

*Featured Article*

# Low Levels of Estrogen Receptor $\beta$ Protein Predict Resistance to Tamoxifen Therapy in Breast Cancer

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## ABSTRACT

**Purpose:** Breast cancer is a hormone-dependent cancer, and the presence of estrogen receptor  $\alpha$  (ER- $\alpha$ ) in tumors is used clinically to predict the likelihood of response to hormonal therapies. The clinical value of the second recently identified ER isoform, called ER- $\beta$ , is less clear, and there is currently conflicting data concerning its potential role as a prognostic or predictive factor.

**Experimental Design:** To assess whether ER- $\beta$  expression is associated with clinical outcome, protein levels were measured by immunoblot analysis of a retrospective bank of tumor cell lysates from 305 axillary node-positive patients. A total of 119 received no adjuvant therapy, and 186 were treated with tamoxifen only. The median follow-up time was 65 months. Univariate and multivariate Cox regression modeling was done to assess the prognostic and predictive significance of ER- $\beta$  expression.

**Results:** Expression of ER- $\beta$  protein did not correlate significantly with any other clinical variables, including ER and progesterone levels (as measured ligand binding assay), tumor size, age, or axillary nodal status. In the untreated population, those patients whose tumors who expressed both receptor isoforms exhibited the most favorable outcome as compared with those patients who had lost ER- $\alpha$  expression. However, there was no association between ER- $\beta$  levels alone and either disease-free or overall survival in the untreated patient population. In contrast, in both univariate and multivariate analyses, high levels of ER- $\beta$  predicted an

improved disease-free and overall survival in patients treated with adjuvant tamoxifen therapy.

**Conclusions:** These findings provide evidence that ER- $\beta$  may be an independent predictor of response to tamoxifen in breast cancer. Furthermore, these results suggest that ER- $\beta$  may influence tumor progression in ways different from those mediated by the ER- $\alpha$  isoform.

## INTRODUCTION

For more than 30 years, the classical estrogen receptor, called ER- $\alpha$ , has been extensively studied as a prognostic and predictive marker in clinical breast cancer, making this nuclear receptor the most valuable target for the treatment of human breast cancer with selective estrogen receptor modulators or the newer generation aromatase inhibitors. Patients with ER- $\alpha$ -positive tumors have a significantly prolonged overall and recurrence-free survival with selective estrogen receptor modulators (1) and aromatase inhibitor therapy (2). Our understanding of ER in breast cancer became less clear with the identification of a second related ER isoform called ER- $\beta$  (3, 4). After the identification of ER- $\beta$ , researchers had to reevaluate the prior simplistic model of estrogen action. However, we are only now beginning to appreciate whether ER- $\beta$  expression in tumors exerts a clinical impact on the progression and treatment of breast cancer.

ER- $\alpha$  and ER- $\beta$  are both ligand-induced transcription factors that can modulate the expression of specific target genes. At present, there is little information available concerning differential induction of gene expression from either ER- $\alpha$  or ER- $\beta$ . On the structural level, both receptor isoforms encode two activation functions, as well as a DNA-binding domain, which recognizes and binds to estrogen response elements within the promoter of target genes (5). The two receptor isoforms also share >50% similarity in their hormone-binding domains and a 95% similarity within their DNA binding domains (4). Because there is less sequence similarity within their hormone-independent activation function-1 (AF-1) domains, it has been suggested that the two receptors might perform distinct functions. In agreement with this possibility is a body of literature showing that ER- $\alpha$  is the dominant receptor in the mouse mammary gland essential for ductal development and hormone response (6–8). We also know that ER- $\beta$  binds estrogen with similar affinity as ER- $\alpha$ , but unlike ER- $\alpha$ , antiestrogen-occupied ER- $\beta$  can activate transcription via nonclassical ER-signaling pathways through its binding to activator protein 1 transcription factors (9). This has led some investigators to speculate that ER- $\beta$  could play a role in tamoxifen resistance through the agonist activity of tamoxifen, and indeed, one study examining ER- $\beta$  RNA expression in a small number of tamoxifen-treated patients supported this idea (10). Although the two receptors do not appear to be expressed within the same cell in normal mouse

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mammary tissue (8), we and others have shown that they are frequently coexpressed in breast tumors (11, 12). Because the two receptors can exist as heterodimers when coexpressed (13, 14), ER- $\beta$  might also modify the activity of ER- $\alpha$  and therefore the clinical outcome of patients given hormonal therapy.

A growing number of studies have analyzed ER- $\beta$  levels in clinical breast tumor samples (11, 12, 15–18), but results have been contradictory. Therefore, currently there is no consensus concerning the role that ER- $\beta$  may play as either a prognostic factor in patients not receiving systemic adjuvant treatment or as a predictive factor for response to treatment. We therefore performed immunoblot analysis with an ER- $\beta$ -specific antibody in 305 patients with axillary node-positive tumors with long-term clinical follow-up. For patients who received tamoxifen therapy, high ER- $\beta$  levels predicted a favorable outcome. We conclude that as with ER- $\alpha$ , ER- $\beta$  is a biomarker of response to selective estrogen receptor modulators.

## MATERIALS AND METHODS

**Tumor Specimens.** A cohort of frozen primary breast tumor specimens from 305 axillary lymph node-positive patients was selected from the tumor bank of The Breast Center, Baylor College of Medicine, for use in the immunoblot study. This study was approved by the Baylor College of Medicine Institutional Review Board. This patient cohort has previously been evaluated for expression of other clinical variables (19, 20). Proteins were extracted from the tumors as described previously (21). Briefly, 30 mg of pulverized tumor powder were solubilized in 300  $\mu$ L of 5% SDS at 90°C for 5 minutes and centrifuged at 13,000  $\times g$  for 5 minutes. Protein concentrations of the supernatants were determined using the bicinchoninic acid method (Pierce, Rockford, IL). Typical protein yields were 2 to 5  $\mu$ g/ $\mu$ L. Supernatants were then stored at –70°C until use in the immunoblot analysis.

