

## Expression of ER- $\alpha$ 36, a Novel Variant of Estrogen Receptor $\alpha$ , and Resistance to Tamoxifen Treatment in Breast Cancer

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### ABSTRACT

#### Purpose

Recently, a 36-kDa variant of estrogen receptor  $\alpha$  (ER- $\alpha$ 66), ER- $\alpha$ 36, has been identified and cloned. ER- $\alpha$ 36 predominantly localizes on the plasma membrane and in the cytoplasm and mediates a membrane-initiated “nongenomic” signaling pathway. Here, we investigate the association between ER- $\alpha$ 36 expression and tamoxifen resistance in patients with breast cancer.

#### Patients and Methods

ER- $\alpha$ 36 protein expression in tumors from 896 women (two independent cohorts, 1 and 2) with operable primary breast cancer was assessed using an immunohistochemistry assay.

#### Results

In the first cohort of 710 consecutive patients, overexpression of ER- $\alpha$ 36 was associated with poorer disease-free survival (DFS) and disease-specific survival (DSS) in patients with ER- $\alpha$ 66-positive tumors who received tamoxifen treatment (chemotherapy plus tamoxifen or tamoxifen alone,  $n = 307$ ). In contrast, ER- $\alpha$ 36 was not associated with survival in patients with ER- $\alpha$ 66-positive tumors who did not receive tamoxifen (chemotherapy alone,  $n = 129$ ) and in patients with ER- $\alpha$ 66-negative tumors whether they received tamoxifen ( $n = 73$ ) or not ( $n = 149$ ). In the second cohort of 186 patients who only received tamoxifen as adjuvant therapy, overexpression of ER- $\alpha$ 36 was significantly associated with poorer DFS and DSS in 156 ER- $\alpha$ 66-positive patients from this cohort, and ER- $\alpha$ 36 remained an independent unfavorable factor for both DFS and DSS in these 156 patients by a multivariate analysis (DFS: hazard ratio [HR] = 5.47; 95% CI, 1.81 to 16.51;  $P = .003$ ; DSS: HR = 13.97; 95% CI, 1.58 to 123.53;  $P = .018$ ).

#### Conclusion

Women with ER- $\alpha$ 66-positive tumors that also express high levels of ER- $\alpha$ 36 are less likely to benefit from tamoxifen treatment.

*J Clin Oncol* 27. © 2009 by American Society of Clinical Oncology

### INTRODUCTION

Estrogen receptor  $\alpha$  (also known as ER- $\alpha$ 66) is one of the most important determinants of susceptibility to endocrine therapy in breast cancer. In general, patients with ER- $\alpha$ 66-positive breast cancer respond favorably to tamoxifen, and tamoxifen has proved to be effective in the treatment of all stages of ER- $\alpha$ 66-positive breast cancers.<sup>1,2</sup> However, approximately 40% of ER- $\alpha$ 66-positive tumors fail to respond to tamoxifen therapy at diagnosis.<sup>3</sup> The exact mechanisms underlying this *de novo* tamoxifen resistance have not been established. A number of hypotheses have been proposed to explain tamoxifen resistance, including altered pharmacology of tamoxifen, modification of the ER- $\alpha$ 66 structure and function, cross-talk between the ER- $\alpha$ 66 pathway and growth factor signaling

pathways, and altered expression of coactivators and/or corepressors.<sup>3-7</sup>

Recently, we have identified and cloned a novel variant of ER- $\alpha$  that has a molecular weight of 36-kDa, and thus we have termed it ER- $\alpha$ 36.<sup>8</sup> The transcript of ER- $\alpha$ 36 is initiated from a previously unidentified promoter in the first intron of the ER- $\alpha$ 66 gene. ER- $\alpha$ 36 differs from ER- $\alpha$ 66 by lacking both transcriptional activation domains (AF-1 and AF-2) but retaining the DNA-binding domain and partial dimerization and ligand-binding domains.<sup>8</sup> It possesses a unique 27-amino acid domain that replaces the last 138 amino acids encoded by the exon 7 and 8 of the ER- $\alpha$ 66 gene. ER- $\alpha$ 36 is predominantly expressed on the plasma membrane and in the cytoplasm, where it mediates membrane-initiated effects of estrogen signaling, such as activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/

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Submitted March 16, 2008; accepted January 15, 2009; published online ahead of print at [www.jco.org](http://www.jco.org) on June 1, 2009.

Supported by the Program for New Century Excellent Talents in University (Grant No. 985-2-067-113 to Dr. Yuntao Xie) and a grant from the National Natural Science Foundation of China (Grant No. 30672419). This study was also supported by National Institutes of Health Grant No. DK070016 (Z.Y.W.), the Susan G. Komen Breast Cancer Foundation Grant No. BCTR81906 (Z.Y.W.), and the Nebraska Tobacco Settlement Biomedical Research Program Award (Grant No. LB-595; Z.Y.W.).

