# Estrogen Receptor β Expression Is Associated with Tamoxifen Response in ERα-Negative Breast Carcinoma

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**Abstract**

**Purpose:** Endocrine therapies, such as tamoxifen, are commonly given to most patients with estrogen receptor (ERα)–positive breast carcinoma but are not indicated for persons with ERα-negative cancer. The factors responsible for response to tamoxifen in 5% to 10% of patients with ERα-negative tumors are not clear. The aim of the present study was to elucidate the biology and prognostic role of the second ER, ERβ, in patients treated with adjuvant tamoxifen.

**Experimental Design:** We investigated ERβ by immunohistochemistry in 353 stage II primary breast tumors from patients treated with 2 years adjuvant tamoxifen, and generated gene expression profiles for a representative subset of 88 tumors.

**Results:** ERβ was associated with increased survival (distant disease-free survival, $P = 0.01$; overall survival, $P = 0.22$), and in particular within ERα-negative patients ($P = 0.003$; $P = 0.04$), but not in the ERα-positive subgroup ($P = 0.49$; $P = 0.88$). Lack of ERβ conferred early relapse (hazard ratio, 14; 95% confidence interval, 1.8-106; $P = 0.01$) within the ERα-negative subgroup even after adjustment for other markers. ERα was an independent marker only within the ERβ-negative tumors (hazard ratio, 0.44; 95% confidence interval, 0.21-0.89; $P = 0.02$). An ERβ gene expression profile was identified and was markedly different from the ERα signature.

**Conclusion:** Expression of ERβ is an independent marker for favorable prognosis after adjuvant tamoxifen treatment in ERα-negative breast cancer patients and involves a gene expression program distinct from ERα. These results may be highly clinically significant, because in the United States alone, ~10,000 women are diagnosed annually with ERα-negative/ERβ-positive breast carcinoma and may benefit from adjuvant tamoxifen.

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Estrogens play an important role for the development and progression of breast carcinoma. Their effects on growth and proliferation are mediated through the two estrogen receptors (ER) α and β (1–3), which function as transcription factors and modulate the expression of target genes in response to estrogens. The biology of ERα has been studied for decades and evaluation of tumor ERα content is a mainstay of clinical practice as a marker associated with prognosis and response to endocrine therapies such as tamoxifen (4).

Tamoxifen is a selective ER modulator and is the most frequently prescribed drug for treatment of breast cancer. Tamoxifen is known to inhibit estrogen-stimulated growth of breast cancer cells by competitively binding to and blocking ERα (4). Patients with tumors lacking ERα in general do not benefit from tamoxifen therapy, although a fraction of ERα-negative tumors do seem to be sensitive to tamoxifen (5–7). The factors responsible for these responders are debated and no means of identifying this group is currently known. Therefore, tamoxifen is not indicated for patients with ERα-negative tumors in the adjuvant or metastatic setting.

Since the discovery of ERβ (3), which has a similar binding affinity as ERα for estrogens (8), several studies have focused on its biological function in relation to ERα. The two ER proteins share a high degree of homology in the DNA-binding regions

but differ considerably in the $NH_2$-terminal activation function 1 region, where interactions with other proteins in the transcriptional machinery takes place, and to a certain degree in the ligand-binding region (9), indicating that these receptors may share some similar functions but may not be entirely redundant. Indeed, they have been shown to respond differently in ligand-induced activation at activator protein 1 sites (10). In addition, ERα and ERβ can exist as a heterodimer (11–13), suggesting a possible role for ERβ as a modulator of ERα activity.

Recently, several studies have measured ERβ in breast cancer specimens and sought to clarify the relationship between ERβ and other clinicopathologic features and its role in response to endocrine treatment; however, some of the results have been conflicting and most focused on ERβ as a resistance marker in ERα-positive tumors (refs. 14–16, and those reviewed in ref. 17). ERβ has been shown to bind tamoxifen (18), and it has been suggested that low levels of ERβ associates with tamoxifen resistance (14). Conversely, Hopp et al. (15) showed that expression of ERβ had a beneficial effect on disease-free and overall survival in a group of 186 tamoxifen-treated tumors; however, they found no such association in their set of 119 untreated patients, suggesting a role for ERβ as a predictive marker for tamoxifen sensitivity but not as a prognostic marker. Importantly, the patients studied by Hopp et al. (15) were predominantly ERα-positive and the numbers were limited; thus, they were unable to perform an analysis stratified by ERα status. To our knowledge, no study has investigated the role of ERβ as a predictive marker for tamoxifen response for patients with ERα-negative tumors.

In the present report, we investigated ERβ protein levels as predictor of therapy response in a large patient set, including both ERα-positive and ERα-negative tumors all treated uniformly with 2 years of adjuvant tamoxifen. Furthermore, we sought to identify a gene expression signature for ERβ status and compare it with the ERα-associated expression signature.