**Immunoblot Analysis.** We have previously shown that MCF-7 human breast cancer cells express full-length ER- $\beta$  protein corresponding to the 530 amino acid isoform (22). Thus, MCF-7 extracts (also stored at –70°C) were used as a standard on each gel to correct for gel to gel variations. MCF-7 cell lysates were prepared the same as the patient tumor samples. ER- $\beta$  expression levels were measured by an investigator that was blinded to all of the clinical information. The extracted proteins were solubilized in sample buffer [0.05 mol/L Tris (pH 6.8) containing 2% SDS, 2.5%  $\beta$ -mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue as tracking dye] and placed in boiling water for 2 minutes, cooled to room temperature, and centrifuged at 13,000  $\times g$  for 1 minute. For immunoblotting, SDS-PAGE was done with precast 8% Tricine-Glycine polyacrylamide gels (Invitrogen, Carlsbad, CA) with 20  $\mu$ g of extracted protein from the tumor lysates and 10  $\mu$ g of protein from the MCF-7 standard extract per gel, and then the proteins were transferred onto nitrocellulose (Schleicher and Schuell, Keene, NH) at 4°C overnight at 20 mA. The immunoblots were blocked with 5% nonfat dry milk in TBST [Tris-buffered saline, 100 mmol/L Tris (pH 7.5), 0.9% NaCl, and 0.1% Tween 20] and then incubated for 1 hour with the primary ER- $\beta$ -specific antibody (1:200 mouse clone anti-14C8, GeneTex, San Antonio, TX), which does not recognize ER- $\alpha$  protein (11), followed by

washing three times in TBST, and then incubated for 1 hour with horseradish peroxidase-labeled antimouse IgG (1:2000; Amersham Pharmacia Biotech, Piscataway, NJ). After extensive washing in TBST, ER- $\beta$  protein was then visualized on a FluorChem digital imaging system ( $\alpha$  Innotech, San Leandro, CA) with an enhanced chemiluminescence detection system. Band intensities were measured densitometrically with the AlphaEaseFC software ( $\alpha$  Innotech), and then ER- $\beta$  levels in tumors were normalized to ER- $\beta$  levels in the MCF-7-positive control lysate (10  $\mu$ g) from the same immunoblot.

**Other Biological Factors.** Total ER and PR levels were measured by ligand-binding assay as described elsewhere (23). Briefly, cytosolic proteins were extracted from tumor tissues that had been pulverized in liquid nitrogen. Iodine-125-labeled estradiol and tritiated-ORG 2058 (Amersham Pharmacia Biotech) addition allowed for the simultaneous determination of both ER and progesterone receptor (PgR) levels in a standard multipoint dextran-coated charcoal assay. Tumors with an ER content of at least 3 fmol/mg protein and with a PgR content of at least 10 fmol/mg protein were considered positive for ER and PgR, respectively. These levels were based on prior studies calibrated to clinical outcome (23).

The two separate PgR isoforms, PgR-A and PgR-B, were previously determined with immunoblot analysis after separation by 8% SDS-PAGE as described, with the mouse anti-PgR1294 (Dako, Carpinteria, CA), which specifically recognizes both PgR-A and PgR-B isoforms on immunoblots (20). AIB1 and HER-2 levels in these same tumors were previously determined with immunoblot analysis after separation by 8 and 7.5% SDS-PAGE, respectively, and then staining with two rabbit polyclonal antisera, anti-RAC3 antibody (19), and anti-HER2 (21). The MCF-7 reference cell line standard was used for normalization of AIB1 levels, and a similarly prepared extract from T47D human breast cancer cells was used for normalization of the PgR isoforms and for HER-2 levels. S-Phase fraction and DNA ploidy were calculated with DNA flow cytometry (24) and is reported as low, intermediate, or high. Briefly, DNA flow cytometry was done on tumor extracts, and the histograms were analyzed by Modfit (Verity Software House, Topsham, ME) with single-cut debris stripping (24). Cut points were determined by calibrating S-phase fraction with clinical outcome in a group of >28,800 patients with breast cancer (low, <6%; intermediate, 6–10%; and high, >10%).

## Statistical Methods

**Descriptive Analysis.** Clinical characteristics were summarized separately in tamoxifen-treated and untreated patients with descriptive statistics and compared with  $\chi^2$  or two sample Wilcoxon rank-sum tests. ER- $\beta$ , ER (ligand-binding assay), PgR-A, PgR-B along with AIB1, and HER-2 were also summarized for treated and untreated patients. Continuous levels of ER- $\beta$  were calculated as the ratio of band intensities measured in densitometry units from Western blots of individual samples normalized to the MCF-7 reference standards. Correlations between continuous levels of ER- $\beta$  and patient clinical characteristics or other molecular markers were evaluated with Spearman's rank correlation ( $r$ ). Spearman's correlation ranges in value from –1 to +1. A value of 0 indicates no association, whereas values near +1 or –1 indicate strong positive or

negative relationships, respectively. All variables in the correlation analysis were analyzed as continuous variables.