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/09/2799-1/\$20.00

DOI: 10.1200/JCO.2008.17.2254

ERK) signaling pathway, and stimulates cell growth.<sup>9</sup> In ER- $\alpha$ 36–overexpressing cells, tamoxifen treatment fails to block the ER- $\alpha$ 36–mediated activation of the MAPK/ERK pathway; instead, it stimulates cell growth.<sup>9</sup> These findings raise an intriguing possibility that ER- $\alpha$ 36 may be involved in de novo tamoxifen resistance in breast cancer. To test this hypothesis, we examined ER- $\alpha$ 36 expression in tumor specimens from 896 patients with primary breast cancer (including two independent cohorts, 1 and 2). We aimed to investigate whether ER- $\alpha$ 36 expression is associated with the clinical outcome of patients with breast cancer treated with tamoxifen.

## PATIENTS AND METHODS

### Study Population

In the first cohort, a total of 907 consecutive patients with operable primary breast cancer were treated at Peking University School of Oncology from December 1994 to December 1999. Paraffin blocks of tumor tissue were available for 769 patients. Among these, we failed to assess ER- $\alpha$ 36 staining in 59 tumor specimens as a result of tissue loss during slide preparation. Therefore, specimens from 710 patients with operable primary breast cancer in the first cohort were analyzed in this study. To further verify the results from the first cohort, an independent second cohort of patients was included in this study. Approximately 3,260 consecutive patients with operable primary breast cancer were treated at Peking University School of Oncology (including the Breast Center and Surgical Department Units I to IV) from January 2000 to December 2006. Among them, 186 patients with available paraffin blocks received only tamoxifen as their adjuvant therapy after surgery. Tumor size was defined as the maximum tumor diameter measured on the tumor specimens at the time of operation. Patients received radical mastectomy, modified radical mastectomy, or breast-conserving surgery; the axillary lymph nodes were routinely dissected at least at levels I and II, and lymph node metastasis was determined based on histologic examination. The majority of patients in the first cohort received adjuvant chemotherapy alone (cyclophosphamide, methotrexate, and fluorouracil or anthracycline-based regimen) or sequential chemotherapy and endocrine therapy; patients from the first cohort who had ER- $\alpha$ 66– and/or progesterone receptor (PgR) –positive tumors usually received adjuvant tamoxifen treatment (20 mg/d) for 5 years after chemotherapy or surgery. Patients from the second cohort received adjuvant tamoxifen treatment (20 mg/d) only after surgery. The follow-up data were available for all patients, with a median follow-up of 7.9 years (range, 0.4 to 11.1 years) for the first cohort; the median follow-up is 4.8 years (range, 0.3 to 8.1) for the second cohort. This study was approved by the Research and Ethical Committee of Peking University School of Oncology.

### Hormone Receptors

ER- $\alpha$ 66 and PgR expression in 609 tumors from cohort 1 were measured by using a dextran-coated charcoal assay as previously described.<sup>10</sup> [<sup>3</sup>H]-estradiol (Amersham, Buckinghamshire, United Kingdom) and [<sup>3</sup>H]-R5020 (Dupont New England Nuclear, Boston, MA) were used as the labeled ligands for ER- $\alpha$ 66 and PgR analysis, respectively. Specimens containing at least 10 fmol/mg of protein were considered ER- $\alpha$ 66 or PgR positive. ER- $\alpha$ 66 and PgR expression in the remaining 101 cases from cohort 1 and in the 186 tumors from cohort 2 were assessed by immunohistochemical assay using an ER- $\alpha$ 66–specific antibody raised against the N-terminal of ER- $\alpha$ 66 epitope (clone: 1D5, Zymed, South Francisco, CA; dilution 1:100) and a PgR specific antibody (clone: 1A6, Zymed; dilution 1:100), respectively. ER- $\alpha$ 66 or PgR immunostaining was considered positive when  $\geq 10\%$  of tumor cells showed positive nuclear staining.

### Immunohistochemistry Assay

Immunohistochemical staining was performed on 4- $\mu$ m thick tumor sections via a two-step assay. Briefly, tissue slides were deparaffinized with xylene and rehydrated through a graded alcohol series. The endogenous peroxidase activity was blocked by incubation in a 3% hydrogen peroxide/meth-

anol buffer for 10 minutes. Antigen retrieval was carried out by immersing the slides in EDTA buffer (pH 8.0) and boiling in a waterbath at 95°C for 25 minutes. The slides were rinsed in phosphate-buffered saline and incubated with normal goat serum to block nonspecific staining. The slides were then incubated with the primary antibody (a polyclonal anti-ER- $\alpha$ 36 antibody raised against the last 20 amino acids as a custom service by Alpha Diagnostic International, San Antonio, TX; dilution 1:50) as described previously<sup>9</sup> overnight at 4°C in a humidified chamber. The sections were incubated with the second antibody (horseradish peroxidase-conjugated goat antirabbit immunoglobulin; 1:100, Dako, Copenhagen, Denmark) for 45 minutes. Diaminobenzidine was used as a chromogen, and sections were counterstained with hematoxylin. The staining intensity in the cytoplasm and the plasma membrane was evaluated. Scoring for ER- $\alpha$ 36 staining was graded as follows: no staining or staining observed in less than 10% of tumor cells was given a score 0; faint/barely perceptible staining detected in  $\geq 10\%$  of tumor cells was scored as 1+; a moderate or strong complete staining observed in  $\geq 10\%$  of tumor cells was scored as 2+ or 3+, respectively. Scores of 0 and 1+ were considered negative, whereas 2+ and 3+ were considered positive. Human epidermal growth factor receptor 2 (HER-2) expression was determined using an immunohistochemistry assay as described previously using an HER-2 specific antibody (clone CB-11, Zymed; dilution 1:100).<sup>11</sup> Only the membrane staining was scored, and a score of 3+ was considered as HER-2 positive. The immunostained slides were evaluated by two pathologists (B.D. and Z.L.) who independently examined the whole slide in a blinded manner. In most cases, the evaluations of the two pathologists were identical; any discrepancies were resolved by re-examination and consensus.

