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Clinicopathological Characteristics of Estrogen Receptor-β-positive Human Breast Cancers

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Recent studies have identified the presence of estrogen receptor (ER)-β in addition to ER-α in human breast cancers, but the clinicopathological characteristics of ER-β-positive tumors remain to be established. In this study, we have conducted an immunohistochemical analysis of ER-α and ER-β expression in human breast cancers. In addition, we investigated the correlation of ER-α and ER-β expression with progesterone receptor (PR) status, determined by enzyme immunoassay, and with various clinicopathological factors. Of 79 tumors, 49 (62%) were positive for ER-α and 24 (30%) were positive for ER-β, and there was no significant association between ER-α and ER-β expression. ER-α-positive tumors were significantly more likely to be PR-positive than were ER-α-negative tumors ($P<0.0001$), but there was no significant association between ER-β expression and PR status. However, the PR values of ER-α-positive and ER-β-positive tumors (65±17 fmol/mg protein, mean±SE) were marginally significantly lower than those of ER-α-positive and ER-β-negative tumors (340±109) ($P=0.08$). ER-β positivity was significantly associated with small tumor size (≤2 cm) and high histological grade ($P<0.05$), and this association was also observed when only ER-α-positive tumors were considered. These results suggest that determination of ER-β status might be clinically useful for further defining the characteristics of ER-α-positive tumors.

Key words: ER-α — ER-β — PR — Immunohistochemical staining — Breast cancer

Although estrogen action has long been considered to be mediated through a single receptor (estrogen receptor [ER]-α), recent studies have identified a second ER (ER-β), which has a similar structure to ER-α.[1, 2] ER-β binds estrogens with high affinity and activates the transcription of reporter genes containing a hormone-responsive element (ERE) in an estrogen-dependent manner.[3] However, the functions of ER-α and ER-β are not identical, since differential activation of ERE-regulated reporter genes by these two receptors has been reported using the anti-estrogen 4-hydroxytamoxifen,[4] and differential activation of activator protein-1-regulated reporter genes has also been recognized.[5] The recent demonstration that ER-α and ER-β form heterodimers suggests a putative cross-talk between the two signaling pathways.[6, 7]

Based on these findings, it is speculated that the relative expression of the ER-α and ER-β receptors may modulate estrogen action, although the function of the heterodimeric receptor is far from clear. Several groups, including ours, have demonstrated the expression of ER-β mRNA in breast cancers and reported the characteristic features of breast cancers that express ER-β mRNA.[8–11] In this regard, Roger *et al.*[12] reported downregulation of ER-β mRNA levels during breast carcinogenesis and Speirs *et al.*[9] showed that coexpression of ER-α and ER-β mRNAs was significantly associated with lymph node metastasis. Furthermore, high levels of ER-β mRNA were demonstrated to be associated with tamoxifen resistance.[10] These results appear to indicate that ER-β plays a role in carcinogenesis, progression and hormone dependency of breast cancers. The above studies, however, were based on the detection of ER-β mRNA using a reverse transcriptase-polymerase chain reaction (RT-PCR) assay, and thus, are vulnerable to the criticism that ER-β mRNA levels do not necessarily represent ER-β protein levels and that contamination of the tumor tissues by ER-β mRNA-expressing lymphocytes might flaw the RT-PCR assay results. Immunohistochemical examination would appear to be superior to RT-PCR in this regard. To date, only a few reports are available on the study of ER-β protein expression in breast cancers by immunohistochemistry.[13, 14] Therefore, in the present study, we conducted an immunohistochemical analysis of ER-β expression in breast cancers, and correlated ER-β expression with various clinicopathological features.