**Univariate Analysis of Disease-Free Survival and Overall Survival.** The disease-free interval was calculated from the date of diagnosis to date of first recurrence or first metastasis (local or distant). Patients without recurrence were censored at the time of last follow-up or death. Overall survival was calculated from the date of diagnosis to date of death from any cause. Patients who were alive at the last follow-up were censored at the last follow-up date. Overall survival included all deaths regardless of cause because cause of death data, especially if reported as “not due to breast cancer,” are sometimes unreliable. This definition of overall survival is the most conservative estimate of patient outcome. Therefore, disease-free survival was the primary outcome sought in this analysis and has more relevance than overall survival. Continuous levels of ER-β were dichotomized *a priori* with statistical criteria. First, the functional form of continuous levels of ER-β was evaluated with Martingale residuals (25). In brief, this method fits an intercept-only Cox proportional hazards model and plots the Martingale residuals from this model against quantitative levels of ER-β. A reasonably linear plot indicates that a variable can be entered as a continuous variable in subsequent analyses, whereas a nonlin-

ear plot indicates some threshold effect. The dichotomization of ER-β was chosen based on where this threshold effect is evident from the plot. In addition, the dichotomization resulted into appropriate equal-sized groups (0 to <3 *versus* ≥3) based on the median level of the distribution of ER-β. Disease-free survival and overall survival were estimated between the two levels of ER-β with the Kaplan-Meier method and compared with the Wilcoxon test, which is more appropriate than the log-rank test when the assumption of proportional hazards is violated (see below). Univariate associations of other tumor and clinical characteristics with disease-free survival and overall survival were assessed separately for treated and untreated patients.

**Multivariate Cox Regression Model for Disease-Free Survival and Overall Survival.** Likewise, all multivariable Cox regression modeling was carried out separately for treated and untreated patients. Clinical characteristics (patient age, tumor size, nodes, S-phase, and ploidy) were dichotomized or trichotomized, as indicated in Table 1, and coded ordinally (*i.e.*, 0, 1, 2) for inclusion as continuous variables in the model. ER (ligand-binding assay), PgR-A, and PgR-B levels were entered as dichotomized variables based on prior published studies (20, 26). The molecular markers, AIB1 and HER-2, were entered as continuous variables in the model.

Table 1 Distribution of patient and tumor characteristics

	Overall (N = 305)	Treated (n = 186)	Untreated (n = 119)	P
Age (y)				
<50	43 (14%)	19 (10%)	24 (20%)	0.015
≥50	262 (86%)	167 (90%)	95 (80%)	
Tumor size (cm)				
0–2	73 (24%)	50 (27%)	23 (19%)	0.191
>2–5	176 (58%)	107 (57%)	69 (59%)	
>5	55 (18%)	29 (16%)	26 (22%)	
Missing	1			
Nodes				
1–3	167 (55%)	103 (55%)	64 (54%)	0.846
>3	137 (45%)	83 (45%)	54 (46%)	
Missing	1			
S phase				
Low (0 to <6%)	68 (22%)	48 (26%)	20 (17%)	0.147
Intermediate (≥6 to ≤10%)	101 (34%)	60 (33%)	41 (35%)	
High (>10%)	131 (44%)	74 (41%)	57 (48%)	
Missing	5			
Ploidy				
Diploid	106 (35%)	63 (35%)	43 (36%)	0.787
Aneuploid	195 (65%)	119 (65%)	76 (64%)	
Missing	4			
ER (fmol/mg) (LBA)				
Positive (≥3)	272 (89%)	176 (95%)	96 (81%)	0.0001
Negative (0 to <3)	33 (11%)	10 (5%)	23 (19%)	
PgR (fmol/mg) (LBA)				
Positive (≥5)	180 (61%)	122 (67%)	58 (53%)	0.018
Negative (0 to <5)	113 (39%)	61 (33%)	52 (47%)	
Missing	12 (-)			
	Median (range)	Median (range)	Median (range)	
AIB1 (IU)	1.12 (0.26–5.73)	1.11 (0.26–5.73)	1.17 (0.30–5.40)	0.136
HER-2 (IU)	1.00 (0–4)	1.00 (0–4)	1.00 (0–4)	0.584
ER-β (IU)	2.68 (0.22–29.71)	2.82 (0.30–10.75)	2.29 (0.22–29.71)	0.212
Median follow-up time (mo)	65	74	50	

Abbreviation: LBA, ligand-binding assay.

Previous studies have reported the nonproportional effect of ER on disease-free survival and overall survival (27). Testing for nonproportionality of ER- $\beta$  was accomplished by including each marker in the model as both fixed and time-dependent variables. Plots of time-varying regression coefficients allowed us to visualize the nature of the nonproportionality and determine an appropriate method of adjustment. For this specific patient population, tests of the proportionality assumption revealed no significant departures from proportionality for both ER- $\beta$  and ER (ligand-binding assay). However, as indicated in our recent study (20), the nonproportional effect of PgR isoforms were accounted for in multivariate analysis. Cox regression models were constructed with forward stepwise selection with the level of significance to enter or stay in the model set at  $P < 0.05$ . After an initial set of significant variables were determined, we included an interaction term between ER- $\beta$  and ER (ligand-binding assay) and found no statistically significant interaction between the two ER isoforms. Hazard ratios from the final models are presented with their 95% confidence intervals.

## RESULTS

**Relationship between ER- $\beta$  and Other Clinical Markers.** The study population consisted of 305 tumor specimens from patients with operable stage II breast cancers with positive axillary lymph nodes; the overall median follow-up time was 65 months (Table 1). Most patients were  $>$  age 50 years and had tumors that were  $<$  5 cm with an intermediate to high S-phase fraction. Approximately 89% of patient's tumors expressed ER (as determined by ligand-binding assay), whereas 61% were positive for PgR at the time of diagnosis. Patients either received no adjuvant therapy after primary surgical treatment (119 patients) or were treated with the selective estrogen receptor modulator tamoxifen after surgical removal of their tumor (186 patients). The biological characteristics of the two groups were similar, except that the treated group was slightly older and more frequently ER and PgR positive.

