

# Estrogen Receptor $\beta$ Protein in Human Breast Cancer: Correlation with Clinical Tumor Parameters<sup>1</sup>

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## ABSTRACT

The recent discovery of a second estrogen receptor (ER), designated ER $\beta$ , raises pressing questions about its role in estrogen regulation of human breast cancer cells and its significance for the prediction of recurrence and treatment responses in clinical breast cancer. Most of what we know about ER $\beta$  expression comes from studies examining a limited number of samples at the RNA level. We have now generated a monoclonal antibody useful for the detection of ER $\beta$  at the protein level in archival, formalin-fixed breast tumors and have examined its expression using immunohistochemistry in a pilot series of 242 breast cancer patients. Coexpression of ER $\beta$  and ER $\alpha$  was found in the majority of the tumors, with 76% of the tumors expressing ER $\beta$  as determined by immunohistochemistry. ER $\alpha$ , but not ER $\beta$ , was strongly associated with progesterone receptor expression, suggesting that ER $\alpha$  is the predominant regulator of this estrogen-induced gene in breast tumors. Although ER $\alpha$  expression was positively correlated with low tumor grade, diploidy, and low S-phase fraction, all biological parameters of a good prognostic profile, ER $\beta$  trended toward an association only with aneuploidy; no association with tumor grade or S-phase fraction was seen for ER $\beta$ . We found that ER $\beta$  expression does cause false positive readings for ER $\alpha$ . These results suggest that ER $\beta$  expression is not a surrogate for ER $\alpha$  in clinical breast tumors and, as such, could be a useful biomarker in its own right.

## INTRODUCTION

Many recent discoveries in the nuclear receptor field have contributed to our understanding of steroid hormone action and the mechanisms by which estrogens exert their effects in breast cancer cells. Until recently, estrogen action was thought to be mediated through a single ER,<sup>3</sup> now called ER $\alpha$  (1). However, the identification of a second ER, called ER $\beta$  (2), casts uncertainty upon this understanding of estrogen action. ER $\alpha$  expression is a very useful clinical biomarker of breast tumor progression, and ER $\alpha$  and PR are now routinely used to estimate patient prognosis and select optimal therapies. Overall, approximately 50–60% of women with ER-positive, advanced breast cancer will receive some degree of benefit from standard endocrine treatment with the antiestrogen tamoxifen, whereas the majority of early ER-positive breast cancers will respond to treatment (3, 4). However, most of the clinical implications of ER expression have been assessed using biochemical ligand binding methods, such as the dextran-coated charcoal assay. What we do not know is whether the new ER $\beta$  subtype will confound our simple interpretation of these clinical ER assays because it has almost the same binding affinity for estradiol as ER $\alpha$  (2).

ER $\alpha$  and ER $\beta$  belong to a superfamily of nuclear hormone recep-

tors that function as transcription factors when they are bound to their respective ligands, and they share common structural and functional features. ER $\alpha$  contains 595 amino acids with a central DNA-binding domain, along with a COOH-terminal hormone-binding domain. We have previously shown that human ER $\beta$  protein is somewhat shorter than ER $\alpha$  (5), with a predicted size of 530 residues. Based on their structural dissimilarities, especially in the NH<sub>2</sub>-terminal AF-1 region, it has been suggested that ER $\beta$  expression may regulate a different set of genes than ER $\alpha$ , a difference that may have important consequences for tumor growth. It is also possible that the biological function of ER $\beta$  is dependent on the coexpression of ER $\alpha$  in certain tissues, including the breast. In fact, ER $\beta$  can function as an inhibitor of ER $\alpha$  activity under certain circumstances *in vitro* (6). We believe that the ultimate way to address these questions and discover the potential clinical significance of ER $\beta$  is to determine its role directly in patients' samples and compare its expression with ER $\alpha$ .

There are now a number of published studies examining ER $\beta$  expression in breast tumors, but the majority of these assessed RNA levels, often using semiquantitative methods that might not accurately reflect ER $\beta$  protein expression. These studies, examining a limited number of tumors, have been contradictory in their conclusions, suggesting that ER $\beta$  is either a poor prognostic factor associated with PR-negative, lymph node-positive tumors (7) or, conversely, a marker of good prognosis associated with negative lymph nodes and low proliferative status (8).

Our first goal was to develop an IHC assay to measure ER $\beta$  protein in archival breast specimens to resolve these apparent discrepancies. To accomplish this goal, we generated a monoclonal antibody to the NH<sub>2</sub>-terminal region of ER $\beta$  and developed an IHC assay useful for formalin-fixed, archival specimens. Because the epitope of this antibody is localized to the NH<sub>2</sub>-terminal region of ER $\beta$ , it is capable of detecting both full-length ER $\beta$  (called ER $\beta$ 1) and various COOH-terminal-truncated isoforms of ER $\beta$  (5, 9), therefore measuring total ER $\beta$  protein in tumors. In the present pilot study of 242 breast tumors, we have determined that ER $\beta$  is coexpressed along with ER $\alpha$  in the majority of specimens, and we have investigated the relationships between ER $\beta$ , ER $\alpha$ , and clinical tumor parameters.