## Materials and Methods

***Patients.*** We studied a cohort of 425 women with stage II breast cancer collected by the participating departments of the South Swedish Health Care Region after approval of the Lund University Hospital Ethics Committee. These women had been part of two randomized trials of adjuvant tamoxifen monotherapy (19, 20), and were selected for this study with the following criteria: 2-year tamoxifen treatment arms ($n$ = 995), complete follow-up data ($n$ = 992), receipt of fresh-frozen sample from primary tumor ($n$ = 783), and uniform method for hormone receptor content determination ($n$ = 537). From these, all the premenopausal women ($n$ = 79) and a random selection of the postmenopausal women ($n$ = 346) were included in the study. Losses due to nonevaluable ERβ immunostaining reduced the final cohort to 353 cases (Table 1). Patients were operated with either modified radical

**Table 1.** Patient and tumor characteristics for the 353 patients

| | All *n* (%) | ERβ | | | *P* | ERα | | *P** |
|---|---|---|---|---|---|---|---|---|
| | | – No (%) | + No (%) | ++ No (%) | | – No (%) | + No (%) | |
| All | 353 | 91 (26) | 192 (54) | 70 (20) | | 105 (30) | 248 (70) | |
| Menopausal status | | | | | | | | |
| Pre | 64 (18) | 18 (20) | 34 (18) | 12 (17) | | 28 (27) | 36 (15) | |
| Post | 289 (82) | 73 (80) | 158 (82) | 58 (83) | 0.65 | 77 (73) | 212 (85) | 0.007 |
| Tumor size, mm | | | | | | | | |
| >20 | 259 (73) | 67 (74) | 140 (73) | 52 (74) | | 83 (79) | 176 (71) | |
| ≤20 | 94 (27) | 24 (26) | 52 (27) | 18 (26) | 0.94 | 22 (21) | 72 (29) | 0.12 |
| Lymph node status | | | | | | | | |
| N≥4 | 83 (24) | 21 (23) | 48 (25) | 14 (20) | | 22 (21) | 61 (25) | |
| N1-3 | 152 (43) | 39 (43) | 83 (43) | 30 (43) | | 33 (31) | 119 (48) | |
| N0 | 118 (33) | 31 (34) | 61 (32) | 26 (37) | 0.67 | 50 (48) | 68 (27) | 0.006 |
| DNA ploidy status | | | | | | | | |
| Nondiploid | 164 (67) | 41 (65) | 44 (33) | 33 (67) | | 51 (75) | 113 (63) | |
| Diploid | 82 (33) | 22 (35) | 90 (67) | 16 (33) | 0.79 | 17 (25) | 65 (37) | 0.09 |
| Missing | 107 | | | | | | | |
| S-phase fraction, % | | | | | | | | |
| High (≥12) | 64 (28) | 14 (23) | 34 (27) | 16 (36) | | 29 (48) | 35 (20) | |
| Low (<12) | 168 (72) | 46 (77) | 94 (73) | 28 (64) | 0.16 | 31 (52) | 137 (80) | <0.001 |
| Missing | 121 | | | | | | | |
| ERBB2 amplification | | | | | | | | |
| Positive | 52 (15) | 11 (12) | 27 (14) | 14 (20) | | 30 (30) | 22 (9) | |
| Negative | 295 (85) | 80 (88) | 160 (86) | 55 (80) | 0.16 | 70 (70) | 225 (91) | <0.001 |
| Missing | 6 | | | | | | | |
| PgR status | | | | | | | | |
| Negative | 175 (50) | 49 (54) | 92 (48) | 34 (49) | | 95 (90) | 80 (32) | |
| Positive | 178 (50) | 42 (46) | 100 (52) | 36 (51) | 0.47 | 10 (10) | 168 (68) | <0.001 |
| ERα status | | | | | | | | |
| Negative | 105 (30) | 32 (35) | 55 (29) | 18 (26) | | | | |
| Positive | 248 (70) | 59 (65) | 137 (71) | 52 (74) | 0.18 | | | |

NOTE: For some variables, the number of patients with missing data is given; however, they are not included in the calculations of the percentages or the $\chi^2$ tests.

*$\chi^2$ test of association was used for 2 × 2 tables and $\chi^2$ test for linear trend for tables with more than two rows and/or columns.

mastectomy or breast conservation surgery in combination with axillary lymph node dissection. Radiotherapy was offered to all patients treated with breast conservation surgery and to patients with lymph node metastases treated with modified radical mastectomy. The median follow-up for patients free from distant recurrence was 5.7 years and for patients alive at the end of the study was 14.5 years.

