Low phosphorylation of estrogen receptor α (ERα) serine 118 and high phosphorylation of ERα serine 167 improve survival in ER-positive breast cancer

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Abstract

Endocrine therapy has become the most important treatment option for women with estrogen receptor (ER)-positive breast cancer. Urgently needed are prognostic assays that can identify those who need additional adjuvant therapy, such as signal transduction inhibitors or chemotherapy, for ER-positive early breast cancer. We examined phosphorylation of ERα serine (Ser) 118, ERα Ser167, p44/42 mitogen-activated protein kinase (MAPK), and Akt and expression of progesterone receptor, amplified in breast cancer 1 (AIB1), human epidermal growth factor receptor 2 (HER2), p53, and Ki67 in ER-positive breast cancers by immunohistochemistry, and analyzed their significance for prognosis. Phosphorylation levels of ERα Ser118, ERα Ser167, MAPK, and Akt were positively correlated. AIB1 expression was significantly associated with phosphorylation of ERα Ser118, MAPK, and Akt, and HER2 expression. Low phosphorylation of ERα Ser118 and high phosphorylation of ERα Ser167 were associated with significantly improved disease-free ($P=0.0003$ and $P=0.0002$ respectively) and overall survival ($P=0.0007$ and $P=0.0016$ respectively) in multivariate analyses. Our data suggest that phosphorylation of ERα Ser118 and ERα Ser167 affects survival in ER-positive breast cancer and could be helpful in distinguishing patients who are likely to benefit from endocrine therapy alone from those who are not.

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Introduction

Endocrine therapy has become the most important treatment option for women with estrogen receptor (ER)-positive breast cancer. Nevertheless, many breast cancer patients with tumors expressing high levels of ER are unresponsive to endocrine therapy, and all patients with advanced disease eventually develop resistance to the therapy. The potential mechanisms behind this intrinsic or acquired endocrine resistance involve ER-coregulatory proteins and crosstalk between the ER pathway and other growth factor signaling networks (Schiff *et al.* 2003, Osborne & Schiff 2005). An understanding of the molecular mechanisms that modulate the activity of the estrogen-signaling network has enabled new ways of overcoming endocrine resistance to be developed. An urgent issue is the discovery of prognostic methods to identify those patients who need additional adjuvant therapy, such as signal transduction inhibitors or chemotherapy, for ER-positive early breast cancer (Johnston *et al.* 2003, Ellis 2004).

ERα is phosphorylated on multiple amino acid residues (Lannigan 2003). In general, phosphorylation of serine residues in the activation function 1 (AF-1) domain of ERα appears to influence the recruitment of coactivators, resulting in enhanced ER-mediated transcription. It has been reported that ERα was significantly phosphorylated on Ser118 in response to either estradiol binding or the activation of the mitogen-activated protein kinase (MAPK) pathway, while Ser167 is phosphorylated by Akt, Rsk, and casein kinase II as well as MAPK (Arnold *et al.* 1994, Kato *et al.* 1995, Joel *et al.* 1998,

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Campbell *et al*. 2001, Clark *et al*. 2001). Murphy *et al*. (2004*a*) reported that in 45 human breast tumor biopsies, phosphorylation of ERα Ser118 correlated with active MAPK. Because MAPK is located downstream of human epidermal growth factor receptor 2 (HER2), it is possible that phosphorylation of ERα Ser118 is in part caused by HER2–MAPK signaling in breast cancer. On the other hand, phosphorylation of ERα Ser167 seems to be controlled by different mechanisms.

We previously analyzed phosphorylation of Ser118 and Ser167 of ERα using immunohistochemistry (IHC) in primary breast tumor specimens from 75 metastatic breast cancer patients who received first-line treatment with endocrine therapy on relapse (Yamashita *et al*. 2005). Our results indicated that patients whose primary breast tumors showed high phosphorylation of ERα Ser167, but not ERα Ser118, responded significantly to endocrine therapy and had a better survival than other patients, suggesting that phosphorylation of ERα Ser167 frequently occurs via estrogen-dependent signaling in human breast cancer.

Amplified in breast cancer 1 (AIB1) is a coactivator of ERα, which is phosphorylated by MAPK that has been activated by signaling from epidermal growth factor receptor (EGFR) or HER2 (Font de Mora & Brown 2000). It has been reported that tamoxifen behaves as an estrogen agonist in breast cancer cells that express high levels of AIB1 and HER2, resulting in *de novo* resistance (Shou *et al*. 2004) and that high AIB1 expression in patients who received tamoxifen adjuvant therapy was associated with inferior disease-free survival (Osborne *et al*. 2003).