## MATERIALS AND METHODS

**Tumor Samples.** Two hundred and sixty-one human breast tumor specimens in the Baylor Breast Cancer Specialized Programs of Research Excellence (SPORE) Tissue Resource were included in this pilot study. Treatment histories and long-term follow-up for disease recurrence and death were not available for these patients. Breast tumor specimens were frozen in liquid nitrogen immediately after excision and sent to a central laboratory for steroid receptor assays and DNA flow cytometry. One paraffin-embedded, ER $\beta$ -positive breast tumor was used to evaluate whole tissue section staining with the ER $\beta$  antibody, and different areas of the slide were photographed to examine for intertumor heterogeneity of ER $\beta$  protein expression.

**Steroid Receptor Assays.** Tumor cytosols were prepared for LBA as described previously (10), using a standard multipoint dextran-coated charcoal assay incorporating <sup>125</sup>I-estradiol and <sup>3</sup>H-R5020 in a single assay, allowing for the simultaneous determination of both ER and PR status. Tumors with an ER or PR content of  $\geq 3$  fmol/mg protein or  $\geq 5$  fmol/mg protein, respectively,

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<sup>3</sup> The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; IHC, immunohistochemistry; LBA, ligand binding assay; SPF, S-phase fraction; TA, tissue array; PBST, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20.

were considered to be positive for receptor expression. The pulverized tissue that remained after LBA assay was stored at  $-70^{\circ}\text{C}$  for future use.

#### Flow Cytometric Evaluation of SPF and DNA Ploidy Measurements.

Flow cytometry was carried out as described previously (11). Briefly, approximately 100 mg of frozen pulverized tumor were homogenized, filtered, and centrifuged. Chicken red cells were added as an internal standard, and the cells were lysed and stained for DNA. DNA-stained nuclei were prepared and run on an Epics V flow cytometer (Coulter Electronics, Hialeah, FL). Approximately 50,000 tumor events were acquired on a single-parameter 256-channel integrated fluorescence histogram. Frequency distributions of cells in G<sub>0</sub>-G<sub>1</sub>, SPF, and G<sub>2</sub>-M phases of the cell cycle were evaluated using a modeling program (MODFIT; Verity Software House, Inc., Topsham, ME). Debris was modeled as an exponential, and SPF was modeled as a single trapezoid. Proliferation status as determined with Ki67 staining has been described previously (12).

**Tumor Histological Grade.** Histological grading of the tumors was performed on complete sections (see below) using the Elston-modified Scarff-Bloom-Richardson system (13). This system utilizes a semiquantitative method to assess degree of differentiation (tubule formation), nuclear pleomorphism, and mitotic activity. Because our tissue sections were prepared from pulverized tissue, it was often not possible to count 10 individual and nonoverlapping fields. Therefore, mitotic activity was scored based on one field with maximum number of mitotic figures, an approach similar to the original Scarff-Bloom-Richardson system (14).

**Generation of a Monoclonal ER $\beta$  Antibody.** RNA from MCF-7 cells was used for reverse transcription-PCR of the NH<sub>2</sub>-terminal fragment (amino acid residues 1–146) of ER $\beta$  (15). This fragment was then cloned into the pET28a+ mammalian expression vector. Glutathione *S*-transferase-fusion recombinant ER $\beta$  protein was purified on a histidine affinity column and used for immunization of female BALB/c mice. Spleen cells from mice producing immunoreactive antibody were fused to NS-1 myeloma cells by standard hybridoma methods. Specific monoclonal antibody to ER $\beta$  as determined by Western blot analysis was purified and concentrated through protein G columns and used for the IHC and Western blot studies shown. The one monoclonal antibody useful for IHC was named 14C8, and it is now commercially available through GeneTex (San Antonio, TX).

For Western blot analysis, MCF-7 total cell lysates were prepared as described previously (16). Briefly, a cell pellet was homogenized in a high-salt buffer [20 mM Tris-HCl (pH 7.5), 2 mM DTT, 0.4 M KCl, and 20% glycerol] containing a mixture of protease inhibitors [10  $\mu\text{g}/\text{ml}$  each of aprotinin, antipain, leupeptin, and pepstatin plus 0.3 mM phenylmethylsulfonyl fluoride (Sigma)]. Homogenates were then centrifuged at  $100,000 \times g$  for 1 h, and the supernatants stored at  $-80^{\circ}\text{C}$  until use. Extract protein (50  $\mu\text{g}$ ) was separated by electrophoresis on 8% SDS-PAGE and transferred onto nylon membranes (Schleicher & Schuell, Keene, NH). The blots were first stained with Ponceau S (Sigma, St. Louis, MO) to confirm uniform transfer of all samples and then incubated in blocking solution (5% nonfat dry milk in PBST). After brief washes with PBST, the filters were then reacted with the 14C8 monoclonal antibody at a dilution of 1:250 or with the NCL-ER-6F11 mouse monoclonal antibody against human ER $\alpha$  (Vector Laboratories, Burlingame, CA) at a dilution of 1:100 for 1 h at room temperature followed by extensive washes with PBST. Blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Amersham) for 1 h, washed with TBST, and developed using the enhanced chemiluminescence procedure (Amersham). As positive controls, 1  $\mu\text{l}$  of *in vitro*-translated extract from two mammalian expression vectors (pSG5) containing the complete open reading frames of full-length ER $\beta$ 1 and ER $\beta$ 2 variant isoform expression cDNA clones (5) was used on the Western blots. As a negative control, *in vitro*-translated ER $\alpha$  (17) was also included.

