

## Estrogen Receptor $\beta$ Expression Is Associated with Tamoxifen Response in ER $\alpha$ -Negative Breast Carcinoma

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

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

**Conclusion:** Expression of ER $\beta$  is an independent marker for favorable prognosis after adjuvant tamoxifen treatment in ER $\alpha$ -negative breast cancer patients and involves a gene expression program distinct from ER $\alpha$ . These results may be highly clinically significant, because in the United States alone, ~10,000 women are diagnosed annually with ER $\alpha$ -negative/ER $\beta$ -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)  $\alpha$  and  $\beta$  (1–3), which function as transcription factors and modulate the expression of target genes in response to estrogens. The biology of ER $\alpha$  has been studied for decades and evaluation of tumor ER $\alpha$  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 ER $\alpha$  (4). Patients with tumors lacking ER $\alpha$  in general do not benefit from tamoxifen therapy, although a fraction of ER $\alpha$ -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 ER $\alpha$ -negative tumors in the adjuvant or metastatic setting.

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

but differ considerably in the NH<sub>2</sub>-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, ER $\alpha$  and ER $\beta$  can exist as a heterodimer (11–13), suggesting a possible role for ER $\beta$  as a modulator of ER $\alpha$  activity.

Recently, several studies have measured ER $\beta$  in breast cancer specimens and sought to clarify the relationship between ER $\beta$  and other clinicopathologic features and its role in response to endocrine treatment; however, some of the results have been conflicting and most focused on ER $\beta$  as a resistance marker in ER $\alpha$ -positive tumors (refs. 14–16, and those reviewed in ref. 17). ER $\beta$  has been shown to bind tamoxifen (18), and it has been suggested that low levels of ER $\beta$  associates with tamoxifen resistance (14). Conversely, Hopp et al. (15) showed that expression of ER $\beta$  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 ER $\beta$  as a predictive marker for tamoxifen sensitivity but not as a prognostic marker. Importantly, the patients studied by Hopp et al. (15) were predominantly ER $\alpha$ -positive and the numbers were limited;

thus, they were unable to perform an analysis stratified by ER $\alpha$  status. To our knowledge, no study has investigated the role of ER $\beta$  as a predictive marker for tamoxifen response for patients with ER $\alpha$ -negative tumors.

In the present report, we investigated ER $\beta$  protein levels as predictor of therapy response in a large patient set, including both ER $\alpha$ -positive and ER $\alpha$ -negative tumors all treated uniformly with 2 years of adjuvant tamoxifen. Furthermore, we sought to identify a gene expression signature for ER $\beta$  status and compare it with the ER $\alpha$ -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 ER $\beta$  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 (%)	ER $\beta$			P	ER $\alpha$		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)	
$\leq$ 20	94 (27)	24 (26)	52 (27)	18 (26)	0.94	22 (21)	72 (29)	0.12
Lymph node status								
N $\geq$ 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 ( $\geq$ 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
ER $\alpha$ 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  $\times$  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.

**ER $\beta$  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-ER $\beta$  antibodies (clone 14C8 from GeneTex, San Antonio, TX, which is pan-specific for ER $\beta$  isoforms, and PPG5/10 from Serotec, Oxford, United Kingdom, which is specific for ER $\beta$ 1), 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 ER $\beta$ -negative (ER $\beta$ -) defined as no to weak staining reaction (over background) in less than 20% of carcinoma cells, ER $\beta$  moderately positive (ER $\beta$ +) as weak staining intensity in 20% to 100%, and ER $\beta$  strongly positive (ER $\beta$ ++) 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 [ER $\alpha$  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. ER $\alpha$  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 ER $\beta$  or ER $\alpha$  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 ER $\alpha$  and ER $\beta$  changed significantly with time, Schoenfeld's test for time dependence was applied. The association of the level of ER $\beta$  with patient outcome, adjusted for other prognostic factors and for interaction between ER $\beta$  and ER $\alpha$ , 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  $\approx$ 15,000 UniGene clusters (build 180) and  $\approx$ 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,<sup>6</sup> accession no. GSE6577. The ER $\beta$ + and ER $\beta$ ++ tumors were grouped together and analyzed as a single ER $\beta$ -positive (ER $\beta$ + /++) entity. Thus, the distribution of the 88 tumors between the four ER $\alpha$ /ER $\beta$  groups was as follows: 10 ER $\alpha$ -/ER $\beta$ -, 36 ER $\alpha$ -/ER $\beta$ + /++, 8 ER $\alpha$ + /ER $\beta$ - and 34 ER $\alpha$ + /ER $\beta$ + /++.