In this study, we examined phosphorylation of ERα Ser118, ERα Ser167, p44/42 MAPK, and Akt, as well as expression of progesterone receptor (PR), AIB1, HER2, p53, and Ki67 in ER-positive invasive breast cancer, because these factors are involved in ER signaling and were predicted to affect prognosis. Correlation between phosphorylation and expression levels of these molecular markers and their significance for survival were analyzed to identify patients who need additional therapy, such as signal transduction inhibitors or chemotherapy, together with endocrine therapy in ER-positive early breast cancer.

Materials and methods

Patients and breast cancer tissues

Breast tumor specimens from 278 female patients with invasive breast carcinoma, who were treated at Nagoya City University Hospital between 1982 and 1999, were included in this study (Table 1). The study protocol was approved by the institutional review board and conformed to the guidelines of the 1975 Declaration of Helsinki. All patients had undergone surgical treatment for primary breast cancer (either mastectomy or lumpectomy) and all primary tumors were ER positive. The samples were chosen from the continuous series of invasive carcinoma tissues. After surgery, 61 patients received no additional therapy. Of the remaining 217 patients, 100 received systemic adjuvant therapy consisting of endocrine therapy alone, 24 received chemotherapy alone, and 93 received combined endocrine therapy and chemotherapy. Patients who were positive for axillary lymph nodes received either oral administration of 5-fluorouracil derivatives for 2 years or a combination of cyclophosphamide, methotrexate, and fluorouracil. Patients were observed for disease recurrence at least once every 6 months for the first 5 years after the surgery and thereafter once every year. The median follow-up period was 96 months.

Table 1 Clinicopathological characteristics of patients and tumors

Factor	No. (%)
Total patients	278
Age at diagnosis (years)	
≤50	99 (36)
>50	179 (64)
Age range (years)	28–91
Tumor size (cm)	
<2	116 (42)
≥2	162 (58)
Number of positive lymph nodes	
0	167 (60)
≥1	111 (40)
Histological grade	
1	64 (23)
2	169 (61)
3	31 (11)
Unknown	14 (5)
HER2	
0	213 (77)
1+	37 (13)
2+	14 (5)
3+	13 (5)
Adjuvant therapy	
None	61 (22)
Endocrine therapy	100 (36)
Tamoxifen	93
LHRH agonist	5
LHRH agonist+tamoxifen	2
Chemotherapy	24 (9)
Combined	93 (33)
Follow-up (months)	
Mean	104
Median	96
Range	2–252

LHRH, luteinizing hormone-releasing hormone.

Immunohistochemical analysis

One 4 μm section of each submitted paraffin block was stained first with hematoxylin and eosin to verify that an adequate number of invasive carcinoma cells were present and that the fixation quality was adequate for IHC analysis. Serial sections (4 μm) were prepared from selected blocks and float-mounted on adhesive-coated glass slides, for staining of phosphorylation of ERα Ser118, ERα Ser167, p44/42 MAPK, and Akt, and expression of ER, PR, AIB1, HER2, p53, and Ki67 as described previously (Yamashita *et al*. 2005). Primary antibodies included monoclonal mouse anti-human ERα antibody (1D5, DAKO, Glostrup, Denmark) at 1:100 dilution for ERα, polyclonal rabbit anti-phospho-ERα (Ser118) antibody (no. 2515, Cell Signaling, Beverly, MA, USA) at 1:25 dilution for phosphorylated ERα Ser118, polyclonal rabbit anti-phospho-ERα (Ser167) antibody (no. 2514, Cell signaling) at 1:25 dilution for phosphorylated ERα Ser167, polyclonal rabbit anti-phospho-p44/42 Map kinase (Thr202/Tyr204) antibody (no. 9101, Cell signaling) at 1:25 dilution for phosphorylated MAPK (extracellular signal-regulated kinase; ERK), polyclonal rabbit anti-phospho-Akt (Ser473) antibody (no. 9277, Cell signaling) at 1:50 dilution for phosphorylated Akt, monoclonal mouse anti-human PR antibody (636, DAKO) at 1:100 dilution for PR, monoclonal mouse anti-AIB-1 antibody (Clone 34, BD Biosciences, San Jose, CA, USA) at 1:50 dilution for AIB1, rabbit anti-human c-erbB-2 oncoprotein antibody (DAKO) at 1:200 dilution for HER2, monoclonal mouse anti-human p53 protein antibody (PAb1801, Novocastra, Newcastle, UK) at 1:50 dilution for p53, and monoclonal mouse anti-human Ki67 antibody (MIB-1, DAKO) at 1:100 dilution for Ki67. The DAKO Envision system (DAKO EnVision labeled polymer, peroxidase) was used as the detection system as described previously except for p53 (Yamashita *et al*. 2005). The streptavidin–biotin system (SAB-PO kit, Nichirei Co., Inc., Tokyo, Japan) was used for detection of the bound antibody of p53.