***ERβ immunohistochemical analysis.*** Formalin-fixed, paraffin-embedded tumor blocks from the 425 cases were used to generate tissue microarrays, with three 0.6-mm-diameter cores taken per tumor. Antigens were retrieved by heat pretreatment [102°C for 30 min with 1 mmol/L Tris EDTA buffer (pH 9)] in the PT Module device (LabVision, Fremont, CA). Immunostainings were carried out with an Autostainer (LabVision) using a cocktail of two monoclonal anti-ERβ antibodies (clone 14C8 from GeneTex, San Antonio, TX, which is pan-specific for ERβ isoforms, and PPG5/10 from Serotec, Oxford, United Kingdom, which is specific for ERβ1), both diluted 1:2,000 from the manufacturers' stock. PowerVision+ (Immunovision Technologies, Daly City, CA) was used for immunodetection according to the manufacturer's instructions. The diaminobenzidine reaction product was enhanced with 0.5% copper sulfate for 5 min at room temperature, and the tissue was counterstained with hematoxylin. Losses due to lack of sufficient invasive tumor cells in the cores or detachment of tissue cores left 353 cases that could be evaluated. The scoring was done by one person (M.L.), blinded to all patient data, with the score ERβ-negative (ERβ−) defined as no to weak staining reaction (over background) in less than 20% of carcinoma cells, ERβ moderately positive (ERβ+) as weak staining intensity in 20% to 100%, and ERβ strongly positive (ERβ++) as intense staining intensity in 20% to 100%, using high-resolution digitized images and a virtual microscopy system (21).

***Determination of other tumor markers.*** Steroid receptor protein [ERα and progesterone receptor (PgR)] determinations using enzyme immunoassay (22), and flow cytometric analysis of S-phase fraction and DNA ploidy analysis (23), were done as part of the routine tumor evaluation. ERα and PgR status, S-phase fraction status, and DNA ploidy status were classified as previously described (22, 24). *ERBB2* amplification was measured using chromogenic *in situ* hybridization analysis (25).

***Statistical analysis.*** The $\chi^2$ test for association and $\chi^2$ test for trend, Mann-Whitney *U* test, and Kruskal-Wallis test were used to assess associations between tumor ERβ or ERα content and other variables. All factors were used as categorized variables in the statistical analysis except for age, which was also analyzed as a continuous variable. The Kaplan-Meier method was used to estimate distant disease-free survival and overall survival and the log-rank test was used to compare survival between two strata. The log-rank test for trend was used to compare survival in more than two strata. To test whether the effects on distant disease-free survival of ERα and ERβ changed significantly with time, Schoenfeld's test for time dependence was applied. The association of the level of ERβ with patient outcome, adjusted for other prognostic factors and for interaction between ERβ and ERα, was assessed in a multivariate analysis using Cox proportional hazards model. All tests were two-sided and *P* values <0.05 were considered significant. Statistical analyses were carried out using Stata 8.0 (Stata Corporation, College Station, TX).

***Microarrays.*** cDNA microarrays with 27,648 spots were produced in the SWEGENE Microarray Facility, Department of Oncology, Lund University. The gene set consisted of 24,301 sequence-verified IMAGE clones (Research Genetics, Huntsville, AL) and 1,296 internally generated clones, together representing ≈15,000 UniGene clusters (build 180) and ≈1,200 unclustered expressed sequence tags, and were PCR amplified using vector-specific primers essentially as previously described (26) with some modifications. Tissue processing for the 88 breast tumor samples, RNA labeling, and microarray hybridization protocols are described in detail in Supplementary Materials and Methods.

***Microarray data analysis.*** Microarray data are available through National Center for Biotechnology Information Gene Expression Omnibus,[6] accession no. GSE6577. The ERβ+ and ERβ++ tumors were grouped together and analyzed as a single ERβ-positive (ERβ+/++) entity. Thus, the distribution of the 88 tumors between the four ERα/ERβ groups was as follows: 10 ERα−/ERβ−, 36 ERα−/ERβ+/++, 8 ERα+/ERβ− and 34 ERα+/ERβ+/++.

Data analysis was done using BioArray Software Environment (27). Data preprocessing and filtering procedures, described in the Supplementary Materials and Methods, left 10,493 informative genes. The genes were ranked based on the signal-to-noise statistic (28), which calculates a correlation score between gene expression and the tumor annotation of interest. To evaluate the significance of the expression signatures between two annotation classes (e.g., ERβ status), 1,000 permutations were run whereby the samples were randomly given an annotation label and the *P* value for a score was calculated as the average number of reporters exceeding the score in the permutation test, divided by the total number of reporters in the gene list. The false discovery rate (FDR) was calculated by random permutations, controlled according to Benjamini et al. (29), and used as an indicator of the robustness of the gene expression profile. An FDR of 0% indicates no false positives, whereas an FDR of 100% indicates complete random signal. To test the dependence of FDR on number of samples for the ERβ analysis in the two ERα status subgroups, two ERβ-negative and two ERβ-positive samples were randomly removed from the ERα-negative cohort, making both these two groups equal in size to the corresponding groups in the ERα-positive cohort. For the reduced ERα-negative data set, genes were ranked and FDRs based on 1,000 permutations were calculated; the procedure was repeated 200 times with different random selections of removed samples. The Significance Analysis of Microarrays algorithm (30) implemented in TIGR Multiexperiment Viewer 3.1 (31) with 1,000 permutations and default settings was also used to generate comparative FDR plots. Hierarchical clustering and data visualization are described in the Supplementary Materials and Methods.

