</td>
<td>0.044 ± 0.075</td>
</tr>
<tr>
<td>SRC-1</td>
<td>0.954 ± 0.137</td>
<td>0.890 ± 0.091</td>
</tr>
<tr>
<td>TIF2</td>
<td>0.862 ± 0.117</td>
<td>0.759 ± 0.016</td>
</tr>
<tr>
<td>AIB1</td>
<td>0.884 ± 0.116</td>
<td>0.542 ± 0.160</td>
</tr>
<tr>
<td>CBP</td>
<td>0.630 ± 0.140</td>
<td>0.489 ± 0.054</td>
</tr>
<tr>
<td>P/CAF</td>
<td>0.666 ± 0.212</td>
<td>0.558 ± 0.282</td>
</tr>
<tr>
<td>N-CoR</td>
<td>1.002 ± 0.225</td>
<td>0.876 ± 0.188</td>
</tr>
<tr>
<td>SMRT</td>
<td>1.396 ± 0.367</td>
<td>1.540 ± 0.399</td>
</tr>
</tbody>
</table>

⁷ Cell lines or tumor samples whose ER measured by enzyme immunoassay were >10 fmol/mg protein were defined as ER positive. Values are means ± SD.

⁸ Tumor samples include 13 ER-positive invasive tumors, 4 ER-positive intraductal tumors, 7 ER-negative invasive tumors, and 2 intraductal tumors.

DISCUSSION

ER in breast cancer tissues has been routinely measured worldwide by various methods, including the ligand-binding assay, enzyme immunoassay, or immunocytochemical assay, as the most reliable predictor of the efficacy of endocrine therapy for patients with breast cancer. However, only half of ER-positive recurrent breast cancers respond to endocrine therapy. ER is known as an estrogen-dependent transcriptional factor for modulating expression levels of target genes in target organs. Recent studies have disclosed a complex machinery of ER-mediated transactivation, including the participation of another ER subtype, ERβ, and cofactors.

It has been indicated that estrogen binds to ERα, ERα undergoes conformational change, binds to estrogen response elements on nuclear target genes, and recruits coactivators and general transcription factors to form an active transcriptional complex, resulting in chromatin remodeling and enhancement of target gene expression. Three related coactivators named as p160s, SRC-1, TIF2, and AIB1 interact with CBP and activate the AF-2 function of ERα. SRC-1 and CBP exhibit autonomous HAT activity. Both p160s and CBP bind to a potent HAT, P/CAF. It has been believed that HAT activity of these coactivators may enable them to remodel chromatin and allow access to the transcriptional machinery. In contrast, in the absence of ligand, ERα associates with corepressors, N-CoR or the related factor SMRT, to mediate transcriptional repression of target genes. It is interesting to note that these corepressors have histone deacetylase activity (1–3).

ERβ is homologous to ERα, particularly in the DNA binding domain (95% amino acid identity). ERβ binds estradiol with a similar affinity to that of ERα. It should be noted that ERα and ERβ may form functional heterodimers (4–6). Three different pathways through which estrogens may activate target genes are possible. However, it is unclear whether specific response elements exist that are selectively recognized only by one or two of the three possible combinations.

These fundamental findings indicate that an abnormality of this machinery may modulate the growth response of breast cancer to estrogen or antiestrogen and may be responsible for the resistance of breast cancer to endocrine therapy. Furthermore, certain changes in the expression levels of ERs and cofactors might influence this machinery and might play important roles in the development and progression of breast cancer. To clarify these hypothetical issues, we systematically screened the mRNA expression levels of ERs and cofactors in normal mammary glands, intraductal carcinomas, invasive ductal carcinomas, and breast cancer cell lines in the present study.

Despite the small number of samples explored, several interesting findings have been made: (a) the expression levels of ERα correlated with those of all cofactors, except for SRC-1 (Table 2); (b) The expression levels of ERβ correlated with those of some coactivators, such as AIB1, CBP, and P/CAF and of a corepressor, N-CoR (Table 2); (c) The expression levels of TIF2 closely correlated with those of CBP and P/CAF (r = 0.666, P < 0.001 and r = 0.754, P < 0.001, respectively), and those of AIB1 also correlated with those of CBP and P/CAF (r = 0.637, P < 0.001 and r = 0.505, P = 0.003). The expression levels of AIB1 also closely correlated with those of