A representative ER- $\beta$  immunoblot is shown in Fig. 1. An ER- $\beta$  band at  $M_r \sim 60,000$  was seen; lower molecular weight bands corresponding to potential ER- $\beta$  splice variants (22) were infrequently observed in this Western blot analysis. ER- $\beta$  levels were heterogeneous among tumors and ranged from a band intensity of 0.22 to a maximum of 29.71 IU, with a median band intensity value of 2.68 (Table 1).

We first correlated ER- $\beta$  levels with the biological markers

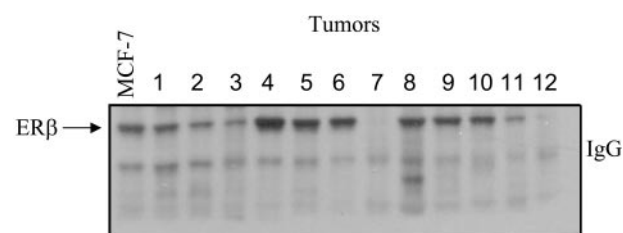


Fig. 1 Representative example of ER- $\beta$  immunoblots from 12 tumor lysates. Lane 1 is the standard used for normalization containing 10  $\mu$ g of MCF-7 cell extract, and the other lanes contain equal amounts of tumor extracts. Human IgG is labeled.

Table 2 Spearman rank correlation of ER- $\beta$  with other variables

Variable	ER- $\beta$
	Correlation ( $P$ )
ER* (LBA)	0.07 (0.214)
PgR* (LBA)	-0.03 (0.587)
PgR-A $\dagger$	-0.007 (0.902)
PgR-B $\dagger$	0.009 (0.873)
Age	0.01 (0.824)
Tumor size	0.05 (0.408)
Nodes	-0.03 (0.606)
S phase	-0.03 (0.645)
Ploidy	-0.02 (0.734)
AIB1	0.11 (0.058)
HER-2	-0.02 (0.712)

Abbreviation: LBA, ligand-binding assay.

\* ER and PgR were previously determined by LBAs.

$\dagger$  PgR-A and PgR-B determined by immunoblot analysis.

and clinical variables available on this group of patients (Table 2). ER- $\beta$  protein expression was only weakly correlated with AIB1 levels (Spearman rank correlation,  $r = 0.11$ ,  $P = 0.058$ ). ER- $\beta$  was not significantly correlated with ER or PgR expression determined by ligand-binding assay, confirming our previous observation with ER- $\beta$  immunohistochemistry in a group of breast cancer patients without clinical follow-up (11). This suggests that the measurement of ER- $\beta$  levels in breast tumors is not just a surrogate for ER- $\alpha$ . There were 141 ER- $\beta$ + patients, with 124 (88%) ER ligand-binding assay positive and 12% ER ligand-binding assay negative (data not shown). Thus, a small percentage of ER- $\beta$ + tumors are not correctly classified with ligand-binding assay. ER- $\beta$  levels were similarly not significantly correlated with age, tumor size, number of positive axillary lymph nodes, S-phase fraction, or DNA ploidy.

**Univariate Analysis of Disease-Free Survival and Overall Survival by ER- $\beta$ .** As described in Materials and Methods, preliminary statistical analyses were used to select dichotomization of ER- $\beta$ ; patients were separated into those with ER- $\beta$  levels of 0 to  $<$ 3 IU versus patients whose breast tumors expressed ER- $\beta$  levels  $\geq$  3. Plots of Martingale residuals confirmed that the relationship between disease-free survival and overall survival were reasonably well represented by the dichotomization (data not shown). We first asked whether there might be differences in clinical outcomes between those tumors expressing both ER isoforms and those expressing a single hormone receptor. Fig. 2 shows the disease-free survival for the untreated study population according to receptor subtype; there were insufficient patients in the treated group to do this subset analysis. Overall, the disease-free survival of the different receptor subtypes were statistically significant in the untreated group ( $P = 0.0124$ ). Those patients who were ER- $\alpha$ + / ER- $\beta$ + had an improved disease-free survival compared with those who were negative for both receptors (ER- $\alpha$ - / ER- $\beta$ - ,  $P = 0.007$ ). Patients whose tumors had lost ER- $\beta$  expression but had retained ER- $\alpha$  expression exhibited no significant change in prognosis ( $P = 0.305$ ) compared with those who expressed both receptors. This result indicates that ER- $\beta$  levels might not appreciably affect ER- $\alpha$  function. However, those patients who had lost ER- $\alpha$  but retained ER- $\beta$  had a significantly worse

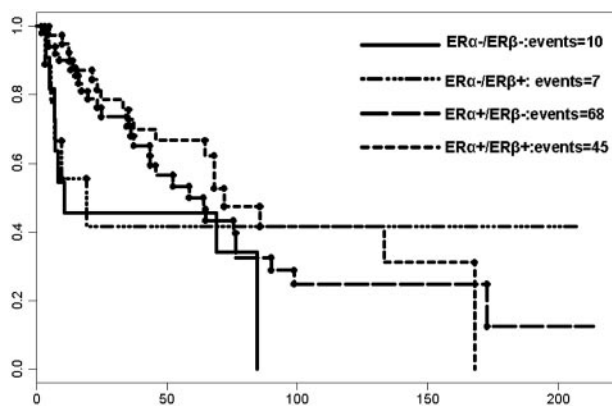


Fig. 2 Kaplan-Meier curve for disease-free survival in overall group. Disease-free survival for both untreated and tamoxifen-treated patients stratified by levels of ER- $\alpha$  and ER- $\beta$  is shown. Overall  $P = 0.012$ ; ER $\alpha$ +/ER $\beta$ + vs. ER $\alpha$ -/ER $\beta$ -:  $P = 0.007$ ; ER $\alpha$ +/ER $\beta$ + vs. ER $\alpha$ -/ER $\beta$ +:  $P = 0.019$ ; ER $\alpha$ +/ER $\beta$ + vs. ER $\alpha$ +/ER $\beta$ -:  $P = 0.305$ .

prognosis (ER- $\alpha$ +/ER- $\beta$ + versus ER- $\alpha$ -/ER- $\beta$ +,  $P = 0.019$ ). This suggests that coexpression of ER- $\beta$  may reflect an intact hormone response pathway in these patients, similar to what has been suggested for the ER- $\alpha$ +/PgR+ breast cancer phenotype, which has been associated with a worse prognosis when ER- $\alpha$  is lost (28).