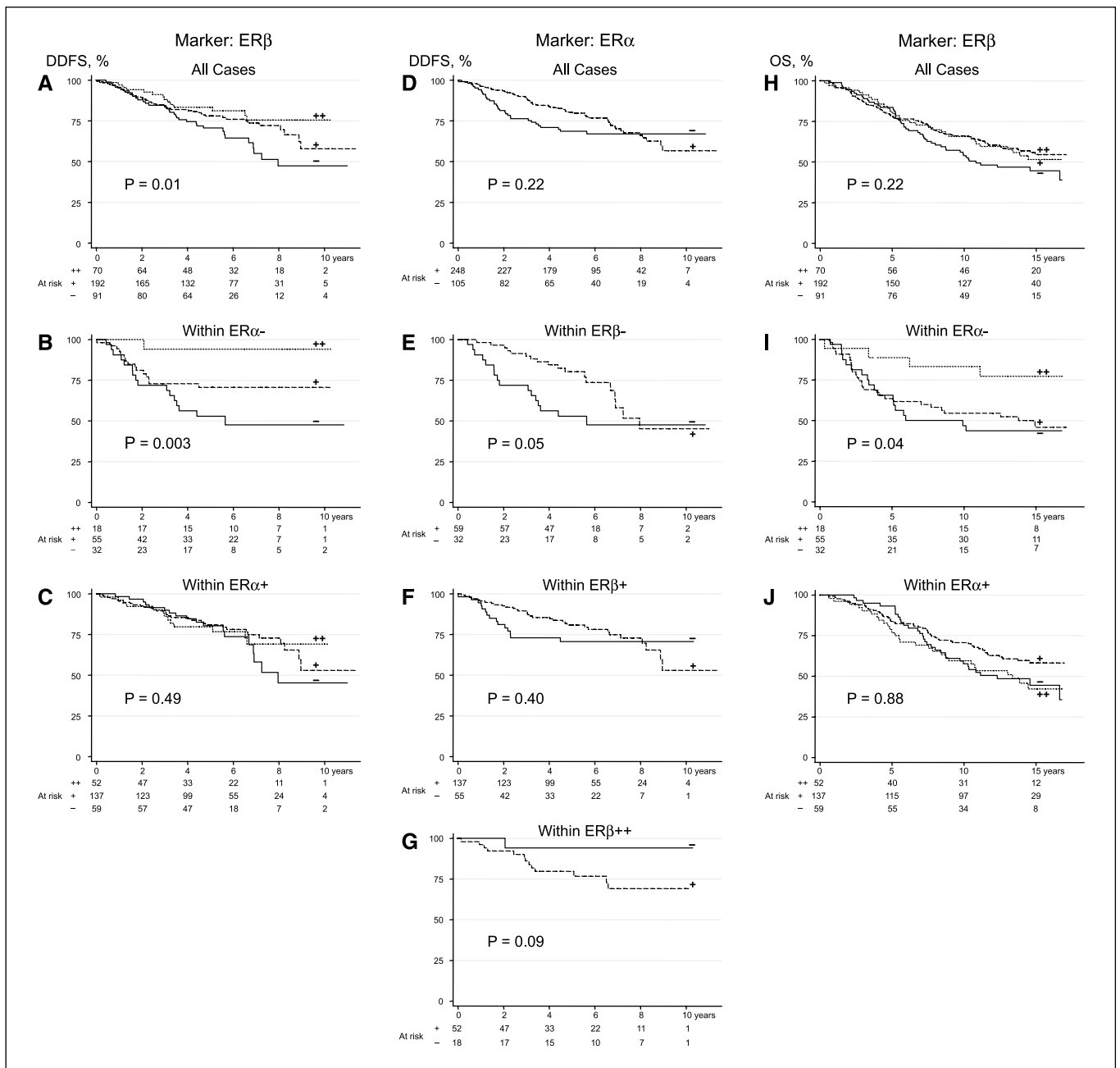
Data analysis was done using BioArray Software Environment (27). Data preprocessing and filtering procedures, described in the Supplementary Materials and Methods, left 10,493 informative genes. The genes were ranked based on the signal-to-noise statistic (28), which calculates a correlation score between gene expression and the tumor annotation of interest. To evaluate the significance of the expression signatures between two annotation classes (e.g., ER $\beta$  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 ER $\beta$  analysis in the two ER $\alpha$  status subgroups, two ER $\beta$ -negative and two ER $\beta$ -positive samples were randomly removed from the ER $\alpha$ -negative cohort, making both these two groups equal in size to the corresponding groups in the ER $\alpha$ -positive cohort. For the reduced ER $\alpha$ -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 Multi-experiment 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 ER $\beta$  and clinicopathologic variables.** We evaluated total ER $\beta$  protein levels by performing immunohistochemistry using a cocktail of two well-characterized monoclonal anti-ER $\beta$  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 ER $\beta$  (ER $\beta$ +, 54%; ER $\beta$ ++, 20%) and 26% were ER $\beta$ - (Table 1; Supplementary Fig. S1). Seventy percent of the tumors were ER $\alpha$  positive, and the association between the expression of ER $\beta$  and ER $\alpha$  was nonsignificant (*P* = 0.18). Although ER $\alpha$  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), ER $\beta$  expression did not correlate significantly with any of the clinical variables tested (Table 1). In a subgroup analysis, increasing ER $\beta$  level was associated with high S-phase fraction within the ER $\alpha$ -negative group (*P* = 0.03) but not with any other markers (data not shown).