IHC scoring

Immunostained slides were scored after the entire slide was evaluated by light microscopy. The expression of ERα, PR, and AIB1 and the phosphorylation of ERα Ser118 and ERα Ser167 were scored by assigning proportion and intensity scores, according to Allred's procedure (Allred *et al*. 1998). In brief, a proportion score represented the estimated proportion of tumor cells staining positive as follows: 0 (none); 1 (<1/100); 2 (1/100–1/10); 3 (1/10–1/3); 4 (1/3–2/3); and 5 (>2/3). Any brown nuclear staining in invasive breast epithelium counted toward the proportion score. An intensity score represented the average intensity of the positive cells as follows: 0 (none); 1 (weak); 2 (intermediate); and 3 (strong). The proportion and intensity scores were then added to obtain a total score, which could range from 0 to 8. Tumors with score ≥3 for ERα were included in this study. The phosphorylation of MAPK and Akt was scored by assigning proportion scores as follows: 0 (none); 1 (<1/100); 2 (1/100–1/10); 3 (1/10–1/3); 4 (1/3–2/3); and 5 (>2/3). Any brown nuclear staining in invasive breast epithelium counted toward the proportion score. HER2 immunostaining was evaluated using the same method as is employed by the Hercep Test (DAKO). To determine the score of HER2 expression, the membrane staining pattern was estimated and scored on a scale of 0–3. The expression status of p53 and Ki67 was assessed according to the estimated proportion of nuclear staining of tumor cells that were positively stained as described previously (Yamashita *et al*. 2006). Scoring criteria were as follows: for p53: score=0, none; score=1, <1/10; score=2, 1/10–1/2; score=3, >1/2; and for Ki67: score=0, none; score=1, <1/100; score=2, 1/100–1/10; score=3, 1/10–1/2; score=4, >1/2.

Statistical analysis

The Mann–Whitney *U* test was used to compare the IHC scores of molecular markers with clinicopathological characteristics. Spearman's rank correlation test was used to study relationships among expression and phosphorylation of molecular markers. Estimation of disease-free and overall survival was performed using the Kaplan–Meier method, and differences between survival curves were assessed with the log-rank test. Cox's proportional hazards model was used for univariate and multivariate analyses of prognostic values.

Results

Correlation between expression and phosphorylation levels of molecular markers and clinicopathological factors

In this study, tumors with score ≥3 for ERα were considered as ER positive and only patients with ER-positive tumors were included. We examined phosphorylation of ERα Ser118, ERα Ser167, MAPK, and Akt and expression of ERα, PR, AIB1, HER2, p53, and Ki67 in 278 invasive breast carcinomas by IHC. The IHC scores for molecular markers were compared among patient subgroups, according to the clinicopathological factors. Phosphorylation of ERα Ser167 was significantly correlated with tumor size ($P=0.010$) and lymph

node status ($P=0.012$). There was a strong association between expression of AIB1 and tumor size ($P=0.033$). Phosphorylation of MAPK and expression of ERα and Ki67 were significantly correlated with histological grade ($P=0.044$, $P=0.036$, and $P=0.011$ respectively). Expression of p53 was strongly correlated with tumor size ($P=0.019$), lymph node status ($P=0.013$), and histological grade ($P=0.0009$).