**Preparation of TAs.** Tissue sections were initially prepared from the pulverized frozen tumor specimens left over from the LBA as described previously (18). TAs were assembled manually as described previously (5). Briefly, an H&E-stained slide from a complete tissue section was used as a guide to mark the area of maximum tumor cellularity on the corresponding formalin-fixed, paraffin-embedded donor block. Then, using a 3-mm dermal biopsy punch (Miltex Instruments Inc.), a 3-mm core of tumor was punched out from the donor block. To create the TA recipient block, an empty paraffin cast was punched out using a stainless steel mold template in a configuration of  $6 \times 5$  (30 samples). Twenty-nine tumor samples and one normal control

tissue (used as a marker of orientation) were arranged using a predetermined map into the paraffin cast. This was then annealed at  $62^{\circ}\text{C}$  for 20 min to create a TA block.

**IHC for ER $\alpha$ , ER $\beta$ , PR, and Ki67.** Four- $\mu\text{m}$ -thick sections of TA were used for IHC. Monoclonal antibodies NCL-ER-6F11 (1:200; Novocastra), 1294 (1:600; Dako, Carpinteria, CA), Mib1 (1:200; Dako), and our in-house monoclonal antibody 14C8 (1:200) were used for ER $\alpha$ , PR, Ki67, and ER $\beta$  IHC, respectively. Heat-induced epitope retrieval was performed using Tris-HCl buffer (pH 9.0) in a pressure cooker for 5 min for each of the markers. Slides were blocked for endogenous peroxidase in a 3% H<sub>2</sub>O<sub>2</sub> solution for 5 min followed by the A/B blocking kit reagents (Vector Laboratories) per the manufacturer's recommendations for endogenous biotin. The linking antibodies step (30 min) used biotinylated rabbit antimouse (E0345; Dako) at a 1:200 dilution for all antibodies except ER $\beta$ , where a dilution of 1:100 was used. The chromagen was applied using 3,3'-diaminobenzidine + solution (Dako) for 15 min, which was intensified with 0.2% osmium tetroxide for 30 s. Slides were counterstained with methyl green and coverslipped with a permanent medium.

Immunostained slides were scored for ER $\alpha$ , ER $\beta$ , and PR as described previously (19). In brief, each entire slide was evaluated by light microscopy. First, a proportion score was assigned, which represented the estimated proportion of positively stained tumor cells (0, none; 1,  $<1/100$ ; 2,  $1/100$  to  $1/10$ ; 3,  $1/10$  to  $1/3$ ; 4,  $1/3$  to  $2/3$ ; and 5,  $>2/3$ ). Next an intensity score was assigned that represented the average intensity of the positive tumor cells (0, none; 1, weak; 2, intermediate; and 3, strong). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8. Mib1/Ki67 was scored by directly counting the percentage of positive cells (average denominator = 500 cells) as described previously (12). Slides were scored without knowledge of the LBA results or prognostic factors.

**Statistical Methods.** Associations between continuous variables were analyzed using nonparametric Spearman rank correlation coefficients. Associations between categorical variables were assessed by Fisher's exact tests. All analyses were performed using SAS (Version 6.12 SAS Institute, Cary, NC) on a Sun SparcStation (Sun Microsystems, Inc., Mountain View, CA).

## RESULTS

**Specificity of ER $\alpha$  and ER $\beta$  Antibodies.** Although the commercially available ER $\alpha$  NCL-ER-6F11 antibody was generated to recombinant ER $\alpha$  protein, because of the high degree of shared homology between the two ERs, we first had to demonstrate its specificity for the  $\alpha$ -receptor subtype. Therefore, *in vitro*-translated ER $\alpha$  and full-length ER $\beta$ 1 extract, along with total cellular extracts from MCF-7 breast carcinoma cells, were prepared and subjected to Western blot analysis with the NCL-ER-6F11 antibody (Fig. 1A). This antibody was indeed specific for the ER $\alpha$  form and did not cross-react with ER $\beta$ ; a band corresponding to the  $M_r$  65,000–66,000 ER $\alpha$  subtype was the only form detected in MCF-7 cells with the NCL-ER-6F11 antibody.