MATERIALS AND METHODS

Tumor samples and immunohistochemical staining of ER-α and ER-β Tumor samples were obtained from 79 breast cancer patients who underwent mastectomy or

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breast conserving surgery in our hospital during the period from March 1998 to December 1999. Informed consent was obtained from every patient prior to the investigation. Immunohistochemical staining of ER-α and ER-β was performed using the avidin-biotin-peroxidase method with a rabbit anti-ER-α polyclonal antibody (H-184, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a chicken anti-ER-β polyclonal antibody (ER-β503),[15] essentially according to the method described previously.[12] In brief, formalin-fixed, paraffin-embedded tissues from the tumor samples were cut into 4-μm-thick serial sections. The sections were deparaffinized, and antigen was retrieved by heating at 120°C for 15 min in 10 m*M* citrate acid buffer (pH=6.0). Endogenous peroxidase activity was blocked by treatment with 0.3% H_2O_2 for 30 min and non-specific binding was blocked with 10% normal goat serum for 10 min. Primary antibodies were incubated with the sections overnight at 4°C, and an avidin-biotin-peroxidase complex technique was used for visualization of the immunostained ER-α and ER-β. Sections of tumors positive for ER-α by enzyme immunoassay (>5 fmol/mg protein) were used as a positive external control. Immunostaining of the normal ductal epithelial cells adjacent to cancer tissues was used as a positive internal control for ER-β. The most actively stained lesions were selected microscopically for calculation of the percentage of stained cells. At least 1000 tumor cells were examined in each case. The results were considered to be positive when more than 20% of cancer cells were stained, according to the report of Järvinen *et al.*[13] The slides were examined by two skilled observers who were blinded as to the clinicopathological features of the patients, and final agreement was reached by concensus using a 2-head microscope when the evaluations differed.

Enzyme immunoassay for progesterone receptor Tumor samples obtained during surgery were used for determination of progesterone receptor (PR) status in all patients except one case, where insufficient tumor sample for PR assay was available. PR levels were measured by enzyme immunoassay (EIA) using the kit provided by Abbott Research Laboratories (Chicago, IL) according to the instructions provided by the manufacturer. The cut-off value for PR positivity was 5 fmol/mg protein.

Statistical analysis The relationship between ER-α or ER-β status and various clinicopathological characteristics was evaluated using the χ^2 test. PR levels were compared between groups according to ER-β status using Student's *t* test. Statistical significance was assumed if $P<0.05$.

RESULTS

ER-α and ER-β expression in breast cancers and correlation with PR status Representative results for ER-α and ER-β immunostaining are shown in Fig. 1. Of 79

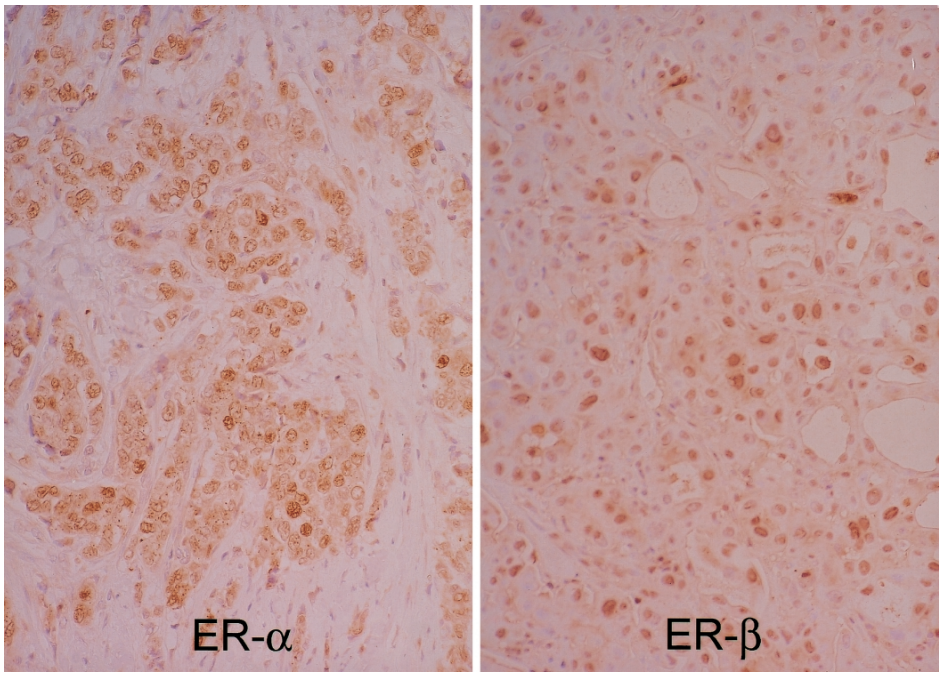

Fig. 1. Immunohistochemical staining of ER-α and ER-β in breast cancers (×200).