Correlation between expression and phosphorylation levels of molecular markers

Links between the IHC scores for phosphorylation of ERα Ser118, ERα Ser167, MAPK, and Akt and expression of ERα, PR, AIB1, HER2, p53, and Ki67 were analyzed using Spearman's rank correlation test (Table 2). Phosphorylation levels of ERα Ser118, ERα Ser167, MAPK, and Akt were strongly and positively correlated ($P<0.0001$ respectively). Expression of ERα was significantly correlated with phosphorylation of ERα Ser118 ($P=0.028$), ERα Ser167 ($P=0.0002$), and Akt ($P=0.008$) and expression of AIB1 ($P=0.014$) and Ki67 ($P=0.017$). Significant and positive association was also found between phosphorylation of ERα Ser118 and expression of AIB1 ($P<0.0001$) and HER2 ($P=0.019$). AIB1 expression was strongly and positively correlated with phosphorylation of MAPK ($P<0.0001$) and Akt ($P<0.0001$) and expression of HER2 ($P<0.0001$) and Ki67 ($P<0.0001$). There was a significant association between Akt phosphorylation and Ki67 expression ($P=0.0008$). The PR expression was positively correlated with Akt phosphorylation ($P=0.025$) and negatively correlated with p53 expression ($P=0.044$).

Low phosphorylation of ERα Ser118 and high phosphorylation of ERα Ser167 improve survival in ER-positive breast cancer

To identify a clinically meaningful cutoff point for levels of phosphorylation and expression of molecular markers that could be used in disease prognosis analysis, various levels of phosphorylation and expression were tested using the Cox's proportional hazards model and the Kaplan–Meier method verified by the log-rank test. When analyzing disease-free and overall survival, the cutoff points for the levels of phosphorylation of ERα Ser118 and ERα Ser167 were set at 6 and 2 respectively, and the cutoff points for the levels of expression of AIB1, HER2, and Ki67 were set at 5, 2 and 1 respectively. Phosphorylation of MAPK and Akt and expression of PR and p53 were not significantly related to survival in the univariate analysis (Tables 3 and 4). Kaplan–Meier analysis showed that low phosphorylation of ERα Ser118 (score 0–6) was strongly associated with increased disease-free and overall survival ($P<0.0001$ and $P=0.0015$ respectively; Fig. 1A and B). By contrast, low phosphorylation of ERα Ser167 (score 0–2) was associated with significantly decreased disease-free and

Table 2 Correlations between immunohistochemistry scores for expression and phosphorylation of molecular markers

	ERα	pERα Ser118	pERα Ser167	PR	AIB1	pMAPK	pAKT	HER2	p53
pERα Ser118	+0.133[a] 0.028*[b]								
pERα Ser167	+0.220 0.0002*	+0.282 <0.0001*							
PR	+0.061 0.31	+0.075 0.21	+0.090 0.14						
AIB1	+0.148 0.014*	+0.450 <0.0001*	+0.111 0.064	+0.040 0.51					
pMAPK	+0.045 0.45	+0.379 <0.0001*	+0.231 0.0001*	+0.073 0.23	+0.248 <0.0001*				
pAKT	+0.159 0.008*	+0.428 <0.0001*	+0.245 <0.0001*	+0.136 0.025*	+0.378 <0.0001*	+0.310 <0.0001*			
HER2	+0.095 0.12	+0.143 0.019*	+0.054 0.37	−0.081 0.18	+0.338 <0.0001*	−0.023 0.71	+0.107 0.075		
p53	+0.009 0.88	−0.053 0.38	+0.054 0.37	−0.122 0.044*	+0.081 0.18	−0.136 0.024*	+0.022 0.72	+0.181 0.0027*	
Ki67	+0.144 0.017*	+0.116 0.056	+0.116 0.055	+0.002 0.97	+0.242 <0.0001*	−0.025 0.68	+0.203 0.0008*	+0.195 0.0012*	+0.368 <0.0001*

*$P<0.05$ is considered significant. pERα, phosphorylated estrogen receptor α; pMAPK, phosphorylated mitogen-activated protein kinase; pAKT, phosphorylated AKT.
[a]Spearman's correlation coefficient.
[b]P, Spearman's rank correlation test.