## Results

***Association between ERβ and clinicopathologic variables.*** We evaluated total ERβ protein levels by performing immunohistochemistry using a cocktail of two well-characterized monoclonal anti-ERβ antibodies, clones 14C8 and PPG5/10, previously shown to be the best-performing antibodies for this application (32). Seventy-four percent of the tumors stained positive for ERβ (ERβ+, 54%; ERβ++, 20%) and 26% were ERβ− (Table 1; Supplementary Fig. S1). Seventy percent of the tumors were ERα positive, and the association between the expression of ERβ and ERα was nonsignificant ($P = 0.18$). Although ERα positivity was associated with several clinical variables such as increasing age ($P = 0.003$), postmenopausal status ($P = 0.007$), a greater number of lymph nodes with metastases ($P = 0.006$), PgR positivity ($P < 0.001$), a low S-phase fraction ($P < 0.001$), and nonamplified *ERBB2* ($P < 0.001$), ERβ expression did not correlate significantly with any of the clinical variables tested (Table 1). In a subgroup analysis, increasing ERβ level was associated with high S-phase fraction within the ERα-negative group ($P = 0.03$) but not with any other markers (data not shown).

***Analysis of distant disease-free survival.*** Among all cases, ERβ expression was significantly associated with an increased distant disease-free survival ($P = 0.01$; Fig. 1*A*). When stratified by ERα status, ERβ level was significantly associated with better

