Coexpression of Estrogen Receptor α and β: Poor Prognostic Factors in Human Breast Cancer?1

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Abstract

The cloning of a second estrogen receptor (ER), ERβ, has prompted a reevaluation of the role of ERs in breast cancer. The aim of this study was to determine the expression of both ER isoforms in normal (n = 23) and malignant (n = 60) human breast tissue by reverse transcription-PCR and correlate this information with known prognostic factors including tumor grade and node status. In normal breast tissue, expression of ERβ predominated, with 22% of samples exclusively expressing ERβ; this was not observed in any of the breast tumor samples investigated. Most breast tumors expressed ERα, either alone or in combination with ERβ. Interestingly, those tumors that coexpressed ERα and ERβ were more node positive (P = 0.02; Fisher’s exact test) and tended to be of higher grade. Because antiestrogens are agonists when signaling through the AP1 element, overexpression of ERβ in tumors expressing both ER subtypes may explain the failure of antiestrogen therapy in some breast cancer patients. Thus, ERβ may be a useful prognostic factor in patients with breast cancer.

Introduction

Since the cloning of the ER2 in 1986 (1, 2), it was believed that only a single receptor (now termed ERα) was responsible for mediating the effects of estrogens on target tissues. Recently, a second ER, referred to as ERβ, has been identified in the rat, human, and mouse (3–5). ERβ is highly homologous to ERα at the DNA (96%) and ligand binding (58%) domains, whereas the A/B domain, hinge region, and F region are not well conserved (4). ERβ binds estrogens with a similar affinity to ERα and activates the expression of reporter genes containing estrogen response elements in an estrogen-dependent manner (3, 6). Curiously, although the discovery of ERβ has prompted a reevaluation of the molecular basis for estrogen action, there have only been a few studies addressing a possible role for ERβ in human breast cancer. This is surprising because alterations in ER signal transduction are believed to contribute to breast cancer progression and the development of a hormone-independent and more aggressive phenotype. Of the studies reported to date, ERβ is expressed in human breast tumors (7, 8) and in chemically transformed human breast epithelial cells (9). ERβ splice variants have also been described in some breast tumors (10, 11). Furthermore, the ratios of ERα:ERβ gene expression appear to alter during carcinogenesis, suggesting that ERα- and ERβ-specific pathways may have definitive roles in this process (12). In addition to mediating gene transcription via the classic estrogen response element, ER subtypes can signal from an AP1 element, enhancer element that requires ligand and the AP1 transcription factors Fos and Jun for transcriptional activation (13). Recent data show that ERα and ERβ signal in opposite ways from an AP1 element. When bound to ERα, 17β-estradiol activates transcription, whereas with ERβ, transcription is inhibited (14). However, when antiestrogens bind to ERβ, they are potent transcriptional activators at an AP1 site, acting as agonists rather than antagonists (14).

Clinically, around two-thirds of all breast cancer patients with ERα+ tumors initially benefit from antiestrogen therapy. However, some of these patients eventually relapse. The mechanisms associated with the acquisition of an antiestrogen-resistant phenotype are incompletely understood. Because estrogens play an important physiological role in the normal breast and are involved in the pathophysiology of breast cancer, the aim of this study was to determine the expression of both ER isoforms in normal and malignant human breast tissue using RT-PCR and to correlate our observations with available clinical information. The use of RT-PCR rather than immunohistochemistry has been necessitated by the lack of any publication to date that has outlined the use of specific antibodies raised against human ERβ.

Materials and Methods

Tumor Samples. Sixty breast tumors were obtained from patients who presented sequentially for surgical removal of a clinically confirmed malignant breast lesion. All patients were staged by standard UICC criteria. Routine histological staging was performed by a consultant pathologist. Details are presented in Table 1. As a source of normal breast tissue, samples were obtained from 23 patients undergoing reduction mammoplasty who had no previous history of breast disease (mean age, 35 years; range, 18–42 years). Ethical approval was obtained, and all patients gave informed consent.

RNA Extraction and cDNA Synthesis. Frozen tissue was pulverized using a mortar and pestle, and total RNA was extracted with Trizol (Life Technologies, Paisley, United Kingdom) according to the manufacturer’s instructions. RNA (1 μg) was used as a template for first-strand synthesis as described previously (15).