Table 3 Univariate and multivariate analysis of factors predicting disease-free survival

	Univariate			Multivariate		
Factor	**RR**	**95% CI**	***P***	**RR**	**95% CI**	***P***
Tumor size (<2 cm, ≥2 cm)	0.47	0.29–0.79	0.0039*	0.87	0.51–1.51	0.63
Lymph node status (0, ≥1)	0.19	0.11–0.32	<0.0001*	0.23	0.13–0.39	<0.0001*
Histological grade (1–2, 3)	0.48	0.27–0.85	0.013*	0.68	0.35–1.30	0.24
pERα Ser118 (0–6, 7–8)	0.34	0.20–0.58	<0.0001*	0.31	0.16–0.59	0.0003*
pERα Ser167 (0–2, 3–8)	2.18	1.36–3.49	0.0013*	2.76	1.61–4.72	0.0002*
PR	0.98	0.91–1.07	0.68			
AIB1 (0–5, 6–8)	0.55	0.34–0.87	0.011*	0.81	0.49–1.35	0.42
pMAPK	1.08	0.90–1.30	0.40			
pAKT	1.05	0.92–1.20	0.43			
HER2 (0–2, 3)	0.35	0.15–0.81	0.014*	0.46	0.19–1.11	0.085
p53	0.73	0.39–1.35	0.31			
Ki67 (0–1, 2–4)	0.58	0.36–0.94	0.026*	0.52	0.30–0.87	0.014*

RR, relative risk; CI, confidence interval. *$P<0.05$ is considered significant.

overall survival ($P=0.001$ and $P=0.001$ respectively; Fig. 1C and D). Combination analysis of phosphorylation status for ERα Ser118 and ERα Ser167 revealed that patients whose tumors showed both low ERα Ser118 phosphorylation and high ERα Ser167 phosphorylation had significantly longer disease-free and overall survival ($P<0.0001$ and $P<0.0001$ respectively), whereas patients with high ERα Ser118 phosphorylation and low ERα Ser167 phosphorylation relapsed and died significantly sooner after surgery (Fig. 1E and F).

Prognostic analysis of disease-free survival in ER-positive breast cancer

Univariate analysis demonstrated significant association between disease-free survival and phosphorylation status of ERα Ser118 ($P<0.0001$) and ERα Ser167 ($P=0.0013$), as well as tumor size ($P=0.0039$), lymph node status ($P<0.0001$), histological grade ($P=0.013$), expression of AIB1 ($P=0.011$), HER2 ($P=0.014$), and Ki67 ($P=0.026$; Table 3). There was no significant association between disease-free survival and phosphorylation of MAPK or Akt or expression of PR or p53. In multivariate analysis, patients with low phosphorylation of ERα Ser118 ($P=0.0003$) and high phosphorylation of ERα Ser167 ($P=0.0002$), as well as patients with negative lymph node status ($P<0.0001$) and low expression of Ki67 ($P=0.014$), had significantly improved disease-free survival (Table 3).

Prognostic analysis of overall survival in ER-positive breast cancer

Univariate analysis showed significant association between overall survival and phosphorylation status of ERα Ser118 ($P=0.0024$) and ERα Ser167

Table 4 Univariate and multivariate analysis of factors predicting overall survival

	Univariate			Multivariate		
Factor	**RR**	**95% CI**	***P***	**RR**	**95% CI**	***P***
Tumor size (<2 cm, ≥2 cm)	0.34	0.17–0.69	0.0030*	0.55	0.26–1.17	0.12
Lymph node status (0, ≥1)	0.15	0.07–0.33	<0.0001*	0.22	0.10–0.49	0.0002*
Histological grade (1–2, 3)	0.39	0.19–0.79	0.0095*	0.58	0.27–1.22	0.15
pERα Ser118 (0–6, 7–8)	0.34	0.17–0.68	0.0024*	0.26	0.12–0.57	0.0007*
pERα Ser167 (0–2, 3–8)	2.58	1.42–4.71	0.0020*	2.87	1.49–5.50	0.0016*
PR	0.93	0.84–1.03	0.15			
AIB1	0.69	0.37–1.27	0.23			
pMAPK	1.04	0.82–1.32	0.75			
pAKT	0.98	0.83–1.16	0.82			
HER2	0.41	0.13–1.34	0.14			
P53	0.62	0.29–1.33	0.22			
Ki67	0.66	0.36–1.22	0.19			

*$P<0.05$ is considered significant.