[6] http://www.ncbi.nih.gov/geo/

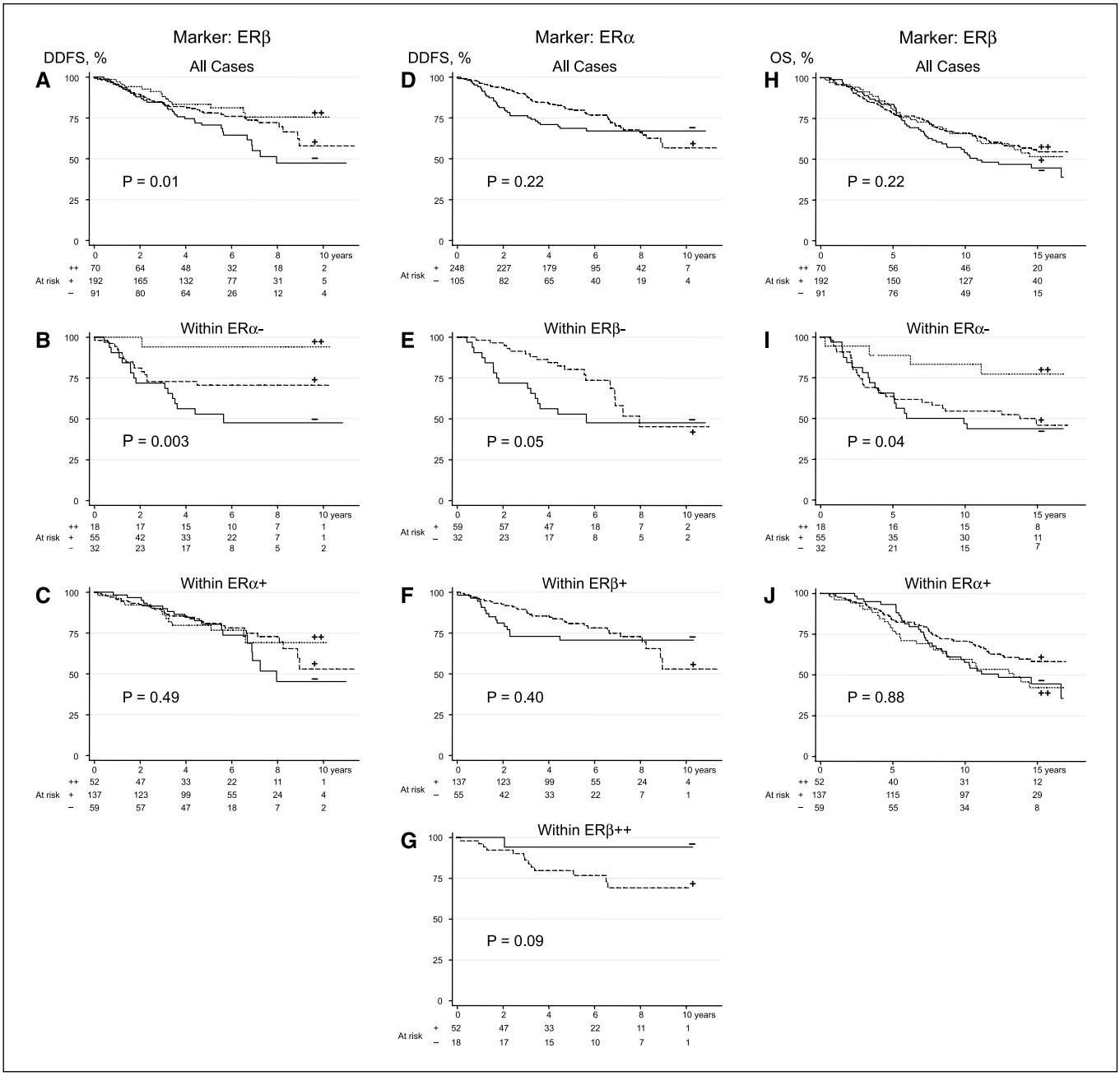


**Fig. 1.** Kaplan-Meier estimates of distant disease-free survival (*DDFS*; *A-G*) and overall survival (*OS*; *H-J*) for ERβ and ERα status in the whole patient group (*All*) and in the two ERα and three ERβ subgroups. Distant disease-free survival according to ERβ status for the whole patient group (*A*), for the ERα-negative group (*B*), and for the ERα-positive group (*C*). ERα effects on distant disease-free survival in all tumors (*D*), in the ERβ− group (*E*), in the ERβ+ group (*F*), and in the ERβ++ group (*G*). Estimates of overall survival for ERβ status in the whole patient group (*H*), in the ERα-negative subgroup (*I*), and in the ERα-positive group (*J*). *P* values were calculated using log-rank test for trend (*A-C* and *H-J*) or the log-rank test (*D-G*). Numbers below each graph, number of patients remaining at risk in each group at each time point.

distant disease-free survival ($P$ = 0.003) in the ERα-negative group (Fig. 1*B*). A multivariate Cox regression analysis of distant disease-free survival, including lymph node status, menopausal status, tumor size, *ERBB2* amplification, ERα status, two dummy variables for ERβ− (versus ++ and + versus ++), and two interaction terms for ERβ with ERα status (Table 2) showed a significantly worse distant disease-free survival for the ERβ− group compared with ERβ++ in the ERα-negative subgroup, with a hazard ratio (HR) of 14 [95% confidence interval (95% CI), 1.8-106; $P$ = 0.01]. Between the ERβ+ and ERβ++ groups in the ERα-negative subgroup, there was a similar but nonsignificant trend (HR, 6.1; 95% CI, 0.79-46; $P$ = 0.08). In contrast, as extrapolated from the model presented in Table 2, there was no effect of ERβ on distant disease-free survival in the ERα-positive group (Fig. 1*C*), neither between the ERβ and ERβ++ groups (HR, 1.2; 95% CI, 0.57-2.5; $P$ = 0.70) nor between the ERβ+ and ERβ++ groups (HR, 1.0; 95% CI, 0.05-2.0; $P$ = 1.00). The Cox regression model also showed that the effect of ERβ++ status (compared with ERβ−) was significantly different in the two ERα subgroups ($P$ = 0.02; Table 2).

Having identified an ERα-dependent effect for ERβ, we tested for the inverse dependency: The correlation of ERα status with distant disease-free survival in the whole patient group and in the different ERβ subgroups (Fig. 1*D-G*). Interestingly, using the Kaplan-Meier method and log-rank test, ERα was significantly associated with a better prognosis exclusively in the ERβ− subgroup ($P = 0.05$; Fig. 1*E*) but not in the whole cohort or any of the other ERβ subgroups (Figs. 1*D* and *F-G*, respectively). From the Cox regression model with interaction between ERα and ERβ (Table 2), the predictive value of ERα for distant disease-free survival in the different ERβ subgroups could be interpreted. In agreement with the results from the Kaplan-Meier analysis, we found that ERα positivity was a significant independent predictive marker for improved distant disease-free survival only within the ERβ− group (HR, 0.44; 95% CI, 0.21-0.89; $P = 0.02$), whereas no significant effect was seen in the other two subgroups (ERβ+: HR, 0.86; 95% CI, 0.45-1.7; $P = 0.70$; ERβ++: HR, 5.2; 95% CI, 0.67-40; $P = 0.12$).