To examine whether ER- $\beta$  levels were associated with prognosis, we separately analyzed the 119 patients who had not received adjuvant selective estrogen receptor modulator therapy, and the Kaplan-Meier estimates are shown in Fig. 3, A and B. These analyses revealed that there were no statistically significant correlations of ER- $\beta$  with either disease-free survival or overall survival ( $P = 0.36$  and  $P = 0.79$ , respectively). Thus, although ER- $\alpha$  expression is a well-established prognostic factor in untreated patients, ER- $\beta$  levels do not appear to provide significant prognostic information in node-positive patients. To examine the effect of ER- $\beta$  expression on the benefit from adjuvant selective estrogen receptor modulator therapy in these patients, we next analyzed the 186 tamoxifen-treated patients (Fig. 4, A and B). Treated patients with higher intensity values of ER- $\beta$  ( $\geq 3$ ) had a modest improvement in disease-free survival and overall survival compared with those with low values (0 to  $<3$ ;  $P = 0.06$  and  $P = 0.07$ , respectively).

Several of the other prognostic factors examined in this patient population that had significant associations with a worse disease-free survival in the untreated group included tumor size ( $P = 0.007$ ), greater than three positive lymph nodes ( $P = 0.0002$ ), high S-phase fraction ( $P = 0.012$ ), and low AIB (Table 3). In the treated group, tumor size ( $P = 0.025$ ), nodes  $> 3$  ( $P > 0.001$ ), and negative ER or PgR status were associated with poor disease-free survival. The univariate results encouraged us to next do multivariate analyses.

**Multivariate Cox Regression Analysis of Disease-Free Survival and Overall Survival.** When ER- $\beta$  and the nine other clinical and biological factors available on these patients were included in a Cox multivariate analysis (Table 4A), only ER negativity [hazard ratio (HR), 2.3; 95% confidence interval (CI), 1.20–4.40;  $P = 0.0121$ ], nodal status  $> 3$  (HR, 3.83; 95%

CI, 2.20–6.68;  $P < 0.0001$ ), and low AIB1 levels (HR, 0.59; 95% CI, 0.41–0.86;  $P = 0.0059$ ) remained in the model and were associated with poor disease-free survival in the untreated group of patients. These results are consistent with previous observations that axillary lymph node status is a powerful prognostic factor in breast cancer (29) and with a recent report from this same patient group that low AIB1 levels are associated with improved prognosis in node-positive patients not receiving adjuvant therapy (19). For overall survival in this group, only ER (HR, 2.20; 95% CI, 1.27–3.79;  $P = 0.0047$ ), and number of nodes (HR, 2.20; 95% CI, 1.43–3.40;  $P = 0.0004$ ) remained as significant prognostic variables in the untreated patients (Table 4B). Thus, ER- $\beta$  is not a prognostic factor in these patients.

We next examined the ability of ER- $\beta$  levels to predict benefit with tamoxifen in the treated patients (Table 5, A and B). This model revealed that patients with lower ER- $\beta$  values are 2.04 times more likely to relapse than patients with higher ER- $\beta$  values (HR, 2.04; 95% CI, 1.22–3.41;  $P = 0.0063$ ; Table 4A), suggesting that ER- $\beta$  is an independent predictive factor for disease-free survival in treated patients. More than three positive nodes (HR, 2.54; 95% CI, 1.54–4.18;  $P = 0.0003$ ), low PgR-A during the first 5 years (HR, 2.38; 95% CI, 1.33–4.26;  $P = 0.0005$ ), and high AIB1 levels (HR 1.68; 95% CI: 1.22, 2.32;  $P = 0.0015$ ) were also significantly associated with a decrease in disease-free survival. Furthermore, ER- $\beta$  was also significantly associated with overall survival (Table 4B). Specifically, patients with lower ER- $\beta$  values were 1.52 times more likely to die than patients with higher ER- $\beta$  values (HR, 1.52; 95% CI, 1.04–2.22;  $P = 0.0296$ ). Tumors  $> 5$  cm (HR, 2.25; 95% CI, 1.26–4.02;  $P = 0.0064$ ) and low PgR-A levels (HR, 1.97; 95% CI, 1.37–2.85;  $P = 0.0003$ ), as well as high AIB1 levels (HR, 1.40; 95% CI, 1.11–1.77;  $P = 0.0043$ ) were also significantly predictive of overall survival in this particular model. These data suggest that breast cancers expressing low levels of ER- $\beta$  may be resistant to tamoxifen treatment.

## DISCUSSION

Estrogen and its receptors play a crucial role in the progression of breast cancer, and ER has been identified as a target for breast cancer treatment for many years. Numerous studies have shown that breast cancers expressing ER- $\alpha$  are more likely to respond to hormonal therapies such as tamoxifen because tamoxifen acts as a competitive inhibitor of ER- $\alpha$  signaling (28). This exploratory study is the first comparing the effect of ER- $\beta$  levels on disease-free survival or overall survival in axillary node-positive breast cancers with long-term clinical follow-up. This cohort was chosen because such patients have higher recurrence rates after surgery than do lymph node-negative patients, resulting in better statistical power due to more events. Surprisingly, we found that although ER- $\beta$  is not a useful prognostic marker in untreated node-positive cancers, it is a significant marker of improved response to tamoxifen. Our data support the hypothesis that ER- $\beta$  expression is an independent marker of response and is not just a surrogate for ER- $\alpha$  in clinical breast cancer.