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

<sup>6</sup> <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.

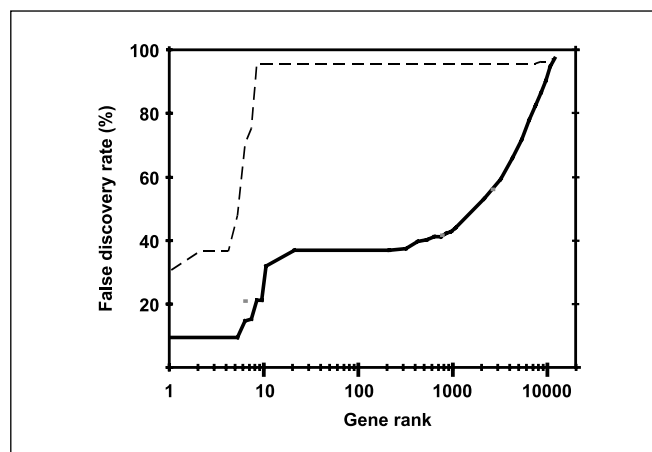
distant disease-free survival ( $P = 0.003$ ) in the ERα-negative group (Fig. 1B). A multivariate Cox regression analysis of distant disease-free survival, including lymph node status, menopausal status, tumor size, *ERBB2* amplification, ERα status, two dummy variables for ERβ- (versus ++ and + versus ++), 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. 1C), 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 ER $\alpha$ -dependent effect for ER $\beta$ , we tested for the inverse dependency: The correlation of ER $\alpha$  status with distant disease-free survival in the whole patient group and in the different ER $\beta$  subgroups (Fig. 1D-G). Interestingly, using the Kaplan-Meier method and log-rank test, ER $\alpha$  was significantly associated with a better prognosis exclusively in the ER $\beta$ - subgroup ( $P = 0.05$ ; Fig. 1E) but not in the whole cohort or any of the other ER $\beta$  subgroups (Figs. 1D and F-G, respectively). From the Cox regression model with interaction between ER $\alpha$  and ER $\beta$  (Table 2), the predictive value of ER $\alpha$  for distant disease-free survival in the different ER $\beta$  subgroups could be interpreted. In agreement with the results from the Kaplan-Meier analysis, we found that ER $\alpha$  positivity was a significant independent predictive marker for improved distant disease-free survival only within the ER $\beta$ - group (HR, 0.44; 95% CI, 0.21-0.89;  $P = 0.02$ ), whereas no significant effect was seen in the other two subgroups (ER $\beta$ +: HR, 0.86; 95% CI, 0.45-1.7;  $P = 0.70$ ; ER $\beta$ ++: HR, 5.2; 95% CI, 0.67-40;  $P = 0.12$ ).