### Statistical Analysis

The correlation between ER- $\alpha$ 36 expression, clinicopathologic characteristics, and adjuvant tamoxifen treatment was determined using Pearson's  $\chi^2$  test. Disease-free survival (DFS) was defined as the time from date of diagnosis to first recurrence (local or distant) or death from breast cancer without a recorded relapse. Disease-specific survival (DSS) was defined as the time from date of diagnosis to death where breast cancer was the primary or underlying cause of death. Patients who were alive at the last follow-up were censored at the last follow-up date, and patients who died from causes other than breast cancer were censored at the time of death. Survival curves were derived from Kaplan-Meier estimates, and the curves were compared using log-rank tests. A Cox regression model was applied to determine whether a factor was an independent predictor of survival in multivariate analysis. All statistical tests were two-sided, and *P* values less than .05 were considered as statistically significant. The statistical analyses were performed using SPSS 13.0 software (SPSS Inc, Chicago, IL).

## RESULTS

### Clinicopathologic Characteristics

The clinicopathologic characteristics of the two cohorts are presented in Table 1. Unlike ER- $\alpha$ 66 that is mainly localized in the cell nucleus, ER- $\alpha$ 36 staining was predominantly observed on the plasma membrane and in the cytoplasm (Fig 1). In the first cohort of 710 patients, 39% of patients exhibited high levels of ER- $\alpha$ 36 expression in their tumors, whereas the remaining 61% of tumors exhibited a low level of expression (see description of cutoff for ER- $\alpha$ 36 expression in Patients and Methods). In the second cohort of 186 patients, 46% of patients exhibited high levels of ER- $\alpha$ 36, and the remaining 54% showed low levels of expression (Table 1). Thirty-nine percent (182 of 465) of ER- $\alpha$ 66–positive and 41% (96 of 237) of ER- $\alpha$ 66–negative tumors exhibited a positive ER- $\alpha$ 36 staining in the first cohort, respectively (Appendix Table A1, online only). ER- $\alpha$ 36 expression was not associated with ER- $\alpha$ 66 expression, tumor size, or lymph node status, but was significantly associated with menopausal status and expression of PgR and HER-2 in the first cohort. Patients with

**Table 1.** Characteristics of Cohort 1 and 2

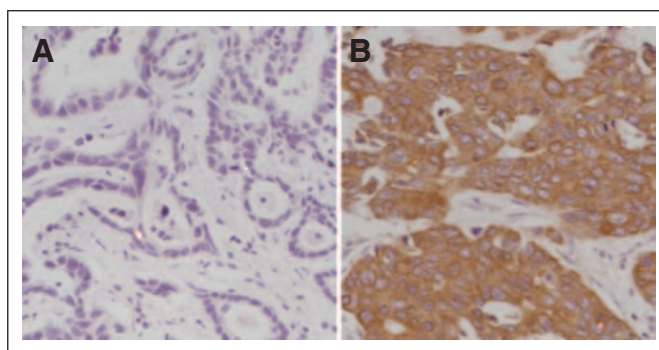
Characteristic	Cohort 1		Cohort 2	
	No.	%	No.	%
Total	710		186	
ER- $\alpha$ 36 status				
Positive	280	39	86	46
Negative	430	61	100	54
Menopausal status				
Premenopausal	350	49	53	28
Postmenopausal	360	51	133	72
Tumor size, cm				
$\leq 2$	441	62	120	65
$> 2$	269	38	66	35
Lymph node status				
0	431	61	147	79
1-3	146	20	20	11
$\geq 4$	133	19	19	10
ER- $\alpha$ 66 status				
Positive	465	66	156	85
Negative	237	34	27	15
Unknown	8		3	
PgR status				
Positive	355	50	124	68
Negative	349	50	58	32
Unknown	6		4	
HER-2 status				
Positive	142	20	36	20
Negative	564	80	149	80
Unknown	4		1	
Histologic grade				
I	153	23	64	38
II	469	70	99	58
III	49	7	6	4
Unknown	39		17	
Adjuvant therapy				
Chemotherapy	280	41	0	
Chemotherapy plus tamoxifen	291	42	0	
Tamoxifen alone	91	13	186	100
No treatment	24	4	0	
Unknown	24		0	

Abbreviations: ER- $\alpha$ 36, estrogen receptor- $\alpha$  36; ER- $\alpha$ 66, estrogen receptor- $\alpha$  66; PgR, progesterone receptor; HER-2, human epidermal growth factor receptor 2.