The effect of ERβ on distant disease-free survival in the ERα-negative group did not change significantly with time ($P = 0.21$; Schoenfeld's test), whereas the effect of ERα expression on distant disease-free survival in the ERβ− group proved to be time dependent ($P = 0.005$) and diminished over a long follow-up time. Furthermore, as the distributions of ERα and PgR concentrations between the ERβ subgroups within the ERα-negative tumors were similar ($P = 0.68$ for ERα and $P = 0.41$ for PgR, Kruskal-Wallis test), we concluded that residual low levels of ERα or PgR protein in the three ERβ subgroups did not contribute to the prognostic effect of ERβ within the ERα-negative tumors.

In addition to ERβ++ status compared with ERβ− in the ERα-negative subgroup, four or more lymph nodes (compared with 0) and menopausal status had independent prognostic value after tamoxifen treatment in the multivariate analysis (Table 2). No other effects were significant; however, *ERBB2* amplification and ERβ+ status (compared with ERβ++) in the ERα-negative subgroup showed a tendency ($P < 0.10$) to carry prognostic information (Table 2). S-phase fraction, age as a continuous variable, PgR, and DNA ploidy status were not included in the multivariate analysis as they showed no significant association with distant disease-free survival in univariate analysis.

**Table 2.** Cox multivariate analysis of distant disease-free survival in 347 tamoxifen-treated patients with stage II breast cancer

| Variable | HR (95% CI) | *P* |
|---|---|---|
| Menopausal status | | |
| Pre versus post | 1.7 (1.1-2.7) | 0.02 |
| Tumor size, mm | | |
| >20 vs ≤20 | 1.3 (0.77-2.1) | 0.36 |
| Lymph node status | | |
| 1-3 vs 0 | 1.2 (0.70-2.2) | 0.45 |
| ≥4 vs 0 | 3.3 (1.9-5.8) | <0.001 |
| ERBB2 | | |
| Amplified vs nonamplified | 1.6 (0.93-2.9) | 0.09 |
| ERβ (in ERα−) | | |
| + vs ++ | 6.1 (0.79-46) | 0.08 |
| − vs ++ | 14 (1.8-106) | 0.01 |
| ERα (in ERβ++) | | |
| + vs − | 5.2 (0.67-40) | 0.12 |
| ERβ × ERα | | |
| (+ vs ++) × (+ vs −) | 0.17 (0.02-1.4) | 0.10 |
| (− vs ++) × (+ vs −) | 0.08 (0.01-0.73) | 0.02 |

NOTE: Data for all variables were available for 347 patients. Multivariate analysis was done using Cox proportional hazards model.

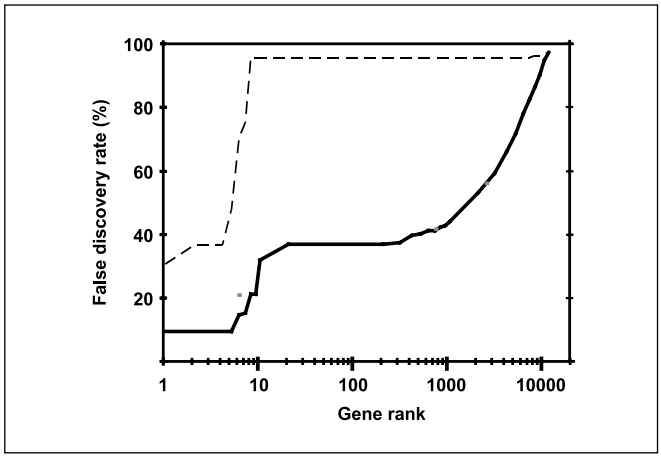


**Fig. 2.** FDRs as a function of gene rank from the signal-to-noise ratio analysis of ERβ status (− versus +/++) from gene expression data from ERα-negative tumors (*dashed line*) and ERα-positive breast tumors (*solid line*). One thousand random permutations were run to estimate the FDRs, which were controlled according to Benjamini et al. (29). *X* axis, gene ranks.

***Analysis of overall survival.*** Using the Kaplan-Meier method and log-rank test, a beneficial effect of ERβ positivity on overall survival was seen among ERα-negative tumors ($P = 0.04$; Fig. 1*I*) but not in the whole tumor group ($P = 0.22$; Fig. 1*H*) or among ERα positives ($P = 0.88$; Fig. 1*J*).

***Gene expression analysis.*** The ERβ gene expression signature in the 88 patients, representative of the original cohort (data not shown), had an FDR of 49% per top 100 discriminator genes ranked by the signal-to-noise ratio analysis score (28), and 50% per top 500 genes, indicating that ERβ was associated with a unique, albeit weak, expression profile. The ERα status–associated gene expression signature from this data set had no false-positive genes in the top 1,000 genes. When stratified by ERα, ERβ was associated with a detectable expression profile within the ERα-negative group (FDR of 40% per top 100 discriminator genes, and 43% per top 500; Fig. 2); however, there was no signal in the ERα-positive group (FDR reached >90% by the top 10 genes; Fig. 2). A similar difference in gene expression signature strength was seen when using the Significance Analysis of Microarrays algorithm (30) to generate FDR curves (data not shown).