PCR Amplification. Primers were obtained from Life Technologies and designed from published gene sequences. Primers used to amplify ERα were 5'-TGCCAGGAGACTCGCTA-3' (nucleotides 894–912) and 5'-TCAACTCTCCCCTTCCTCCTC-3' (nucleotides 1139–1157), giving an amplified product of 263 bp. For ERβ, primer sequences were 5'-TGTTACGAGATTGGAATGTGA-3' (nucleotides 484–505) and 5'-CCTTGAATTCTGCAGAGAAA-3' (nucleotides 936–956), giving an amplified product of 472 bp. To check cDNA integrity, fragments of glyceraldehyde phosphate-dehydrogenase, a standard housekeeping gene, were amplified in parallel; this was consistently positive (data not shown). The PCR reaction contained 2 units of BIOTAQ; 10× PCR buffer containing 1.5 mm MgCl2 (both from Bioline, London, United Kingdom); 0.5 μM of each oligonucleotide primer; 200 μM each of dATP, dCTP, dGTP, and dTTP; 1 μl of nascent cDNA; and sterile distilled water to bring the volume to 50 μl. As a positive control for ERα, cDNA from the ERα MCF-7 human breast cancer cell line was used; for ERβ, human testis cDNA was used. Negative controls included the substitution of RNA or cDNA with distilled water or the substitution of cDNA with an irrelevant cDNA (synthesized from human tibialis anterior muscle). These were consistently negative. All transcripts were analyzed in parallel on at least two separate occasions in a thermal cycler (Hybaid OmniGene, Teddington,
Table 1 Pathoclinical details of breast tumors used in this study

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>No.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Total no. in study</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Mean age (range) (yr)</td>
<td>63 (32–86)</td>
<td></td>
</tr>
<tr>
<td>Tumor histology</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Duct origin</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Miscellaneousa</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Tumor grade</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Lymph node status</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Menopausal status</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

a This group comprised three lobular carcinomas, two mixed lobular-ductal carcinomas and one medullary carcinoma.

Table 2 Expression of ERα and ERβ mRNA in normal breast and breast tumors

<table>
<thead>
<tr>
<th>ER status</th>
<th>Normal breast</th>
<th>Breast tumor</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>3 (13)</td>
<td>16 (27)</td>
<td>0.249</td>
</tr>
<tr>
<td>ERβ</td>
<td>5 (22)</td>
<td>0 (0)</td>
<td>0.0011</td>
</tr>
<tr>
<td>ERα + ERβ</td>
<td>3 (13)</td>
<td>30 (50)</td>
<td>0.0002</td>
</tr>
<tr>
<td>None</td>
<td>12 (52)</td>
<td>14 (23)</td>
<td>0.017</td>
</tr>
</tbody>
</table>

* Tumor versus normal tissue.

United Kingdom) with the following cycle: (a) a denaturation step of 94°C for 2 min; (b) 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and (c) a final primer extension step of 72°C for 5 min. PCR products were analyzed by electrophoresis through a 1.2% agarose gel and visualized by ethidium bromide staining under UV illumination.

**Restriction Enzyme Digests.** Restriction digests were performed on representative PCR products to confirm their identity. Amplified product (5 μl) was digested overnight at 37°C with either AvaII (ERα) or HinfI (ERβ), which yielded discrete fragments of 197 and 66 bp (ERα) or 321 and 151 bp (ERβ). Digested products were then electrophoresed through a 2% agarose gel and visualized as described above.

**Statistical Analysis.** Statistical analysis was performed using the Arcus software package for Windows (Research Solutions, Cambridge, United Kingdom). Fisher’s exact test was used to test the difference between the groups. Results were considered to be significant at P ≤ 0.05.