ER- $\alpha$ 36–positive tumors tended to be older and were more likely to have HER-2–positive and PgR–negative tumors than those with ER- $\alpha$ 36–negative tumors in this group (Appendix Table A1). In contrast, no associations were observed between ER- $\alpha$ 36 and clinicopathologic characteristics in the second cohort of 186 patients, possibly due to a selection bias (Appendix Table A2, online only).

### ER- $\alpha$ 36 Expression and Survival in Cohort 1

In the first cohort of 710 patients, ER- $\alpha$ 36 expression was significantly associated with survival in ER- $\alpha$ 66–positive patients; patients with both ER- $\alpha$ 66– and ER- $\alpha$ 36–positive tumors had poorer DFS and DSS than did those with ER- $\alpha$ 66–positive/ER- $\alpha$ 36–negative tumors (data not shown). We stratified these 465 ER- $\alpha$ 66–positive patients according to adjuvant treatments. Among these, 307 patients received tamoxifen treatment (chemotherapy plus tamoxifen or tamoxifen alone), whereas 129 patients did not receive tamoxifen (chem-



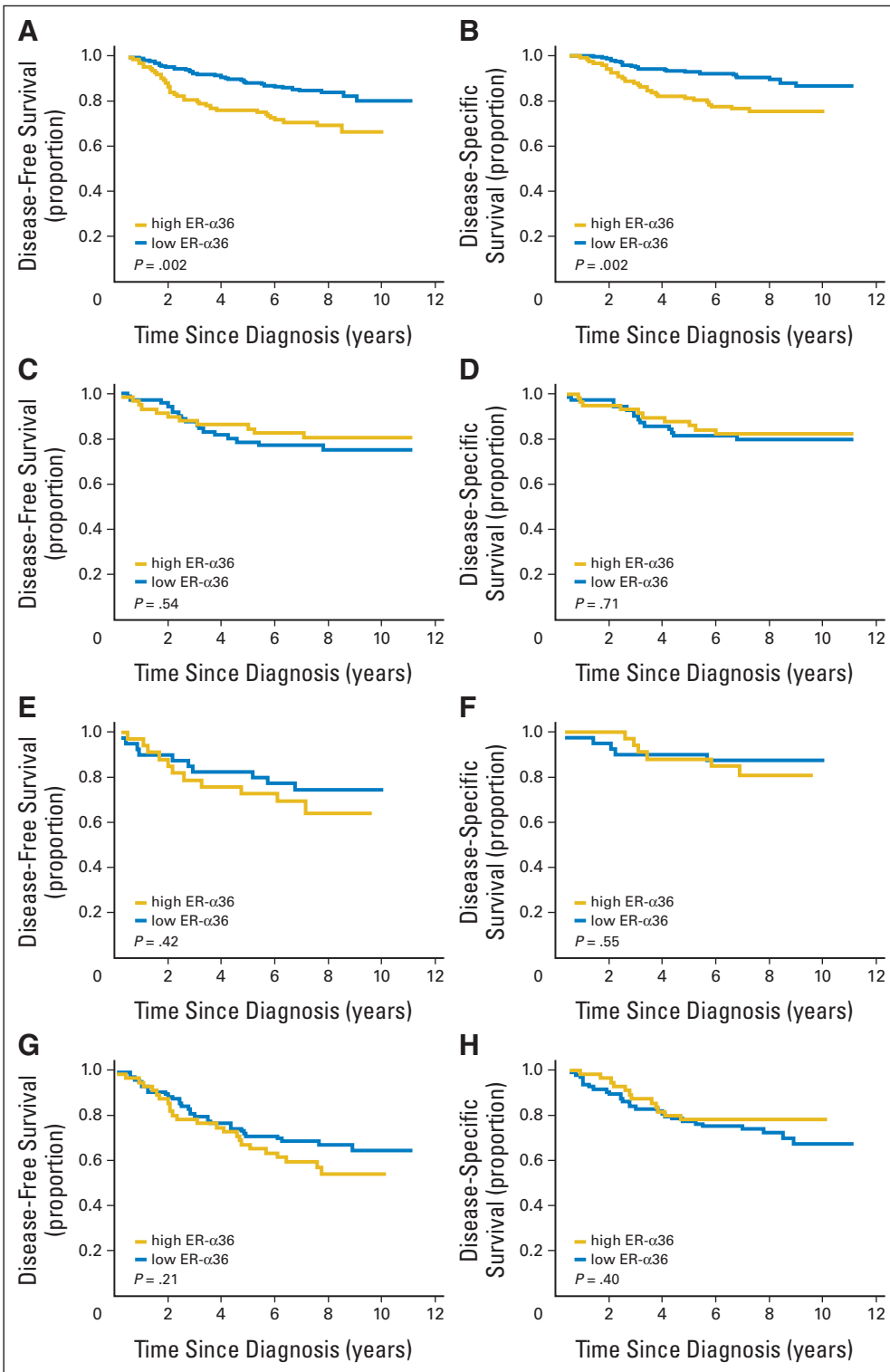
**Fig 1.** Immunohistochemical staining for estrogen receptor (ER)- $\alpha$ 36 expression in primary breast cancers. ER- $\alpha$ 36 showed (A) negative staining and (B) positive staining (magnification  $\times 200$ ).