Fig. 3 Changes in the relative mRNA expression levels of ERα and cofactors from normal mammary glands (N) to intraductal carcinomas (D) and from D to invasive ductal carcinomas (I). In general, the expression levels increase from N to D and decrease from D to I. *, a statistically significant difference.
TIF2 ($r = 0.754$, $P < 0.001$). In contrast, those of SRC-1 did not correlate with those of CBP and P/CAF. These findings indicate coexpression of coactivators, such as TIF2, AIB1, CBP, and P/CAF, but not SRC-1 in target organs. SRC-1 expression levels may be independent of those of other coactivators; (d) the expression levels of some members involved in the ER-mediated transcriptional machinery, such as ER-$\alpha$, TIF2, AIB1, CBP, N-CoR, and SMRT, seems to be up-regulated during the development of intraductal carcinomas from normal mammary glands (Fig. 3); and (e) the expression levels of ER-$\alpha$ and N-CoR seem to be down-regulated during the progression of intraductal carcinomas to invasive ductal carcinomas (Fig. 3).

Although a positive correlation between AIB1 expression levels or AIB1 gene amplification and ER-$\alpha$ expression levels in breast cancer has been reported already (12, 13), the present study demonstrates, for the first time, a positive correlation of expression levels among ER-$\alpha$ and other cofactors, such as TIF2, CBP, P/CAF, N-CoR, and SMRT in breast cancer (Table 2). Interestingly, the expression levels of these cofactors were up-regulated during the development of intraductal carcinomas from normal mammary glands. The expression levels of ER-$\alpha$ were also up-regulated at the same time (Fig. 3). These findings suggest that some epigenetic or genetic factors may promote ER-$\alpha$ expression in normal mammary glands, and this may result in the coinduction of several cofactors during breast tumorigenesis. Furthermore, the expression levels of ER-$\alpha$ and a cofactor, N-CoR, were simultaneously down-regulated during breast cancer progression from intraductal carcinomas to invasive ductal carcinomas, in particular, node-positive ones (Fig. 3). Clonal selection of ER-negative and more aggressive tumor cells, which express a low level of N-CoR, may occur during breast cancer progression. Because the number of samples was limited and only the mRNA expression levels of these factors were investigated in the present study, our speculation should be carefully evaluated. However, it has been already accepted that ER-$\alpha$ is up-regulated during breast tumorigenesis and down-regulated during breast cancer progression (9). Further analysis of the roles of these cofactors in the development and progression of breast cancer may provide us with a better understanding of hormone responsiveness in breast cancer.

Recent studies have attempted to elucidate the roles of ER-$\beta$ in the development and progression of breast cancer or ovarian cancer. It is suggested that the relative expression ratios of ER-$\alpha$:ER-$\beta$ increase during breast or ovarian tumorigenesis (10, 18, 19). However, in the present study, these ratios in intraductal and invasive ductal carcinomas were not higher than those in normal mammary glands (data not shown). This may be attributable to differences in the methods used to measure the expression levels of ERs (the other investigators used a multiplex RT-PCR with both ER-$\alpha$ and ER-$\beta$, but we used a multiplex RT-PCR with respective ER and $\beta$-actin) and/or attributable to the differences in explored samples. No other relationship among ER-$\beta$ expression levels and clinicopathological factors in breast cancer tissues has been reported yet. The present study also revealed a novel finding that ER-$\beta$ expression levels correlated with some cofactors, such as AIB1, CBP, P/CAF, and N-CoR (Table 2). Because of a lack of information on functional roles of ER-$\beta$ in breast cancer, the importance of this correlation is unknown.

Recently, additional members of possible coactivators, such as REA (20), Tip60 (21), and p68 (22), have been cloned and proved to take part in ER-mediated transactivation. In addition, some ER-related nuclear receptors, such as SHP (23) and ERR3 (24), have been reported to interact with ERs and regulate ER-mediated transactivation function. These new members of the ER-mediated transcriptional machinery might also play some roles in the development and progression of breast cancer.

In conclusion, our screening of mRNA expression levels of ERs and cofactors by the multiplex RT-PCR in tissue samples and cell lines revealed a possible coinduction of ERs and several cofactors during the development of breast cancer and a possible down-regulation of ER-$\alpha$ and a corepressor, N-CoR, during the progression of breast cancer. These findings suggest that ERs and cofactors may synergistically play certain roles in the development and progression of breast cancer.

**REFERENCES**


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