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

In addition to ER $\beta$ ++ status compared with ER $\beta$ - in the ER $\alpha$ -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



**Fig. 2.** FDRs as a function of gene rank from the signal-to-noise ratio analysis of ER $\beta$  status (- versus +/++) from gene expression data from ER $\alpha$ -negative tumors (dashed line) and ER $\alpha$ -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.

amplification and ER $\beta$ + status (compared with ER $\beta$ ++) in the ER $\alpha$ -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.

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

**Gene expression analysis.** The ER $\beta$  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 ER $\beta$  was associated with a unique, albeit weak, expression profile. The ER $\alpha$  status-associated gene expression signature from this data set had no false-positive genes in the top 1,000 genes. When stratified by ER $\alpha$ , ER $\beta$  was associated with a detectable expression profile within the ER $\alpha$ -negative group (FDR of 40% per top 100 discriminator genes, and 43% per top 500; Fig. 2); however, there was no signal in the ER $\alpha$ -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 ER $\beta$ -associated expression profile within the ER $\alpha$ -positive tumors was due to the ER $\beta$  signal being masked by the much stronger ER $\alpha$ -associated profile, we used the top 50 and top 100 ER $\beta$  genes generated from the ER $\alpha$ -negative subgroup and tested whether they could separate the tumors according to ER $\beta$  status within the ER $\alpha$ -positive subgroup using hierarchical clustering analysis. The resulting tumor dendrogram showed a scattered distribution not correlating to ER $\beta$  levels (data not shown), whereas using these genes within the ER $\alpha$ -negative group displayed ER $\beta$ -associated tumor clusters (result for the top

**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 $\leq$ 20	1.3 (0.77-2.1)	0.36
Lymph node status		
1-3 vs 0	1.2 (0.70-2.2)	0.45
$\geq$ 4 vs 0	3.3 (1.9-5.8)	<0.001
ERBB2		
Amplified vs nonamplified	1.6 (0.93-2.9)	0.09
ER $\beta$ (in ER $\alpha$ -)		
+ vs ++	6.1 (0.79-46)	0.08
- vs ++	14 (1.8-106)	0.01
ER $\alpha$ (in ER $\beta$ ++)		
+ vs -	5.2 (0.67-40)	0.12
ER $\beta$ $\times$ ER $\alpha$		
(+ vs ++) $\times$ (+ vs -)	0.17 (0.02-1.4)	0.10
(- vs ++) $\times$ (+ 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.

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

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

We compared the extent of overlap of genes comprising the ER $\beta$  and ER $\alpha$  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 ER $\beta$  signature generated from within the ER $\alpha$ -negative tumors, which could be considered a "purer" ER $\beta$  profile, showed a similar degree of nonoverlap with the ER $\alpha$  gene list from all cases: 1% in top 100 and 7% in top 1,000.