otherapy alone), the remaining 29 patients either did not receive adjuvant therapy or the adjuvant therapy information was not available. In the 307 patients with ER- $\alpha$ 66–positive tumors who received tamoxifen treatment, patients with ER- $\alpha$ 36–positive tumors had poorer DFS and DSS than did those with ER- $\alpha$ 36–negative tumors (5-year DFS: 76% *v* 88%,  $P = .002$ ; and 5-year DSS: 81% *v* 92%,  $P = .002$ , respectively; Figs 2A and 2B). Multivariate analysis revealed that overexpression of ER- $\alpha$ 36 was an independent unfavorable factor for both DFS and DSS (DFS: hazard ratio [HR] = 1.92; 95% CI, 1.17 to 3.14;  $P = .009$ ; DSS: HR = 2.48; 95% CI, 1.40 to 4.40;  $P = .002$ ) in these 307 ER- $\alpha$ 66–positive patients after adjustment for menopausal status, histologic grade, tumor size, lymph node status, PgR status, and HER-2 status (Table 2). In addition, among these 307 patients, patients with both ER- $\alpha$ 36 and HER-2–positive tumors had more unfavorable survival than did patients with ER- $\alpha$ 36–positive/HER-2–negative tumors (data not shown). However, this was not the case for patients with ER- $\alpha$ 36–positive/PgR–negative tumors as compared with patients with both ER- $\alpha$ 36– and PgR–positive tumors. Among the 129 patients with ER- $\alpha$ 66–positive tumors who received chemotherapy alone, although ER- $\alpha$ 36 expression was not significantly associated with DFS and DSS (Fig 2C and D), overexpression of ER- $\alpha$ 36 seemed to be a favorable factor of DFS and DSS in this subgroup in a multivariate analysis (Table 2). Furthermore, when the patients with ER- $\alpha$ 66–positive tumors who received tamoxifen as their only adjuvant therapy ( $n = 79$ ) were analyzed, overexpression of ER- $\alpha$ 36 expression was significantly associated with poorer DFS and DSS in this relatively small group (data not shown).

Among the 237 patients with ER- $\alpha$ 66–negative tumors, 15 patients either did not receive adjuvant therapy or the therapy information was not available. The remaining 222 ER- $\alpha$ 66–negative tumors were included for survival analysis. ER- $\alpha$ 36 expression was not significantly associated with DFS or DSS regardless of whether the patients received tamoxifen treatment (chemotherapy plus tamoxifen or tamoxifen alone,  $n = 73$ ; Figs 2E and 2F) or not (chemotherapy alone,  $n = 149$ ; Figs 2G and 2H). However, in 149 ER- $\alpha$ 66–negative patients who received chemotherapy alone, overexpression of ER- $\alpha$ 36 tended to be a favorable factor of DSS in a multivariate analysis (Table 2).

### ER- $\alpha$ 36 Expression and Survival in Cohort 2 Who Received Only Tamoxifen Treatment

The results from cohort 1 suggested that ER- $\alpha$ 36 might be associated with tamoxifen resistance in patients with ER- $\alpha$ 66–positive



**Fig 2.** Kaplan-Meier estimate of disease-free survival (DFS) and disease-specific survival (DSS) in cohort 1, according to estrogen receptor (ER)- $\alpha$ 36 expression. (A) DFS and (B) DSS in 307 ER- $\alpha$ 66-positive patients who received tamoxifen treatment (chemotherapy plus tamoxifen or tamoxifen alone); (C) DFS or (D) DSS in 129 ER- $\alpha$ 66-positive patients who did not receive tamoxifen therapy (chemotherapy alone); (E) DFS or (F) DSS in 73 ER- $\alpha$ 66-negative patients who received tamoxifen therapy (chemotherapy plus tamoxifen or tamoxifen alone); (G) DFS or (H) DSS in 149 ER- $\alpha$ 66-negative patients who did not receive tamoxifen therapy (chemotherapy alone).

breast cancer who received tamoxifen treatment. To verify this finding, an independent second cohort of 186 patients who received tamoxifen as their only adjuvant therapy was included for analysis. Univariate analysis showed that patients with ER- $\alpha$ 36-positive tumors had poorer DFS and DSS than did patients with ER- $\alpha$ 36-negative tumors in these 186 patients (data not shown). When the

analysis was restricted to patients with ER- $\alpha$ 66-positive tumors receiving only tamoxifen in this cohort ( $n = 156$ ), overexpression of ER- $\alpha$ 36 was significantly associated with poorer DFS and DSS (5-year DFS: 71%  $\nu$  93%,  $P < .001$ ; and 5-year DSS: 83%  $\nu$  98%,  $P = .001$ , respectively; Figs 3A and 3B). Furthermore, a multivariate analysis revealed that ER- $\alpha$ 36 was an independent unfavorable factor for both