We next analyzed a number of different monoclonal antibodies that we generated to a glutathione *S*-transferase-fusion protein of the NH<sub>2</sub>-terminal region of ER $\beta$ . Although all of these antibodies reacted with recombinant ER $\beta$  protein on Western blot analyses, only one, the 14C8 ER $\beta$ -specific antibody, demonstrated a strong nuclear signal in formalin-fixed material (data not shown). Because we have shown that ER $\beta$  variant isoforms exist in breast tumor cell lines (5), we included *in vitro*-translated ER $\beta$ 1 and the COOH-terminal splicing variant ER $\beta$ 2, along with ER $\alpha$  and extracts from MCF-7 cells, to examine the specificity of the 14C8 monoclonal ER $\beta$  antibody in immunoblot analysis (Fig. 1B). Our antibody reacted specifically with ER $\beta$ , but not with ER $\alpha$ , and detected full-length ER $\beta$ 1 of approximately  $M_r$  58,000–60,000 in MCF-7 cells. Because our antibody was generated to a fusion protein of the NH<sub>2</sub>-terminal region of ER $\beta$ , it also detected the COOH-terminal-truncated ER $\beta$ 2 isoform. This result suggests that our antibody is capable of detecting total ER $\beta$  expres-

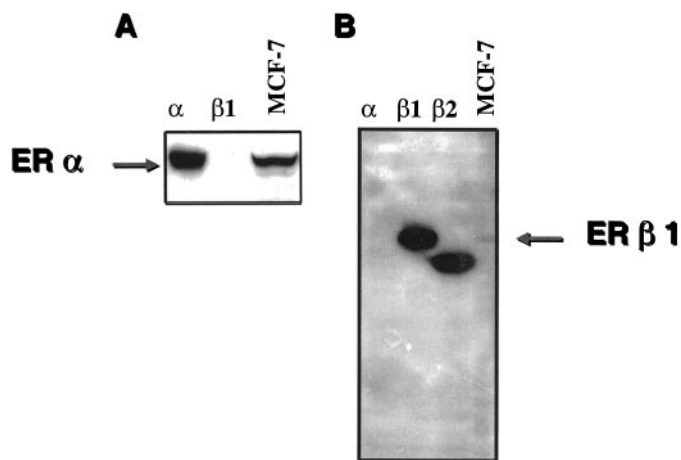


Fig. 1. Western blot comparison of ER $\alpha$  NCL-ER-6F11 antibody and our 14C8 ER $\beta$  antibody. Lysate (5  $\mu$ l) from *in vitro* translation reactions primed with pSG5 expression plasmid containing either ER $\beta$ 1 ( $\beta$ 1) or ER $\beta$ 2 ( $\beta$ 2) and cell lysate (50–100  $\mu$ g) prepared from the MCF-7 breast carcinoma cell line were electrophoresed on SDS-PAGE gels and transferred to nylon filters. The blots were incubated with the NCL-ER-6F11 antiserum diluted 1:500 (A) or ER $\beta$  antiserum diluted 1:200 (B). Binding of primary antibody was detected with horseradish peroxidase-conjugated secondary antibody.

sion in cells because most of the ER $\beta$  variants described arise from alternative splicing at the COOH terminus (9).

We next performed immunohistochemical analysis on a panel of whole tissue sections from primary breast tumors; representative photographs from one tumor are shown in Fig. 2. To look for heterogeneity of ER $\beta$  expression in the same tumor using whole sections, we examined three different areas of the same tumor and found the same pattern of staining throughout the tissue (compare Fig. 2, A–C). Thus, the expression of ER $\beta$  appears to be homogeneous. This finding suggests that the use of TAs would be representative of a given tumor sample, and in our experience, we have not seen significant heterogeneity of ER $\beta$  expression within a tumor to be concerned about sampling error with this antibody.

**Validation of TAs.** TAs offer the advantage of rapid staining and scoring of a large number of tumors. An obvious question, however, is whether the results obtained from TAs are indeed representative of results obtained from larger specimens as suggested by the homogeneity of staining obtained and shown in Fig. 2. To address this question, we arrayed 261 human breast tumors on 9 tissue blocks and stained for ER $\alpha$  using the NCL-ER-6F11 and ER $\beta$  antibodies. Representative IHC staining of four breast tumor specimens for ER $\alpha$  and ER $\beta$  is shown in Fig. 3. For comparison, ER $\alpha$  had previously been determined in larger sections from these same tumors using IHC with the NCL-ER-6F11 antibody (19). ER $\alpha$  results were obtained in the TAs from 237 of the 261 tumors (91%). Complete concordance of total IHC scores between TA and the previously stained, larger sections was observed in 46% of cases, and concordance within one IHC score was observed in 82% of cases. The Spearman correlation coefficient was 0.76. When results were classified as ER positive (ER+) or ER negative (ER–) using our previously defined criteria (total IHC score  $> 2 =$  ER+), only 12 cases (5%) were discordant (11 were ER+ on larger sections but ER– on TA; 1 was ER– on larger sections but ER+ on TA).