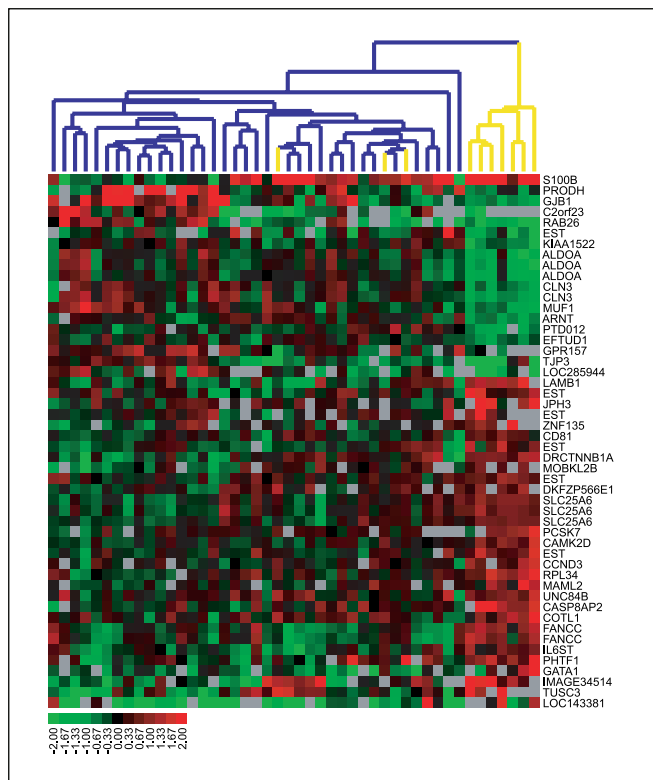
## Discussion

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

Although ER $\alpha$  positivity is a well-established predictor of response to tamoxifen and ER $\alpha$ -negative patients are considered nonresponders, it has been noted that 5% to 10% of ER $\alpha$ -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 ER $\alpha$  measurements result in miscategorization of what are actually ER $\alpha$ -positive tumors, or that tamoxifen acts through mechanisms independent of ER $\alpha$ . Our results support the latter and suggest that an ER $\alpha$ -independent alternative mechanism of action of tamoxifen is via ER $\beta$  (of note, 17% of ER $\alpha$ -negative tumors were strongly positive for ER $\beta$ ). Furthermore, there was a similar distribution of ER $\alpha$  and PgR protein values in the three ER $\beta$  subgroups within the ER $\alpha$ -negative tumors, suggesting that misclassification of ER $\alpha$ -positive tumors did not confound our results.

Although ER $\alpha$  did not have significant predictive value for tamoxifen response in the entire patient group over the complete long follow-up period, ER $\alpha$  was predictive within the ER $\beta$ -negative group ( $P = 0.05$ , Fig. 1E; HR, 0.44; 95% CI, 0.21–0.89;  $P = 0.02$ , extrapolated from Table 2). Moreover, it appeared that ER $\alpha$  negativity may confer a better outcome within the ER $\beta$  strongly positive group ( $P = 0.09$ , Fig. 1G; 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 ER $\alpha$  or ER $\beta$ , 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); ER $\beta$  can function as an inhibitor of ER $\alpha$  activity under certain conditions (34); and when coexpressed with ER $\alpha$ , ER $\beta$  has been suggested to be associated with tumor characteristics indicative of a poorer prognosis (35).

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



**Fig. 3.** Hierarchical clustering analysis of ER $\alpha$ -negative tumors using the top 50 signal-to-noise ratio – ranked genes in the ER $\beta$  expression profile within ER $\alpha$ -negative tumors. Blue, ER $\beta$ + / ++ tumors; yellow, ER $\beta$  – 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).

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

We have previously shown that ER $\alpha$  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 ER $\alpha$  status (39, 40). The present study is the first to identify an ER $\beta$ -associated gene expression profile in human tumor biopsies. The lack of ER $\beta$ -associated signal in ER $\alpha$ -positive tumors suggests that ER $\beta$  seems to be less influential on gene expression, and hence tumor biology, in cancers also expressing ER $\alpha$ . However, this does not necessarily rule out that ER $\beta$  under certain conditions can have some modulating effect on ER $\alpha$ . Our results indicate that the ER $\beta$  gene expression signature was markedly different from the ER $\alpha$  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 ER $\beta$ , in the absence of ER $\alpha$ , is not simply a surrogate marker for ER $\alpha$ , but rather ER $\beta$  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 ER $\beta$  in breast cancer specimens to date. It will be important to confirm our results in other cohorts containing large numbers of ER $\alpha$ -negative tumors treated uniformly with tamoxifen. It remains to be tested whether a predictive effect of ER $\beta$  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 ER $\beta$  does not have prognostic value (15) and is specifically a predictive marker for therapeutic response to tamoxifen. In this regard and suggesting that the ER $\beta$  benefit may indeed be related to antiestrogen therapy, a recent study of ER $\alpha$ -negative breast tumor patients that received varied therapies (<35% received unspecified hormonal therapies alone or in combination) found no recurrence-free survival benefit for ER $\beta$  expression (16).

In the present study, we categorized ER $\beta$  status into three groups with the goal of understanding the influence of different levels of ER $\beta$  expression on survival for tamoxifen-treated patients. Further refinement will be needed to identify optimal and standardized methods for determining ER $\beta$  content, scoring, and thresholds, and whether different ER $\beta$  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 ER $\alpha$ -negative/ER $\beta$ -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 ER $\alpha$ -negative/ER $\beta$ -positive breast tumors.

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