ER- $\alpha$  expression and PgR levels are two of the most clinically useful biological markers for determining the likelihood of response to tamoxifen in breast cancer patients (ref. 26

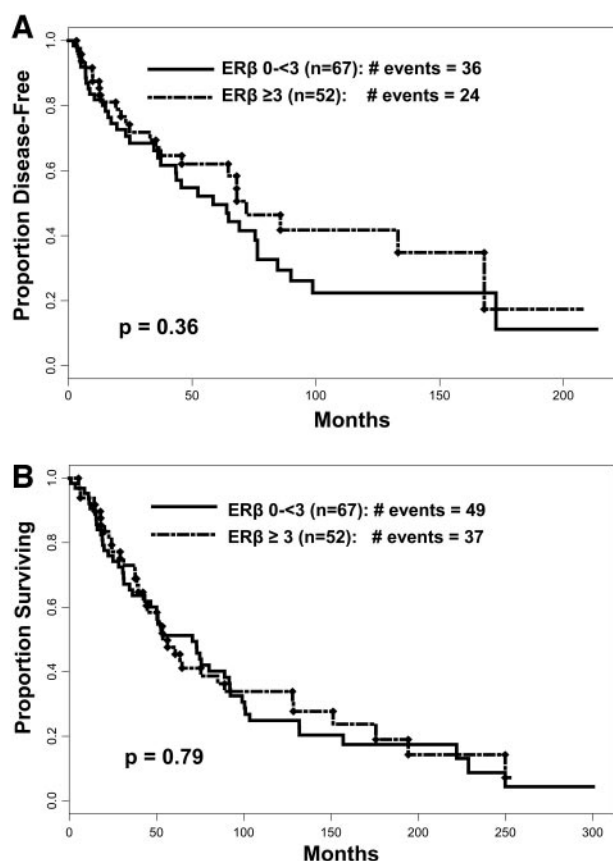


Fig. 3 Kaplan-Meier survival curve of disease-free survival (A) and overall survival (B) for untreated patients stratified by low and high levels of ER- $\beta$  protein. The number of events in each group and *P* values are also shown.

and reviewed in ref. 30). In recent years, several protein-based studies have attempted to assess the prognostic and/or predictive value of ER- $\beta$  protein expression in breast cancer (summarized in Table 6). However, many of these previous studies were hampered by small sample size or lack of long-term clinical follow-up. These studies also provide inconsistent correlations of ER- $\beta$  with other important clinical variables. For instance, some of the studies have detected significant correlations between ER- $\alpha$  and ER- $\beta$  levels (16, 17, 31), but others did not show any association between the two receptor isoforms (32, 33). These differences of course could reflect the different ER- $\beta$  antibodies that were used in the assorted studies and the various cut points for determining ER- $\alpha$  and ER- $\beta$  positivity.

In this current study, we did not find any correlation between ER- $\beta$  levels determined by immunoblot analysis and total ER levels as determined by ligand-binding assay, which was surprising because the two receptors have similar binding affinities (34). However, this finding is in agreement with our other patient study without clinical follow-up that measured ER- $\beta$  levels by immunohistochemistry with the same antibody as this study (11). These combined results suggest that ligand-binding assay is imprecise for measuring ER- $\beta$  protein. Although ER- $\beta$  is generally expressed at lower levels than ER- $\alpha$

(11), this difference probably does not fully explain the apparent inconsistency between ligand-binding assay and ER- $\beta$  protein measurements. In the present study, we measured full-length ER- $\beta$  protein, but others have reported that a number of COOH-terminal truncated forms of ER- $\beta$  exist in breast cancers (17, 35). It is possible that these smaller isoforms might not be accurately measured in ligand-binding assay. However, we only found infrequent expression of putative ER- $\beta$  variants in our study with an ER- $\beta$  antibody to the NH<sub>2</sub> terminus, which should detect these smaller forms. Thus, expression of ER- $\beta$  variants does not entirely explain our observed discrepancy between total ligand binding in the tumors and protein-based measurements for ER- $\beta$  in these samples.

Previous studies have shown that elevated ER- $\beta$  levels might predict a better response to tamoxifen (12, 18, 36). Our study confirms these results and shows in a multivariate analysis, including nine other clinical variables, that high ER- $\beta$  expression independently predicts tamoxifen response in a relatively homogenous group of treated patients. It has been determined that tamoxifen is a potent transcriptional activator with ER- $\beta$  at an activator protein 1 enhancer element, which predicts that ER- $\beta$  may act to enhance the agonist activity of tamoxifen (9). This ER- $\beta$  activity could