tumors, 49 (62%) were positive for ER-α and 24 (30%) were positive for ER-β, and there was no significant correlation between ER-α and ER-β expression (Table I). ER-α-positive tumors were significantly ($P<0.0001$) more likely to be PR-positive than ER-α-negative tumors but there was no significant association between ER-β expression and PR status (Table I). Although there was a trend towards ER-β-negative tumors being more likely to be PR-positive in either ER-α-positive or -negative tumors, a statistically significant association was not observed (Table I). In order to analyze the influence of ER-β on PR expression, we compared the PR values between ER-β-positive and -negative tumors. In this analysis, only ER-α-positive tumors were considered. PR values (65±17 fmol/mg protein, mean±SE) of ER-β-positive tumors ($n=15$) tended to be lower than those of ER-β-negative tumors (340±109, $n=30$, $P=0.08$).

Correlation of ER-α and ER-β expression with clinicopathological parameters ER-α status did not exhibit a significant correlation with tumor size, but ER-β-positive tumors were of significantly smaller size (≤2 cm, $P<0.05$, Table II). Low histological grades were significantly more common in ER-α-positive than -negative tumors ($P<0.001$). In contrast, high histological grades were significantly more common in ER-β-positive than -negative tumors ($P<0.05$). ER-α and ER-β status was not influenced by menopausal status or lymph node status. When only ER-α-positive tumors were considered, ER-β positivity was significantly associated with small tumor size and high histological grade ($P<0.05$, Table II).

DISCUSSION

Using immunohistochemical analysis, we were able to demonstrate ER-β expression in 30% of breast cancers, and found that ER-β expression did not correlate significantly with ER-α expression. This observation is inconsistent with the report of Järvinen *et al.*[13] who reported a significant correlation, based on immunohistochemical analysis, between ER-α and ER-β expression. The reason for this discrepancy can be explained, at least in part, by the difference in the antibodies used for immunohistochemical staining of ER-β. Järvinen *et al.*[13] used a rabbit polyclonal antibody raised against C-terminal amino acid residues 467–485 of ER-β, which meant that their antibody could detect wild-type ER-β but not ER-β variants lacking these residues. In contrast, the antibody used in our study was prepared by immunizing a chicken with whole ER-β,[15] and thus, the resultant antibody could detect both wild-type ER-β and ER-β variants. Since ER-β variants, and not wild-type ER-β, are predominantly expressed in breast cancers,[16] we suggest that an antibody that can detect such variants is preferable to a wild-type-specific antibody.

A strong positive correlation between ER-α expression and PR positivity was observed in our study, consistent with the well-established theory that ER-α induces PR. In contrast, there was no significant association between ER-β expression and PR positivity (Table I), and all the ER-α-negative and ER-β-positive tumors were PR-negative (Table I), suggesting that ER-β, unlike ER-α, does not induce PR. In addition, we found that PR values tended to

Table I. Relationship between ER-α and ER-β Expression and PR Status in Breast Cancers

	PR status[a]			
	Positive	Negative	Unknown	Total
ER-α status				
ER-α positive	40 (51)[b, c]	8 (10)	1	49 (62)
ER-α negative	3 (4)	27 (35)	0	30 (38)
ER-β status				
ER-β positive	13 (17)	10 (13)	1	24 (30)
ER-β negative	30 (38)	25 (32)	0	55 (70)
ER-α/ER-β status				
ER-α positive/ER-β positive	13 (17)	4 (5)	1	18 (23)
ER-α positive/ER-β negative	27 (34)	4 (5)	0	31 (39)
ER-α negative/ER-β positive	0 (0)	6 (8)	0	6 (8)
ER-α negative/ER-β negative	3 (4)	21 (27)	0	24 (30)

a) PR was determined by enzyme immunoassay (EIA) in all tumors except one in which available tumor tissue was insufficient for EIA.
b) Numbers in parentheses represent percentage data.
c) $P<0.0001$ compared between ER-α-positive and -negative tumors.