**Table 2.** Results of Multivariate Analyses of ER- $\alpha$ 36 Positive Versus ER- $\alpha$ 36 Negative for DFS and DSS in Four Subgroups in Cohort 1

Group	No.*	DFS			DSS		
		HR	95% CI	P	HR	95% CI	P
ER- $\alpha$ 66 positive/TAM+	307	1.92	1.17 to 3.14	.009	2.48	1.40 to 4.40	.002
ER- $\alpha$ 66 positive/TAM-	129	0.84	0.38 to 1.85	.67	0.79	0.34 to 1.83	.58
ER- $\alpha$ 66 negative/TAM+	73	1.55	0.62 to 3.85	.35	1.29	0.37 to 4.51	.69
ER- $\alpha$ 66 negative/TAM-	149	0.95	0.53 to 1.68	.86	0.52	0.25 to 1.05	.07

Abbreviations: ER- $\alpha$ 36, estrogen receptor- $\alpha$  36; DFS, disease-free survival; DSS, disease-specific survival; HR, hazard ratio; ER- $\alpha$ 66, estrogen receptor- $\alpha$  66; TAM+, chemotherapy plus tamoxifen or tamoxifen alone; TAM-, chemotherapy alone without tamoxifen treatment.

\*Twenty-nine ER- $\alpha$ 66-positive patients and 15 ER- $\alpha$ 66-negative patients in cohort 1 either did not receive adjuvant therapy or the adjuvant therapy information was not available, and the ER- $\alpha$ 66 status is unknown in eight patients in cohort 1.

DFS and DSS (DFS: HR = 5.47; 95% CI, 1.81 to 16.51;  $P = .003$ ; DSS: HR = 13.97; 95% CI, 1.58 to 123.53;  $P = .018$ ) in these 156 patients after adjustment for menopausal status, histologic grade, tumor size, lymph node status, PgR status, and HER-2 status (Table 3). Negative PgR and more than four positive lymph nodes also remained independent unfavorable factors for both DFS and DSS, with more than four positive lymph nodes having the most influence (Table 3).

## DISCUSSION

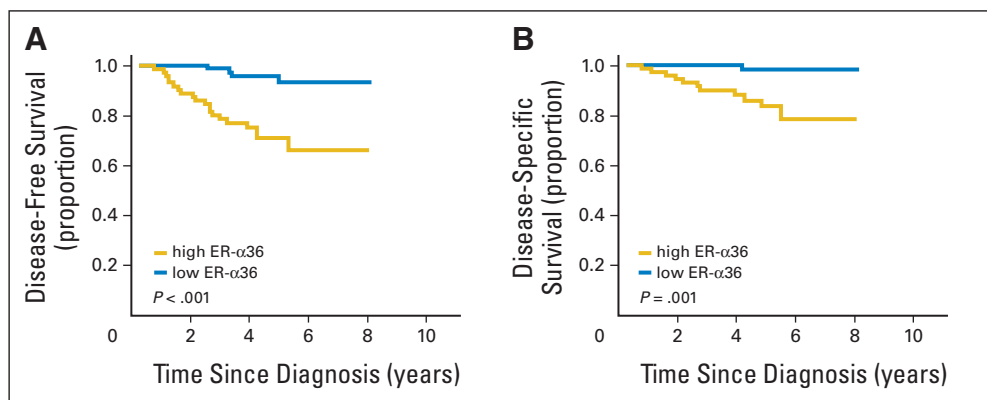
In the present study, we examined ER- $\alpha$ 36 expression in specimens from 896 patients with breast tumor. We report here that patients with ER- $\alpha$ 66-positive breast tumors that also express high levels of ER- $\alpha$ 36 are less likely to benefit from tamoxifen treatment than patients who do not. In patients with ER- $\alpha$ 66-positive breast tumors who received adjuvant tamoxifen treatment, ER- $\alpha$ 36 expression was associated with poorer survival and remained as an independent unfavorable factor of DFS and DSS in multivariate analyses in this group. These findings are replicated in two independent cohorts. However, in patients with ER- $\alpha$ 66-positive tumor who did not receive tamoxifen treatment but received chemotherapy alone, high levels of ER- $\alpha$ 36 seemed to be a favorable factor for survival in a multivariate analysis, indicating that patients with both ER- $\alpha$ 66- and ER- $\alpha$ 36-positive tumors may be less likely to benefit from tamoxifen treatment but may benefit from chemotherapy. The notion that ER- $\alpha$ 36 expression may predict the benefits of chemotherapy is supported in ER- $\alpha$ 66-negative patients who received chemotherapy alone. Overexpression of ER- $\alpha$ 36 tended to exhibit a favorable DSS in this subgroup in a

multivariate analysis. Interestingly, a 21-gene assay has recently been developed and has been shown to accurately predict distant recurrence in patients with ER- $\alpha$ 66-positive tumors treated with adjuvant tamoxifen.<sup>12</sup> Patients with high recurrence score (RS) are less likely to benefit from tamoxifen treatment but gain a large chemotherapy benefit.<sup>13</sup> Although our present findings may have potential clinical implications, they should not be translated into clinical practice until the data from prospective studies are available.