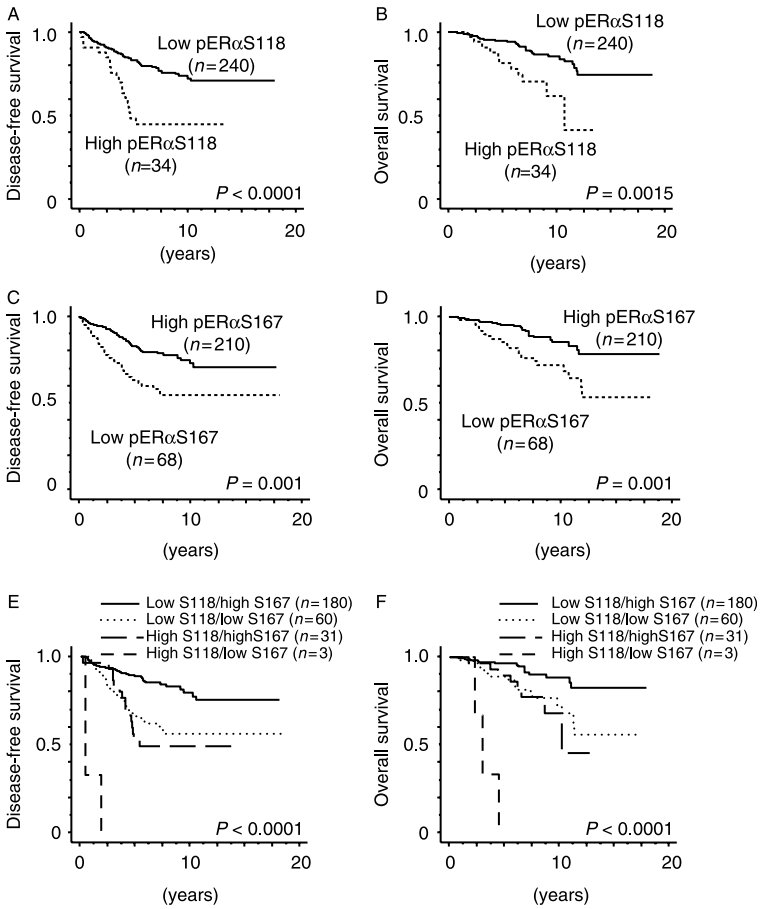

Figure 1 Effect of phosphorylation of ERα Ser118, ERα Ser167, and combination subgroups of ERα Ser118 and ERα Ser167 on disease-free (A, C, and E) and overall (B, D, and F) survival.

($P=0.0020$), as well as tumor size ($P=0.0030$), lymph node status ($P<0.0001$) and histological grade ($P=0.0095$; Table 4). There was no significant association between overall survival and phosphorylation of MAPK or Akt or expression of PR, AIB1, HER2, p53, or Ki67. In multivariate analysis, patients with low phosphorylation of ERα Ser118 ($P=0.0007$) and high phosphorylation of ERα Ser167 ($P=0.0016$), as well as patients with negative lymph node status ($P=0.0002$) had significantly improved overall survival (Table 4). We conclude that the phosphorylation levels of ERα Ser118 and ERα Ser167 are independent prognostic factors of disease-free and overall survival in ER-positive breast cancer.

Discussion

Using IHC technology, we investigated the phosphorylation of ERα Ser118, ERα Ser167, p44/42 MAPK, and Akt and expression of ERα, PR, AIB1, HER2, p53, and Ki67 in ER-positive breast cancer. Our results indicate that those whose tumors showed low phosphorylation of ERα Ser118 and high phosphorylation of ERα Ser167 had a better disease-free and overall survival.

Our study demonstrated that phosphorylation of ERα Ser118 was positively associated with phosphorylation of MAPK and expression of AIB1 and HER2, and that high phosphorylation of ERα Ser118 and high expression of AIB1 and HER2 significantly reduced disease-free survival in ER-positive breast cancer. Murphy *et al.* demonstrated that phosphorylation of ERα Ser118 correlated with active MAPK in 45 human breast tumor biopsies. Their result is consistent with our results that phosphorylation of ERα Ser118 and MAPK was strongly and positively correlated (Murphy *et al.* 2004*a*). Our data also showed that significant and positive association was found between phosphorylation of ERα Ser118 and expression of AIB1 and HER2. Gee *et al.* (2001) showed that phosphorylation of MAPK was associated with poor response to anti-hormonal therapy and decreased patient survival. It has been reported that ERα is phosphorylated on Ser118 by MAPK (Kato *et al.* 1995), which also