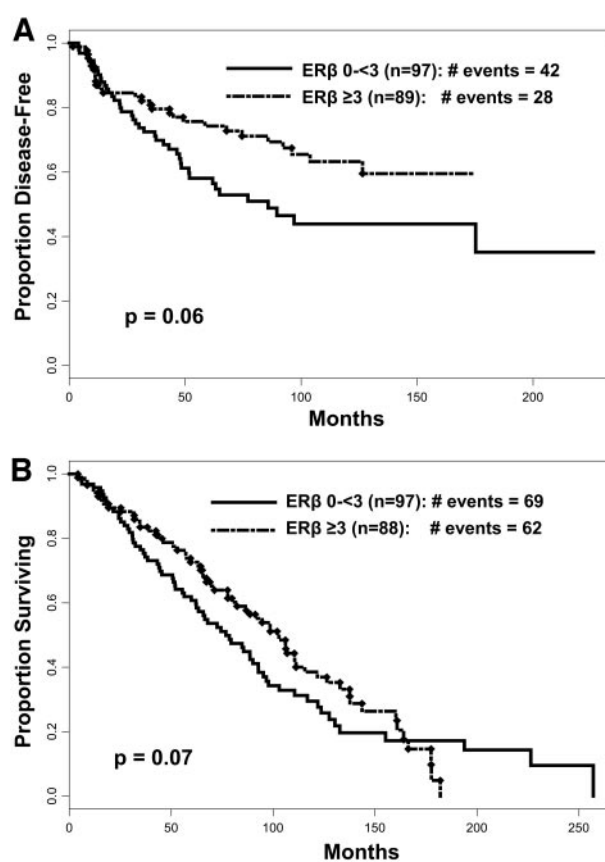


Fig. 4 Kaplan-Meier survival curves for disease-free survival (A) and overall survival (B) for tamoxifen-treated patients stratified by low and high levels of ER- $\beta$  protein. The number of events in each group and *P* values are also shown.

Table 3 Univariate association of clinical and tumor characteristics with disease-free survival

	Treated		Untreated	
	HR (95% CI)	P	HR (95% CI)	P
Age (y)				
<50	1.00	0.998	1.00	0.976
≥50	0.999 (0.48–2.09)		0.99 (0.52–1.87)	
Tumor size (cm)				
0–2	1.00	0.007	1.00	0.025
>2–5	1.68 (1.15–2.45)		1.63 (1.06–2.51)	
>5	2.82 (1.33–5.99)		2.67 (1.13–6.28)	
Nodes				
1–3	1.00	0.0002	1.00	<0.0001
>3	2.54 (1.57–4.12)		3.28 (1.92–5.64)	
S phase				
Low (0 to <6%)	1.00	0.012	1.00	0.197
Intermediate (≥6 to ≤10%)	1.49 (1.09–2.04)		1.25 (0.89–1.76)	
High (>10%)	2.22 (1.19–4.14)		1.57 (0.79–3.11)	
Ploidy				
Diploid	1.00	0.895	1.00	0.016
Aneuploid	0.97 (0.58–1.60)		1.97 (1.13–3.42)	
ER (fmol/mg) (LBA)				
Positive (≥3)	1.00	0.480	1.00	0.002
Negative (0 to <3)	1.44 (0.52–3.96)		2.28 (1.15–4.55)	
ER-β				
Positive (≥3)	1.00	0.060	1.00	0.360
Negative (0 to <3)	1.69 (0.98–2.92)		1.36 (0.81–2.29)	
PgR (fmol/mg) (LBA)				
Positive (≥5)	1.00	0.089	1.00	0.039
Negative (0 to <5)	1.53 (0.94–2.48)		1.79 (1.03–3.11)	
AIB1 (IU)	1.39 (1.01–1.91)	0.044	0.70 (0.49, 0.98)	0.039
HER-2 (IU)	1.12 (0.92–1.37)	0.242	1.22 (0.99–1.50)	0.058

Abbreviation: LBA, ligand-binding assay.

potentially then provide a molecular mechanism of resistance to tamoxifen. Similarly, ER-β can activate a RARα1 promoter reporter construct in the presence of tamoxifen (37). Our results do not support these reporter-based predictions that ER-β expression confers resistance to tamoxifen.

In our multivariate analyses of disease-free survival and overall survival in the treated cohort, low levels of the PgR-A isoform and elevated AIB1 were also highly predictive of tamoxifen resistance, confirming our previous data showing that both of these molecular markers are predictive of responsiveness in these patients (19, 20). It is interesting to note that HER2 did not enter the Cox model in our study. Some, but not all, prior studies indicate that high HER-2 is associated with tamoxifen resistance (38). Perhaps measurement of HER2 by itself is not a strong predictive marker and thus did not enter our model. A combination of high HER-2 and high AIB1 predicted tamoxifen resistance better than either alone in this same patient population in an earlier study (19).

An interesting observation was made when we examined the untreated patient group and separated these patients into distinct receptor subgroups. Of course, these types of exploratory subset analyses can only be considered preliminary and should not have an impact on clinical decisions, but they are useful for hypothesis generation and testing. Thus, it is tempting to speculate about the discordant receptor phenotypes we observed. For instance, there were no significant differences between those tumors that expressed both receptors and those that had only lost ER-β expression. There has been speculation that

ER-β might serve a tumor suppressor role in breast cancer, acting as a modulator of ER-α activity. In support of this speculation is data showing that ER-β can indeed modulate ER-α activity in the uterus (39), that it may be involved in terminal differentiation of the mouse mammary gland epithelium (40), and that it can inhibit MCF-7 proliferation when overexpressed in these cells (41). However, one plausible inter-

Table 4 Cox regression model of ER-β  
A. Disease-free survival: untreated group

Variable	HR (95% CI)	P
ER (LBA)		0.0121
Positive (≥3)	1.00	
Negative (0 to <3)	2.30 (1.20–4.40)	
Node group		<0.0001
1–3	1.00	
>3	3.83 (2.20–6.68)	
AIB1	0.59 (0.41–0.86)	0.0059

B. Overall survival: untreated group

Variable	HR (95% CI)	P
ER (LBA)		0.0047
Positive (≥3)	1.00	
Negative (0 to <3)	2.20 (1.27–3.79)	
Node group		0.0004
1–3	1.00	
>3	2.20 (1.43–3.40)	

Abbreviation: LBA, ligand-binding assay.