Table II. Relationship between ER-α and ER-β Expression and Clinicopathological Characteristics of Breast Cancers

	All tumors		All tumors		ER-α-positive tumors	
	ER-α-positive	ER-α-negative	ER-β-positive	ER-β-negative	ER-β-positive	ER-β-negative
Menopausal status						
Premenopausal	32 (41)[a)]	19 (24)	17 (22)	34 (43)	14 (28)	18 (37)
Postmenopausal	17 (21)	11 (14)	7 (9)	21 (26)	4 (8)	13 (27)
Tumor size						
≤2cm	15 (19)	9 (11)	12 (15)	12 (15)[b)]	9 (18)	6 (13)[b)]
>2cm	34 (43)	21 (27)	12 (15)	43 (55)	9 (18)	25 (51)
Histology						
DCIS[c)]	2 (3)	2 (3)	0 (0)	4 (5)	0 (0)	2 (4)
IDC[d)]	47 (59)	28 (35)	24 (30)	51 (65)	18 (37)	29 (59)
Histological grade						
1	12 (15)	1 (1)[d)]	3 (4)	10 (13)[b)]	3 (6)	9 (19)[b)]
2	26 (33)	10 (13)	7 (9)	29 (36)	7 (14)	19 (39)
3	11 (14)	19 (24)	14 (18)	16 (20)	8 (16)	3 (6)
Lymph node metastasis						
Negative	31 (39)	15 (19)	12 (15)	34 (43)	9 (18)	22 (45)
Positive	17 (22)	15 (19)	11 (14)	21 (27)	8 (17)	9 (18)
Unknown	1 (1)	0 (0)	1 (1)	0 (0)	1 (2)	0 (0)

a) Numbers in parentheses represent percentage data.
b) $P<0.05$, compared between ER-β-positive and -negative tumors.
c) DCIS, ductal carcinoma *in situ*; IDC, invasive ductal cancer.
d) $P<0.001$, compared between ER-α-positive and -negative tumors.

be lower in ER-α-positive and ER-β-positive tumors, compared with ER-α-positive and ER-β-negative tumors ($P=0.08$), and that all tumors ($n=10$) with high levels of PR (>200 fmol/mg protein) were exclusively ER-α-positive and ER-β-negative (data not shown). These results suggest that ER-β may inhibit the ER-α-dependent induction of PR. These findings are consistent with the report of Weihua *et al.*[17] that PR is up-regulated in the uterus of ER-β knockout mice. The inhibitory effect of ER-β on PR induction might be mediated through competition with ER-α in binding to the ERE of the *PR* gene, or through formation of heterodimers with ER-α. If this is the case, it is possible that ER-β exerts its inhibitory effects on both PR induction and on other ER-α functions. The most abundant splicing variant of ER-β in breast cancers is ER-β2.[18] Since ER-β2, like wild-type ER-β, forms heterodimers with ER-α, we speculate that ER-β2 also exerts negative effects on ER-α functions.

We found that ER-β-positive tumors were of significantly higher histological grade, while ER-α-positive tumors were of significantly lower histological grade. Among ER-α-positive tumors, ER-β-positive tumors were also more likely to be of high histological grade than ER-β-negative tumors (Table II). Recent studies have also shown that tumors expressing high ER-β mRNA are less likely to respond to tamoxifen than tumors with low ER-β mRNA expression, even if the tumors are ER-α-positive.[10] Taken together, these results suggest that ER-β might be useful in further defining the characteristics of ER-α-positive tumors in terms of biological aggressiveness and hormone-responsiveness. Roger *et al.*[12] showed immunohistochemically that ER-β is down-regulated during breast carcinogenesis. In the present study, we found that ER-β-positive tumors were significantly more likely to be small tumors (≤2 cm) than were ER-β-negative tumors, suggesting that ER-β is further down-regulated during tumor progression. Since these observations were obtained by immunohistochemistry, they require confirmation by more quantitative methods in future studies.

In conclusion, we have demonstrated in the present study that 30% of breast cancers expressed ER-β, and that ER-β positivity is associated with small tumor size and high histological grade. Our results suggest that determination of ER-β status might be clinically useful in further defining the characteristics of ER-α-positive tumors. Elucidation of ER-β function is necessary for a more comprehensive understanding of estrogen action in breast cancers.

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