phosphorylates AIB1 (Font de Mora & Brown 2000). Furthermore, a recent study showed that AIB1 knockdown reduced EGF-induced HER2 phosphorylation (Lahusen *et al*. 2007). Because MAPK is located downstream of HER2, our study suggests that phosphorylation of ERα Ser118 is in part caused by HER2–MAPK signaling in human breast cancer and that the HER2–MAPK–AIB1–ERα Ser118 pathway may contribute to poor prognosis and endocrine therapy resistance in ER-positive breast cancer. Previous studies have shown that HER2-induced MAPK and ERα activation leads to tamoxifen resistance (Kurokawa *et al*. 2000). Data from these clinical trials demonstrated that the antiproliferative response to endocrine therapy was impaired in ERα-positive/HER2-positive primary breast cancers (Dowsett *et al*. 2001). It was also reported that ligand-dependent phosphorylation at Ser118 and Ser167 of ERα was lost in tamoxifen-resistant MCF-7 Her2/neu cells (Likhite *et al*. 2006). Sarwar *et al*. (2006) reported that phosphorylation of ERα Ser118 in 301 breast cancer tissues was higher in more differentiated tumors, whereas no significant correlation was found between phosphorylation of ERα Ser118 and clinicopathological factors, such as tumor size, lymph node status, and histological grade in our present study. Patients with ERα-negative tumors (6.3%) were included in their study. Although phosphorylation of ERα Ser118 was not associated with survival, there was a positive correlation between phosphorylation of MAPK staining and ERα Ser118 staining in their studies. They also demonstrated that phosphorylation of ERα Ser118 was elevated in tumor biopsies taken from patients who had relapsed following tamoxifen treatment. *In vitro* studies have demonstrated that phosphorylation of ERα Ser118 is stimulated by both estrogen-dependent and -independent pathways, so that a similar phenomenon may occur in clinical breast cancers.

Murphy *et al*. (2004*b*) analyzed phosphorylation of ERα Ser118 by IHC in 117 breast cancer tissues and showed that phosphorylation of ERα Ser118 is a marker of better prognosis in patients treated with tamoxifen. Their result is opposite to ours. However, expression status of ERα in their study was analyzed by ligand-binding assay not by IHC and there were four cases (3.4%) with ERα-negative tumors. Moreover, immunohistochemically determined phosphorylation status of ERα Ser118 was evaluated as positive if any nuclear staining was detectable and negative in the absence of any detectable nuclear staining. We set at the cutoff at a score of 6 by Allred's methods. Cutoff points for IHC analysis should be investigated in further studies.

We previously found that patients whose primary breast tumors showed high phosphorylation of ERα Ser167 responded significantly better to endocrine therapy than those that did not (Yamashita *et al*. 2005). In this study, we demonstrated that high phosphorylation of ERα Ser167 improves survival in ER-positive breast cancer. Moreover, our present data indicate that high phosphorylation of ERα Ser167 is associated with significantly increased disease-free survival in ER-positive breast cancer patients who received endocrine therapy alone as adjuvant therapy. Since our previous and present studies demonstrate that phosphorylation of ERα Ser167 is predictive of response to endocrine therapy and improved survival in ER-positive breast cancer, it is suggested that phosphorylation of ERα Ser167 may occur frequently in response to estradiol binding. By contrast, phosphorylation of ERα Ser118 was not predictive of response to endocrine therapy, and high phosphorylation of this residue correlated with poor prognosis, indicating that ERα Ser118 phosphorylation occurs frequently without estradiol.

Several studies have reported that phosphorylation of Akt predicts worse outcome and tamoxifen resistance in ER-positive breast cancer (Perez-Tenorio & Stal 2002, Kirkegaard *et al*. 2005, Tokunaga *et al*. 2006*a*,*b*). Our data did not indicate correlation between Akt phosphorylation and prognosis, although phosphorylation of Akt was strongly and positively associated with phosphorylation of ERα Ser118, ERα Ser167, and MAPK, and expression of AIB1. It was reported that estradiol rapidly activates Akt via the HER2 signaling pathway (Stoica *et al*. 2003). Akt might be activated via growth factor signaling pathways both estrogen dependently and -independently in breast cancer.

In conclusion, the present study has demonstrated for the first time that low phosphorylation of ERα Ser118 and high phosphorylation of ERα Ser167 significantly improve disease-free and overall survival in ER-positive breast cancer. Our data suggest that phosphorylation of ERα Ser118 and ERα Ser167 affects survival in ER-positive breast cancer and could be helpful in distinguishing patients who are likely to benefit from endocrine therapy alone from those who are not.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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