To put these results in perspective, we examined the inter-rater reliability of scoring the same slides from the larger sections. Complete concordance of total IHC scores from two independent pathologists was observed in 72% of cases, and concordance within one IHC score was observed in 99% of cases. The Spearman correlation coefficient was 0.92. Only two cases had different qualitative out-

comes, and in each case, the results differed by only one score (IHC = 3 and 2, ER+ and ER–, respectively).

Therefore, we conclude that the scoring system is reproducible between observers, and although there is a slight loss of sensitivity, TAs yield results comparable with those obtained on larger specimens.

**Distribution of ER $\beta$  Expression.** Results for ER $\beta$  were obtained from 242 tumors on the TAs. Nuclear staining was observed in 184 (76%) of the cases. Although the rate of staining is very similar to that observed for ER $\alpha$ , the pattern is somewhat different (Table 1), with higher levels of ER $\alpha$  expression detected in the tumors. The Spearman correlation coefficients between ER $\beta$  and ER $\alpha$  were relatively modest ( $r_{sp} = 0.33$  and 0.36, respectively, for TA and larger samples).

**Relationships between ER Expression and Other Prognostic Factors.** Spearman correlation coefficients between ER $\beta$  and ER $\alpha$  and other prognostic factors are displayed in Table 2. ER $\beta$  expression was positively correlated with ER $\alpha$  determined by IHC ( $r_{sp} = 0.33$ ), but this relationship was not seen with ER LBAs ( $r_{sp} = 0.09$ ). This result is consistent with the report by Dotzlaw *et al.* (20) showing that expression of ER $\beta$  RNA using reverse transcription-PCR amplification was not significantly correlated with ER by LBA in breast tumors. Similarly, ER $\beta$  expression was positively correlated with PR determined by IHC ( $r_{sp} = 0.25$ ), but not with PR by LBA ( $r_{sp} = 0.05$ ). The very strong, positive correlations between ER $\alpha$  by

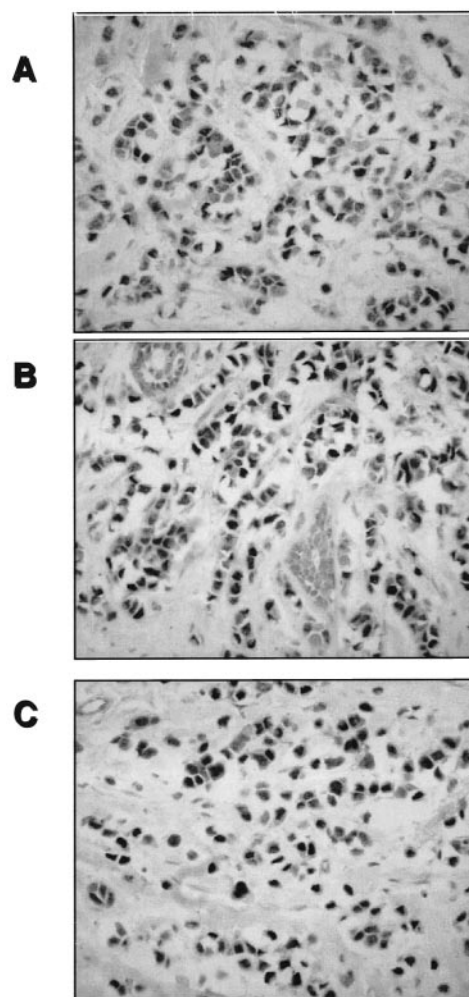


Fig. 2. IHC of a representative breast tumor using a whole tissue section and our ER $\beta$ -specific 14C8 antibody. A–C represent three different areas of the same tumor staining homogeneously positive for ER $\beta$  protein expression.