**Results**

**Expression of ERα and ERβ in Normal and Malignant Human Breast Tissues.** Sixty breast tumor samples and 23 samples of normal breast tissue were analyzed and compared for expression of both ER subtypes by RT-PCR. A significant difference in the expression of one or both receptor subtypes was observed between these tissue groups. As outlined in Table 2, compared with normal breast tissue, a significantly higher proportion of breast tumors coexpressed ERα and ERβ (50%; P = 0.0002). Although 27% of breast tumors exclusively expressed ERα, this was not statistically significant when compared with normal breast tissue, in which 13% of samples exclusively expressed this subtype. Expression of ERβ alone was observed in a proportion of normal breast samples (22%; P = 0.0011 versus breast tumors). Expression of this subtype was not observed in any of the breast tumors. These either coexpressed ERα and ERβ or expressed only ERα. A total of 52% of normal breast samples failed to express either ER subtype, compared with only 23% of breast tumors (P = 0.017 versus breast tumors). A representative gel showing transcripts for ERα and ERβ and their restriction-mapped products is illustrated in Fig. 1.

**Associations with Clinical Information.** Expression of ER subtypes within the tumor group was further analyzed. Fifty percent of this group coexpressed both ER subtypes (P = 0.000017; Table 3). To determine whether coexpression of ER subtypes in breast tumors was associated with clinical parameters, correlations were sought with tumor grade, lymph node metastasis, and menopausal status. When compared with tumor grade, there was a significant association with grade II tumors (P = 0.02); although not statistically significant, there was a suggestion of a trend toward an association with grade III tumors as well (Table 3). Similarly, menopausal status did not correlate with the expression of both subtypes (data not shown). However, those tumors that coexpressed ERα and ERβ were mostly lymph node positive (P = 0.02; Table 3).

**Discussion**

Over the years, clinical, epidemiological, and laboratory data indicate that estrogens are important mitogenic stimulants in breast cancer as well as having an important role in normal breast physiology. Estrogen acts by binding to its cognate receptors, ERα (described in 1986) and ERβ (identified some 10 years later). In the present study, we have evaluated and compared the coexpression of ERα and ERβ.

**Fig. 1.** Representative agarose gel showing RT-PCR products for ERα (A) and ERβ (B). Lane 1, 100-bp ladder; Lane 2, positive control; Lane 3, restriction-digested PCR products; Lanes 4–7, breast cancer samples; Lane 8, negative control. A, single and double arrowheads indicate restriction-digested products of 197 and 66 bp, respectively. B, single and double arrowheads indicate restriction-digested products of 321 and 151 bp, respectively. This confirms the identity of the PCR products.

Table 3 Node status and grade of tumors expressing ER subtypes

<table>
<thead>
<tr>
<th>ER subtype</th>
<th>No. (%)</th>
<th>LN+</th>
<th>LN−</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>16 (27)</td>
<td>5</td>
<td>11</td>
<td>2</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>ERβ</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ERα + ERβ</td>
<td>30 (50)*</td>
<td>19*</td>
<td>11</td>
<td>2</td>
<td>15*</td>
<td>13*</td>
</tr>
<tr>
<td>None</td>
<td>14 (23)</td>
<td>4</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>4</td>
</tr>
</tbody>
</table>

* P = 0.000017.

b P = 0.02.

P = nonsignificant versus other receptor subtypes.
in breast tumors and normal human breast tissue obtained from reduction mammoplasties using RT-PCR.

Our data show that compared with normal breast tissue, a significantly higher proportion of breast tumor samples expressed both ER subtypes. Furthermore, 50% of all breast tumors analyzed coexpressed ERα and ERβ. The only related study published to date has shown that expression of ERβ does not correlate with that of ERα (7). The ability of both ER subtypes to form DNA-binding homo- and heterodimers (6, 16, 17) points to three possible pathways of estrogen signaling. In those tissues that exclusively express either ERα or ERβ, signaling would be via the specific receptor, whereas in those tissues expressing both subtypes, signaling would be mediated by ERα/ERβ heterodimers. Thus, coexpression of ER subtypes within the same breast tumor offers an intriguing possibility whereby ERα and ERβ proteins may interact, leading to differential responses to estrogens/antiestrogens. It has been shown that ER subtypes can signal not only from the classic estrogen response element but also from an AP1 enhancer element (13). The downstream effects of AP1 signaling are both receptor and ligand specific. With ERα, 17β-estradiol activates transcription, whereas with ERβ, the reverse is true (14). However, when complexed with ERβ, antiestrogens including tamoxifen, raloxifene, and the pure antiestrogen ZM 164384 are potent transcriptional activators at an AP1 site, acting paradoxically as agonists rather than antagonists (14). Clinically, antiestrogens, particularly tamoxifen, are currently the first-line therapy for treatment of hormone-dependent breast cancer. Around 70% of all breast cancer patients with ERα+ lesions initially benefit from antiestrogen therapy. However, most of these patients eventually relapse. Mechanisms associated with the acquisition of an antiestrogen-resistant phenotype are poorly understood. Our results indicate that coexpression of both ER subtypes is significantly higher in breast tumors. Furthermore, those tumors with the ERα+/ERβ− phenotype were node positive (P < 0.02), and although only grade II tumors showed statistical significance, there was a trend toward an association with more poorly differentiated tumors. Both of these features point to a poorer prognosis. Speculatively, the overexpression/activation of ERβ in tumors that express both ER subtypes and the subsequent interaction with ligand-bound AP1 elements may explain the failure of antiestrogen therapy in some breast cancer patients.