We also found that negative PgR and more than four lymph node metastases were independent unfavorable factors for survival in patients who only received tamoxifen treatment. These findings are concordant with previous studies that PgR status is an important determinant for tamoxifen treatment, patients with ER- $\alpha$ 66-positive/PgR-positive tumors are more likely to benefit from tamoxifen treatment than those with ER- $\alpha$ 66-positive/PgR-negative tumors.<sup>14,15</sup> It is also well documented that four lymph node metastases is an unfavorable factor for patients who received tamoxifen treatment.<sup>15,16</sup>

In this study, we did not observe a correlation between ER- $\alpha$ 66 and ER- $\alpha$ 36 expression. Approximately 40% of ER- $\alpha$ 66-positive or -negative tumors expressed high levels of ER- $\alpha$ 36, suggesting that ER- $\alpha$ 36 expression is independent of ER- $\alpha$ 66 expression. The transcript of the ER- $\alpha$ 36 isoform is initiated from a previously unidentified promoter in the first intron of the ER- $\alpha$ 66 gene, suggesting that ER- $\alpha$ 36 expression is regulated by a different promoter from ER- $\alpha$ 66.<sup>8</sup>

HER-2 is a member of the epidermal growth factor receptor family. The HER-2 protein is overexpressed in 25% to 30% of breast cancers, where it is a marker of poor prognosis and an indicator of trastuzumab treatment.<sup>17-19</sup> Laboratory and clinical evidence indicate



**Fig 3.** Kaplan-Meier estimate of disease-free survival (DFS) and disease-specific survival (DSS) in the 156 estrogen receptor (ER)- $\alpha$ 66-positive patients who only received tamoxifen in cohort 2. High levels of ER- $\alpha$ 36 expression were significantly associated with poorer (A) DFS and (B) DSS in this group.

**Table 3.** Multivariate Analyses of Disease-Free Survival and Disease-Specific Survival in 156 ER- $\alpha$ 66–Positive Patients Who Only Received Tamoxifen Treatment in Cohort 2

Factor	DFS			DSS		
	HR	95% CI	P	HR	95% CI	P
ER- $\alpha$ 36 status, positive v negative	5.47	1.81 to 16.51	.003	13.97	1.58 to 123.53	.018
Menopausal status, post v pre	3.88	0.89 to 16.96	.07	6.21	0.67 to 57.35	.11
Tumor size, > 2 v $\leq$ 2 cm	1.34	0.42 to 4.32	.62	1.19	0.22 to 6.48	.84
PgR status, negative v positive	3.31	1.32 to 8.31	.011	4.27	1.26 to 14.48	.012
Histologic grade, 3 v 1 and 2	2.65	0.52 to 13.51	.24	4.65	0.37 to 58.08	.23
Lymph node status						
1-3 v 0	2.37	0.49 to 11.41	.28	7.86	1.21 to 51.08	.031
$\geq$ 4 v 0	10.77	4.34 to 26.75	< .001	10.42	2.83 to 38.36	< .001
HER-2 status, positive v negative	0.97	0.37 to 2.56	.96	2.34	0.67 to 8.23	.19

Abbreviations: ER- $\alpha$ 66, estrogen receptor- $\alpha$  66; DFS, disease-free survival; DSS, disease-specific survival; HR, hazard ratio; ER- $\alpha$ 36, estrogen receptor- $\alpha$  36; PgR, progesterone receptor; HER-2, human epidermal growth factor receptor 2.

that breast cancers that overexpress HER-2 are particularly less responsive to tamoxifen treatment.<sup>20-22</sup> ER- $\alpha$ 36 expression was associated with HER-2 expression in the first cohort of 710 consecutive patients; ER- $\alpha$ 36 positive breast tumors more frequently expressed high levels of HER-2 compared with ER- $\alpha$ 36–negative breast tumors. In agreement with this finding, our *in vitro* experiments demonstrated that block of HER-2 function using the HER-2–specific inhibitor AG825 in established breast cancer cells suppressed ER- $\alpha$ 36 expression (Wang et al, unpublished data), suggesting that ER- $\alpha$ 36 expression is positively regulated by the HER-2 signaling pathway. Thus it is possible that a signaling pathway mediated by HER-2 activates ER- $\alpha$ 36 expression, which in turn confers tamoxifen resistance in HER-2–overexpressing tumors.

ER- $\alpha$ 36 is predominantly located on the plasma membrane and in the cytoplasm and mediates membrane-initiated estrogen signaling.<sup>9</sup> Membrane-initiated estrogen signaling has been linked to rapid responses to estrogen and generally activates signaling pathways like MAPK/ERK, phosphatidylinositol-3-kinase, and protein kinase C pathways.<sup>23-26</sup> Thus it raises a possibility that ER- $\alpha$ 36 is a potential therapeutic target because tamoxifen cannot block ER- $\alpha$ 36–mediated membrane-initiated estrogen signaling pathways.