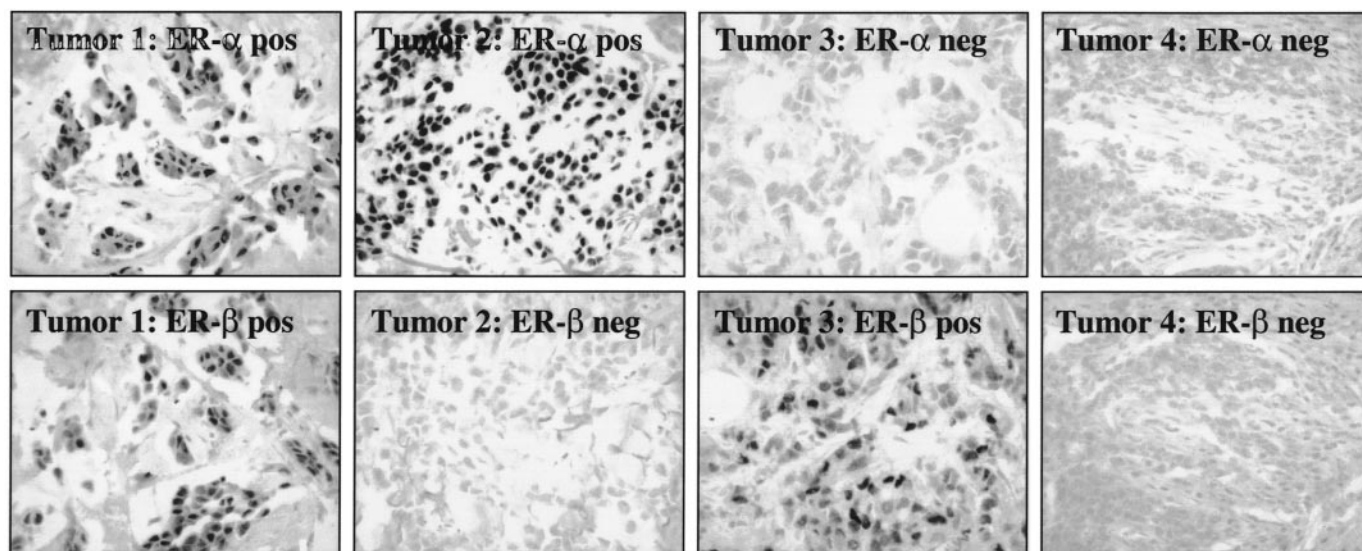


Fig. 3. IHC of four representative breast tumors from the TA using the ER $\alpha$  NCL-ER-6F11 antibody or our ER $\beta$ -specific 14C8 antibody. Tumors representative of the different subgroups are included: *Tumor 1*, ER $\alpha$  positive/ER $\beta$  positive; *Tumor 2*, ER $\alpha$  positive/ER $\beta$  negative; *Tumor 3*, ER $\alpha$  negative/ER $\beta$  positive; and *Tumor 4*, ER $\alpha$  negative/ER $\beta$  negative.

Table 1 Distribution of ER $\alpha$  and ER $\beta$  as determined by IHC in 242 primary breast cancer cases

ER IHC score	Patients			
	ER- $\beta$ positive		ER- $\alpha$ positive	
	No.	%	No.	%
0	58	24	54	23
2	0	0	4	2
3	48	20	14	6
4	39	16	17	7
5	36	15	18	8
6	44	18	46	19
7	14	6	45	19
8	3	1	39	16

IHC and ER by LBA, both in this study and in our previous larger study (19), suggest that the ER status of breast tumors as defined by the standard clinical dextran-coated charcoal assay fails to accurately reflect the levels of ER $\beta$  present in breast tumors.

No significant relationships were observed between ER $\beta$  expression and tumor grade, proliferation determined by Ki67 staining, SPF, or DNA ploidy. In contrast, ER $\alpha$  expression was significantly correlated with each of these prognostic factors, as we have previously described for ER by LBA (21), providing additional evidence that the samples in this pilot study are representative of clinical breast cancer. The different results for ER $\beta$  and ER $\alpha$  suggest that ER $\beta$  expression is not a surrogate for ER $\alpha$ , and is not correlated with the same clinical parameters as ER $\alpha$ . As such, ER $\beta$  could predict different biological features of breast tumors, but unfortunately, ER $\beta$ -specific functions or signaling pathways that may be clinically important for tumor progression have not yet been elucidated.

**ER $\alpha$  Expression Is the Major Determinant of PR Expression in Breast Tumors.** Although the study was relatively small, we next investigated the biological importance of ER $\alpha$  and ER $\beta$  coexpression and interactions with other prognostic factors. We defined tumors to be positive for either ER if its IHC scores were greater than 2. This corresponds to our published definition for ER $\alpha$  (19), which was based on correlations with clinical outcome, but is admittedly arbitrary for ER $\beta$ . Using these definitions, 9% of the tumors (21 of 234) were negative for both receptors, 14% (32 of 234) were positive only

for ER $\alpha$ , 15% (36 of 234) were positive only for ER $\beta$ , and 62% (145 of 234) were positive for both receptors. Examples of all four categories are seen in Fig. 1. We found (Table 3) that receptor-negative tumors rarely (5% by LBA and 14% by IHC) expressed the PR. However, PR expression by IHC was more closely associated with ER $\alpha$  expression than ER $\beta$  expression: PR was expressed in 88% of ER $\alpha$ -positive/ER $\beta$ -negative tumors but in only 6% of the ER $\alpha$ -negative/ER $\beta$ -positive tumors ( $P < 0.0001$ ). Similar results were found for PR by LBA.