To investigate whether the absence of an ERβ-associated expression profile within the ERα-positive tumors was due to the ERβ signal being masked by the much stronger ERα-associated profile, we used the top 50 and top 100 ERβ genes generated from the ERα-negative subgroup and tested whether they could separate the tumors according to ERβ status within the ERα-positive subgroup using hierarchical clustering analysis. The resulting tumor dendrogram showed a scattered distribution not correlating to ERβ levels (data not shown), whereas using these genes within the ERα-negative group displayed ERβ-associated tumor clusters (result for the top

50 ERβ genes are shown in Fig. 3). The successful ERβ discrimination in the ERα negatives was not due to a difference in distribution of residual ERα concentration in the ERβ groups, because the distribution of ERα values in the three ERβ subgroups within the ERα-negative tumors were similar ($P$ = 0.75) as were the distribution of PgR concentrations ($P$ = 0.58). Moreover, the ERβ signal in ERα-negative tumors was still present even when top 400 ERα signature genes were removed before identifying the ERβ signature (FDR of 42% per top 100 genes).

Furthermore, to test whether the lack of signal for ERβ in the ERα-positive group was due to the lower number of samples in this group compared with the ERα-negative group (42 ERα positives versus 46 ERα negatives), samples were randomly removed from the ERα-negative cohort, to make the two ERβ groups equal in size to the corresponding groups in the ERα-positive cohort. In none out of the 200 randomly reduced ERα-negative cohorts was the FDR, for top 10 up to top 1,000 genes or more, equal to or larger than that of the ERα-positive cohort, indicating that the difference in ERβ signal strength in the two ERα subgroups is robust.

We compared the extent of overlap of genes comprising the ERβ and ERα expression signatures. The two expression profiles generated from all 88 cases were substantially different: No genes were overlapping among the respective top 100 ranked genes; moreover, among the top 1,000 ranked genes, the overlap was only 6%. The ERβ signature generated from within the ERα-negative tumors, which could be considered a "purer" ERβ profile, showed a similar degree of nonoverlap with the ERα gene list from all cases: 1% in top 100 and 7% in top 1,000.

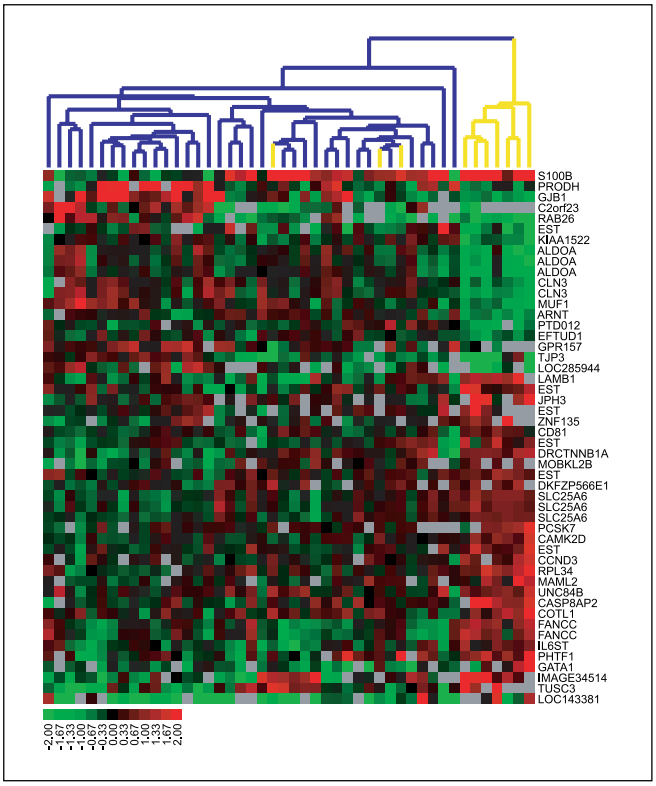


**Fig. 3.** Hierarchical clustering analysis of ERα-negative tumors using the top 50 signal-to-noise ratio – ranked genes in the ERβ expression profile within ERα-negative tumors. Blue, ERβ+/++ tumors; yellow, ERβ− tumors. Hierarchical clustering presents the clustered samples in columns and the clustered genes in rows. Pseudocolored representation of gene expression ratios: red, high expression; green, low expression, relative to the median expression for each gene. Gray, missing data. Colorbar scale is given in relative $\log_2$ (ratio).

## Discussion

To our knowledge, this is the first study to show an ERα status – specific survival benefit of ERβ expression in tamoxifen-treated breast cancer patients. In light of a previous study showing no prognostic value of ERβ in untreated patients (15), our results suggest that ERβ is a predictive marker for response to tamoxifen in ERα-negative patients.