pretation is that ER- $\beta$  is less active transcriptionally in the breast when bound to estrogen, and therefore, when functioning as a heterodimer with ER- $\alpha$ , estrogen-induced transcriptional activity is not biologically impacted. In support of this concept is our observation that the ER- $\alpha$ -/ER $\beta$ + subgroup was similar in outcome to the ER- $\alpha$ -/ER- $\beta$ - group and did not exhibit as favorable an outcome compared with those patients expressing both receptor isoforms. This could predict that ER- $\beta$  alone may have very little functional activity or consequences for untreated breast cancer patients, a suggestion that is consistent with the lack of a significant breast phenotype in ER- $\beta$  knockout animals (42). In contrast, ER- $\beta$  might serve as a predictive factor and target because of its ability to bind tamoxifen and ER- $\alpha$  in the treated population. The agonist activity of tamoxifen has been located within the AF-1 domain of ER- $\alpha$  (43), and the ER- $\alpha$ :ER- $\beta$  heterodimer might diminish the agonist activity of ER- $\alpha$  when bound to tamoxifen.

A number of antibodies recognizing different epitopes of ER- $\beta$ , from both commercial and private sources, have been used for measuring ER- $\beta$  in clinical samples. Speirs *et al.* (15) have evaluated some of these antibodies for immunohistochemistry and immunoblot analysis of breast tumors and cell lines. As expected, the antibodies differed in their suitability for these two methods, and there was considerable variability in the immunostaining that was obtained. An interesting observation reported in this study (15) was the occurrence of varying degrees of cytoplasmic staining in breast tumors. This may be consistent with other observations that ER- $\beta$  and ER- $\alpha$  might localize to both the nuclear and mitochondrial/cytoplasmic compartments

Table 5 Cox regression model of ER- $\beta$

A. Disease-free survival: tamoxifen-treated group

Variable	HR (95% CI)	P
ER- $\beta$		0.0063
$\geq 3$ IU	1.00	
0 to <3 IU	2.04 (1.22–3.41)	
Node group		0.0003
1–3	1.00	
>3	2.54 (1.54–4.18)	
PgR-A		0.0005
<5 y		
$\geq 1$ IU	1.00	
0 to <1 IU	2.38 (1.33–4.26)	
>5 y		
$\geq 1$ IU	1.00	
0 to <1 IU	0.58 (0.21–1.65)	
AIB1	1.68 (1.22–2.32)	0.0015

B. Overall survival: tamoxifen-treated group

Variable	HR (95% CI)	P
ER- $\beta$		0.0296
$\geq 3$ IU	1.00	
0 to <3 IU	1.52 (1.04–2.22)	
Tumor size group		0.0064
0–2 cm	1.00	
>2–5 cm	1.50 (1.12–2.01)	
>5 cm	2.25 (1.26–4.02)	
PgR-A		0.0003
$\geq 1$ IU	1.00	
0 to <1 IU	1.97 (1.37–2.85)	
AIB1	1.40 (1.11–1.77)	0.0043

Table 6 Summary of ER- $\beta$  protein studies in breast tumors

Author (ref.)	No. of tumors (method)	Clinical correlations with ER- $\beta$ (P)
Murphy <i>et al.</i> (36)	27 (IHC)	Response to Tam (0.046)
Jensen <i>et al.</i> (32)	29 (IB)	High proliferation (<0.05)
Saunders <i>et al.</i> (49)	51 (IHC)	None reported
Omoto <i>et al.</i> (17)	57 (IHC)	Better DFS (0.084)
Skiris <i>et al.</i> (50)	65 (IHC)	Low grade (0.04)
Iwase <i>et al.</i> (12)	77 (IHC)	Response to Tam (0.088)
Miyoshi <i>et al.</i> (33)	79 (IHC)	High grade (<0.05)
Omoto <i>et al.</i> (16)	88 (IHC)	Better DFS (0.0318)
Jarvinen <i>et al.</i> (51)	92 (IHC)	Low grade (0.0003)
Mann <i>et al.</i> (18)	118 (IHC)	Response to Tam (0.0077)
Fuqua <i>et al.</i> (11)	242 (IHC)	Grade (0.90)
Skiris <i>et al.</i> (31)	319 (IHC)	ER- $\alpha$ (0.03), PgR (0.02)

Abbreviations: IHC, immunohistochemistry; IB, immunoblot; Tam, tamoxifen.

in some cells, including MCF-7 human breast cancer cells (44–48). A putative ER- $\beta$  internal mitochondrial targeting peptide has also been identified with computer homology searching, which led to speculation that ER- $\beta$  might play a role in mitochondrial DNA transcription (45). Unfortunately, however, there is currently neither a consensus on the use of the various ER- $\beta$  antibodies, nor a standardization and optimization of immunohistochemistry techniques to measure ER- $\beta$  in breast tumors. This is a similar situation to that first encountered in measuring ER- $\alpha$  (23) and which remains problematic in the measurement of PgR in clinical samples. In addition, in the majority of protein-based published clinical studies, cytoplasmic staining of ER- $\beta$  was not reported (16, 17, 31, 32, 36, 49, 50). Because of these uncertainties, we used immunoblot analysis of ER- $\beta$  for this study, with the additional benefit that only full-length and not variant forms of the receptor were evaluated. Thus, our study was not designed to examine the cellular distribution of ER- $\beta$  in tumors but the potential role of total ER- $\beta$  protein in tumor progression.

In conclusion, these results provide additional data indicating that measurement of ER- $\beta$  protein in breast cancer patients may help to predict tamoxifen responsiveness. Of course, validation and prospective evaluation of this potential marker are needed, as is the development of a clinically useful assay methodology. To determine why ER- $\beta$  is a predictive marker because it does not correlate with other clinically useful tumor variables and how it precisely functions in mammary gland and breast cancer biology requires further study.

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