In this study, we revealed that approximately 40% of ER- $\alpha$ 66–positive breast cancer patients express high levels of ER- $\alpha$ 36 in their tumors, and this subset of patients are less likely to benefit from tamoxifen treatment compared with those with ER- $\alpha$ 66–positive/ER- $\alpha$ 36–negative tumors. Although aromatase inhibitors are increasingly

used for postmenopausal women with ER- $\alpha$ 66–positive tumors,<sup>27</sup> tamoxifen still remains as a first-line therapy for the foreseeable future, especially in premenopausal women in whom aromatase inhibitors are unlikely to be effective. Therefore, early identification of patients with ER- $\alpha$ 66–positive breast cancer who may be resistant to tamoxifen treatment is extremely important in a clinical setting, because these patients may select an alternative endocrine therapy or other types of therapy from the diagnosis.

#### AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

#### AUTHOR CONTRIBUTIONS

**Conception and design:** Yuntao Xie

**Financial support:** Zhaoyi Wang, Yuntao Xie

**Provision of study materials or patients:** Tao Ouyang, Jinfeng Li, Tianfeng Wang, Zhaoqing Fan, Tie Fan, Benyao Lin, Yuntao Xie

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**Manuscript writing:** Zhaoyi Wang, Yuntao Xie

**Final approval of manuscript:** Liang Shi, Bin Dong, Zhongwu Li, Yunwei Lu, Tao Ouyang, Jinfeng Li, Tianfeng Wang, Zhaoqing Fan, Tie Fan, Benyao Lin, Zhaoyi Wang, Yuntao Xie

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## Appendix

**Table A1.** Associations Between ER- $\alpha$ 36 Expression, Clinicopathologic Characteristics, and Adjuvant Therapy in the First Cohort of 710 Patients

Characteristic	No. of Patients	ER- $\alpha$ 36 Expression				P*
		Positive		Negative		
		No.	%	No.	%	
Total	710	280	39	430	61	
Menopausal status						.006
Pre	350	120	43	230	53	
Post	360	160	57	200	47	
Tumor size, cm						.16
$\leq 2$	441	165	59	276	64	
$> 2$	269	115	41	154	36	
Lymph node status						.89
0	431	169	60	262	61	
1-3	146	60	22	86	20	
$\geq 4$	133	51	18	82	19	
ER- $\alpha$ 66 status						.73
Positive	465	182	65	283	67	
Negative	237	96	35	141	33	
Unknown	8	2		6		
PgR status						.024
Positive	355	126	45	229	54	
Negative	349	153	55	196	46	
Unknown	6	1		5		
HER-2 status						.005
Positive	142	71	25	71	17	
Negative	564	209	75	355	83	
Unknown	4			4		
Histologic grade						.72
1	153	61	23	92	23	
2	469	192	70	277	70	
3	49	20	7	29	7	
Unknown	39	7		32		
Adjuvant therapy						.43
Chemotherapy	280	113	42	167	40	
Chemotherapy plus tamoxifen	291	112	42	179	43	
Tamoxifen alone	91	39	14	52	13	
No treatment	24	6	2	18	4	
Unknown	24	10		14		

Abbreviations: ER- $\alpha$ 36, estrogen receptor- $\alpha$  36; ER- $\alpha$ 66, estrogen receptor- $\alpha$  66; PgR, progesterone receptor; HER-2, human epidermal growth factor receptor 2.  
\*P values were derived from  $\chi^2$  test.

ER- $\alpha$ 36 and Tamoxifen Resistance in Breast Cancer

**Table A2.** Associations Between ER- $\alpha$ 36 Expression and Clinicopathologic Characteristics in the Second Cohort of 186 Patients Who Only Received Tamoxifen

Characteristic	No. of Patients	ER- $\alpha$ 36 Expression				P*
		Positive		Negative		
		No.	%	No.	%	
Total	186	86	46	100	54	
Menopausal status						.62
Pre	53	23	27	30	30	
Post	133	63	73	70	70	
Tumor size, cm						.65
$\leq 2$	120	54	63	66	66	
$> 2$	66	32	37	34	34	
Lymph node status						.039
0	147	64	75	83	83	
1-3	20	8	9	12	12	
$\geq 4$	19	14	16	5	5	
ER- $\alpha$ 66 status						.90
Positive	156	73	85	83	86	
Negative	27	13	15	14	14	
Unknown	3			3		
PgR status						.61
Positive	124	57	66	67	70	
Negative	58	29	34	29	30	
Unknown	4			4		
HER-2 status						.40
Positive	36	19	22	17	17	
Negative	149	67	80	82	83	
Unknown	1			1		
Histologic grade						.60
1	64	29	36	35	40	
2	99	48	59	51	58	
3	6	4	5	2	2	
Unknown	17	5		12		

Abbreviations: ER- $\alpha$ 36, estrogen receptor- $\alpha$  36; ER- $\alpha$ 66, estrogen receptor- $\alpha$  66; PgR, progesterone receptor; HER-2, human epidermal growth factor receptor 2.  
\*P values were derived from  $\chi^2$  test.