Although ERα positivity is a well-established predictor of response to tamoxifen and ERα-negative patients are considered nonresponders, it has been noted that 5% to 10% of ERα-negative tumors do benefit from adjuvant tamoxifen (5–7). Several theories have been put forward to explain these cases: Failures or inconsistencies in the performance or evaluation of ERα measurements result in miscategorization of what are actually ERα-positive tumors, or that tamoxifen acts through mechanisms independent of ERα. Our results support the latter and suggest that an ERα-independent alternative mechanism of action of tamoxifen is via ERβ (of note, 17% of ERα-negative tumors were strongly positive for ERβ). Furthermore, there was a similar distribution of ERα and PgR protein values in the three ERβ subgroups within the ERα-negative tumors, suggesting that misclassification of ERα-positive tumors did not confound our results.

Although ERα did not have significant predictive value for tamoxifen response in the entire patient group over the complete long follow-up period, ERα was predictive within the ERβ-negative group ($P$ = 0.05, Fig. 1*E*; HR, 0.44; 95% CI, 0.21-0.89; $P$ = 0.02, extrapolated from Table 2). Moreover, it appeared that ERα negativity may confer a better outcome within the ERβ strongly positive group ($P$ = 0.09, Fig. 1*G*; HR, 5.2; 95% CI, 0.67-40; $P$ = 0.12, Table 2). These findings raise an intriguing hypothesis that the best tamoxifen response is achieved when a tumor is expressing either ERα or ERβ, but not both. A biological explanation for this could be that the two receptors modulate the function of each other, so that when coexpressed the effect of tamoxifen becomes less pronounced or, alternatively, is growth promoting. This postulation is supported by the following observations: The two receptors can be coexpressed within individual breast carcinoma cells (33); they can form heterodimers (11–13); ERβ can function as an inhibitor of ERα activity under certain conditions (34); and when coexpressed with ERα, ERβ has been suggested to be associated with tumor characteristics indicative of a poorer prognosis (35).

Despite the similar utility of ERβ or ERα as markers for benefit from tamoxifen therapy in this study group, the two receptors show many dissimilarities. Within all cases, ERα expression correlates strongly with the majority of other standard clinicopathologic markers, whereas ERβ expression did not, corroborating the results of recent studies (15, 16). ERβ did, however, correlate with high S-phase fraction within the ERα-negative group, which is consistent with the literature showing an association between ERβ and the proliferation marker Ki67 (16, 36). An ERβ-dependent high proliferation

rate of these tumor cells may render them more sensitive to tamoxifen therapy (37).

We have previously shown that ERα status in breast cancer is associated with a robust gene expression signature (38), and the most readily apparent subdivision of breast cancers based on gene expression data is according to ERα status (39, 40). The present study is the first to identify an ERβ-associated gene expression profile in human tumor biopsies. The lack of ERβ-associated signal in ERα-positive tumors suggests that ERβ seems to be less influential on gene expression, and hence tumor biology, in cancers also expressing ERα. However, this does not necessarily rule out that ERβ under certain conditions can have some modulating effect on ERα. Our results indicate that the ERβ gene expression signature was markedly different from the ERα gene expression signature as the genes included in the two profiles showed minimal overlap. However, due to the limited sample size in this study, these findings call for further validation in data sets including larger number of tumors. Together, our data suggests that ERβ, in the absence of ERα, is not simply a surrogate marker for ERα, but rather ERβ may affect growth and proliferation of breast cancer cells through modulation of different downstream target genes.

As far as we are aware, this is the largest survival study of ERβ in breast cancer specimens to date. It will be important to confirm our results in other cohorts containing large numbers of ERα-negative tumors treated uniformly with tamoxifen. It remains to be tested whether a predictive effect of ERβ exists for patients treated with other selective ER modulators or other types of endocrine therapies, such as aromatase inhibitors. Moreover, analysis of large series of untreated patients is necessary to confirm that ERβ does not have prognostic value (15) and is specifically a predictive marker for therapeutic response to tamoxifen. In this regard and suggesting that the ERβ benefit may indeed be related to antiestrogen therapy, a recent study of ERα-negative breast tumor patients that received varied therapies (<35% received unspecified hormonal therapies alone or in combination) found no recurrence-free survival benefit for ERβ expression (16).

In the present study, we categorized ERβ status into three groups with the goal of understanding the influence of different levels of ERβ expression on survival for tamoxifen-treated patients. Further refinement will be needed to identify optimal and standardized methods for determining ERβ content, scoring, and thresholds, and whether different ERβ variants modulate response dissimilarly.

From our data, it can be estimated that in the United States alone, ~10,000 patients per year will be diagnosed with ERα-negative/ERβ-positive breast carcinoma. Given the relatively low toxicity and cost of tamoxifen, our results have striking clinical implications that motivate further studies to explore the efficacy of tamoxifen to treat ERα-negative/ERβ-positive breast tumors.

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