**ER $\alpha$  Expression Is the Major Determinant of Some, but not All, Breast Cancer Prognostic Factors.** High tumor grade and high SPFs were significantly related to ER $\alpha$  expression, and the same strong relationships were observed when patients were further stratified by ER $\beta$  status (Table 3). For example, 24% of tumors with both receptors had high SPF, compared with 25% of tumors in the ER $\alpha$ -positive/ER $\beta$ -negative subset of tumors; conversely, 60% of tumors lacking both receptors had high SPF, compared with 67% of the ER $\alpha$ -negative/ER $\beta$ -positive subset. Based on these data, one could speculate that coexpression of ER $\beta$  may have little impact on the prognosis of patients with breast tumors, at least as assessed by established factors such as proliferation and tumor grade.

However, the relationships with DNA ploidy appear to be more complex. There was a trend ( $P = 0.12$ ) for tumors with both receptors to be more aneuploid (59%), compared with ER $\alpha$ -positive/ER $\beta$ -negative tumors (44%). Similarly, the rate of aneuploidy was marginally higher ( $P = 0.20$ ) in ER $\alpha$ -negative/ER $\beta$ -positive tumors (82%), compared with tumors that lack both receptors (65%), suggesting that ER $\beta$ -positive tumors might be more aggressive biologically, similar in characteristics to the receptor-negative breast tumors. Therefore,

Table 2 Spearman correlation coefficients between ER expression and other prognostic factors

Factor	ER $\beta$	ER $\alpha$
ER $\alpha$ (IHC)	0.33 ( $P = 0.0001$ )	
ER (LBA)	0.09 ( $P = 0.15$ )	0.76 ( $P = 0.0001$ )
PR (IHC)	0.25 ( $P = 0.0001$ )	0.58 ( $P = 0.0001$ )
PR (LBA)	0.04 ( $P = 0.49$ )	0.44 ( $P = 0.0001$ )
Tumor grade	-0.01 ( $P = 0.90$ )	-0.40 ( $P = 0.0001$ )
Ki67	-0.09 ( $P = 0.16$ )	-0.36 ( $P = 0.0001$ )
SPF	-0.08 ( $P = 0.25$ )	-0.34 ( $P = 0.0001$ )
Ploidy	0.08 ( $P = 0.20$ )	-0.11 ( $P = 0.10$ )

Table 3 Comparison of combined ER status with standard prognostic factors

ER status was determined by IHC; Ps were determined by Fisher's exact tests.  $\alpha$  represents ER $\alpha$ , and  $\beta$  represents ER $\beta$ .

Factor	$\alpha$ -/ $\beta$ - No. (%)	$\alpha$ -/ $\beta$ + No. (%)	$\alpha$ +/ $\beta$ - No. (%)	$\alpha$ +/ $\beta$ + No. (%)	$\alpha$ -/ $\beta$ - vs. $\alpha$ -/ $\beta$ +P	$\alpha$ +/ $\beta$ - vs. $\alpha$ +/ $\beta$ +P	$\alpha$ -/ $\beta$ - vs. $\alpha$ +/ $\beta$ -P	$\alpha$ -/ $\beta$ + vs. $\alpha$ +/ $\beta$ +P	$\alpha$ -/ $\beta$ + vs. $\alpha$ +/ $\beta$ -P	$\alpha$ -/ $\beta$ - vs. $\alpha$ +/ $\beta$ +P
PR+ by IHC	1/21 (5%)	2/36 (6%)	28/32 (88%)	134/145 (92%)	1.00	0.48	<0.0001	<0.0001	<0.0001	<0.0001
PR+ by LB	3/21 (14%)	4/36 (11%)	25/32 (78%)	115/145 (79%)	0.70	1.00	<0.0001	<0.0001	<0.0001	<0.0001
ER+ by LB	8/21 (38%)	11/36 (31%)	31/32 (97%)	139/145 (96%)	0.58	1.00	<0.0001	<0.0001	<0.0001	<0.0001
Grade 3	14/21 (67%)	30/36 (83%)	9/32 (28%)	27/145 (19%)	0.20	0.23	0.01	<0.0001	<0.0001	<0.0001
Aneuploid	13/20 (65%)	27/33 (82%)	14/32 (44%)	86/145 (59%)	0.20	0.12	0.16	0.016	0.002	0.81
High SPF	9/15 (60%)	16/24 (67%)	8/32 (25%)	35/145 (24%)	0.74	1.00	0.027	<0.0001	0.003	0.006

the specific measurement of ER $\beta$ , combined with the measurement of ER $\alpha$  by IHC, might provide useful clinical information in certain breast cancer patients, a possibility that will require a larger study with clinical follow-up information to validate.

## DISCUSSION

ER and PR are measured in breast tumor specimens for prognostication of disease recurrence and prediction of treatment response. In guidelines published by the American Society of Clinical Oncology Tumor Marker Expert Panel (22), ER and PR were the only biomarkers recommended for routine use in the management of patients with breast cancer. The ER assay is most useful if the tumor is ER negative; these patients seldom respond to endocrine therapy. However, predicting the probability of response in ER-positive patients is more difficult. Overall, approximately 50–60% of women with ER-positive, advanced breast cancer will receive some degree of benefit from endocrine treatment (3). With the discovery of the second ER subtype, ER $\beta$ , it was reasonable to hope that an accurate measurement of the two forms might provide additional prognostic or predictive clinical information.