A distinct pattern of expression of ER subtypes was observed between normal breast tissue and breast tumors. Both tissue sets expressed ERα. Although it appeared that a greater number of breast cancer samples expressed this subtype, this did not reach significance. The number of normal breast samples expressing ERα (13%) is slightly higher than the 7% reported by immunohistochemistry (18) but may be a reflection of the higher sensitivity of the RT-PCR over immunohistochemistry. Exclusive expression of ERβ was not observed in any of the breast tumor samples. Instead, this subtype was significantly associated with normal breast tissue and was found in 22% of these samples. This observation parallels a recent study by Leygue et al. (12) in which the relative expression of ERα/ERβ was determined using multiplex RT-PCR. These authors showed that ERβ expression was reduced in tumor compared to adjacent normal breast tissue, and although this did not reach statistical significance, there was a suggestion of a trend. Although our RT-PCR conditions were qualitative rather than quantitative, we too noted that in those samples that coexpressed both receptor subtypes, expression of the ERα product was always greater than that of ERβ (see Fig. 1). High levels of ERβ have recently been reported in the normal human breast epithelial cell line HBL100 and in chemically transformed human breast epithelial cells (9). Although we did not observe expression of just the ERβ subtype in breast tumors, this has been reported in both ERα+ and ER− breast cancer cell lines (7). However, it is possible that the acquisition of an ERβ+ phenotype may be an artifact of in vitro culture, which is not observed in primary tumors.

The age distribution of our study populations should also be considered. All normal breast samples were obtained from premenopausal women undergoing reduction mammoplasty for cosmetic purposes (mean age, 35 years; range, 18–42 years), whereas the majority of breast tumors were from postmenopausal patients. Although only six tumors were obtained from premenopausal subjects (mean age, 44 years; range, 32–51 years), four of these six subjects had ERα+/ERβ− phenotype (with the remainder expressing ERα). This somewhat contradicts previously reported findings, which show that in comparison with breast tumors from postmenopausal women, generally fewer premenopausal patients have ER− lesions (19). However, greater numbers of samples must be studied before any significance can be attributed to this observation.

In accord with all studies that evaluate gene expression, the detection of a gene transcript gives no indication of whether the transcript will go on to be transcribed and translated into an active peptide. Nevertheless, a positive relationship between ERα mRNA and the ligand binding assay has been observed in this laboratory, whereas others have shown associations between ERα mRNA and immunohistochemistry (20). Trends toward associations between ERβ status by ligand binding assay and RT-PCR have also been reported (12). However, there have been no reports confirming that the expression of ERβ mRNA parallels that of protein, which may reflect the difficulties in raising specific ERβ antibodies to date.

In conclusion, overexpression of ERβ in tumors expressing both ER subtypes and the subsequent interaction of ligand-bound receptors with AP1 elements may help explain the failure of antiestrogen therapy in some breast cancer patients. Current diagnostic procedures for breast cancer use immunological techniques designed to detect only ERα. With the imminent development of ERβ-specific antibodies, our results suggest that it may be relevant to determine the expression of both ER subtypes. This may be helpful in predicting the response of breast tumors to endocrine therapy. This information may permit the subsequent development of selective ER modulators to target tumors with a particular ER phenotype.

Acknowledgments

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References


4 V. Speirs, unpublished observation.
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