An accurate assessment of ER $\beta$  expression requires the availability of specific antibodies useful for routinely fixed clinical material. Only recently have antibodies to ER $\beta$  become available (23, 24), but unfortunately, most of these have not proven useful for IHC or worked only in frozen material (8). Although we had previously developed two antibodies to ER $\beta$  that were specific in Western blot analyses (5), neither of these antibodies worked in paraffin-embedded sections. Therefore, we developed a third new antibody, now called 14C8, which is capable of recognizing ER $\beta$  protein in archival samples. Because the antibody was prepared to the NH<sub>2</sub>-terminal region of the protein, it is capable of recognizing putative COOH-terminal-truncated forms as well as full-length ER $\beta$ , thus presumably detecting total translated ER $\beta$  protein, unlike many of the other commercially available ER $\beta$  antibodies (8).

We found that the majority of breast tumors coexpressed both receptors, which is in agreement with a number of other published studies. In a study of 60 tumors, Speirs *et al.* (7) hypothesized that breast tumors coexpressing both receptors, as opposed to those only expressing ER $\alpha$ , were more frequently associated with poor prognostic biomarkers, such as positive axillary nodes and higher tumor grade. This is certainly a viable hypothesis, given that the two receptors can form functional heterodimers on DNA (25) and that the heterodimer may be preferentially formed as opposed to homodimers (26). However, our results do not completely agree with this hypothesis. For instance, when comparing high tumor grade and high SPF with ER status, we found little difference between these two clinical parameters in tumors coexpressing both receptors *versus* those only expressing ER $\alpha$ . These results could lead us to hypothesize that ER $\beta$  expression might have little clinical impact on ER $\alpha$  function. However, we also found that tumors expressing both receptors tended to be more aneuploid, and tumors expressing only ER $\beta$  exhibited even

slightly higher rates of aneuploidy. Our observed association between ER $\beta$  expression and tumor aneuploidy undoubtedly needs to be validated in a larger data set, but it does suggest an intriguing biological association for ER $\beta$  in breast tumors that has not been previously appreciated.

We know very little about the specific function of ER $\beta$  in the breast. Studies of ER $\beta$  knockout mice suggest that, although these mice have impaired ovarian function, breast development and function are not compromised (27). It has also been suggested that ER $\beta$  may function to inhibit the induction of PR by ER $\alpha$ , at least in the normal rodent mammary gland (28). However, this does not appear to be the case in human breast tumors, where there was no difference between the induction of PR in tumors expressing both receptors compared with those expressing only ER $\alpha$ . Our results strongly support the conclusion that ER $\alpha$  rather than ER $\beta$  is the predominant regulator of PR expression in clinical breast cancers, in agreement with reports of an inverse correlation of ER $\beta$  RNA expression and levels of the PR (20).

The failure of ER $\beta$  IHC measurements to correlate with ER or PR LBA is another relatively surprising finding from our study, especially because ER $\beta$  has a similar binding capacity for estradiol as ER $\alpha$  (2). Again, there are controversial data in the literature concerning this question, with one study also reporting no correlation between ER $\beta$  RNA and ER LBA (20), and one study reporting a good correlation between ER $\beta$  protein (using a COOH-terminal-specific ER $\beta$  antibody), and PR IHC (8). These discordant results highlight the necessity of developing reliable ER $\beta$ -specific antibodies useful for clinical studies. In addition, because there are a number of COOH-terminal-truncated forms of ER $\beta$  (9), it is uncertain what forms may be measured when using these different assays. It is possible that expression of these ER $\beta$  variant forms, especially in the ER $\alpha$ -negative/ER $\beta$ -positive subgroup of breast tumors, is indicative of a particular "bad" prognosis, equivalent to the truly ER-negative group.

Finally, RNA-based ER $\beta$  studies have also raised the possibility that the presence of ER $\beta$  in breast tumors may be a marker of endocrine therapy resistance. In a limited but provocative study of 17 breast cancer patients with treatment response follow-up, ER $\beta$  was significantly elevated in the tamoxifen-resistant group of tumors (29). This would be consistent with our observed inverse correlation with PR because PR is a known marker of endocrine responsiveness. We did not have endocrine response data on our pilot study of 242 breast tumors, but a predictive clinical study is currently under way in tumors archived in the Baylor Breast Tumor Bank.

In summary, our results suggest that ER $\alpha$  rather than ER $\beta$  expression is correlated with most, but perhaps not all, prognostic factors in breast cancer. Furthermore, we present data suggesting that ER $\beta$  is not just a surrogate for ER $\alpha$  in breast cancer prognosis and may have distinct but as yet unknown functions. Assessment of the ultimate clinical utility of ER $\beta$  IHC in breast cancer prognosis and its possible usefulness for the prediction of treatment response awaits its examination in large clinical studies.

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