Phosphorylation of Estrogen Receptor-α at Ser^{167} Is Indicative of Longer Disease-Free and Overall Survival in Breast Cancer Patients

Jie Jiang,[1,2] Naveed Sarwar,[1] David Peston,[2] Elena Kulinskaya,[3] Sami Shousha,[2] R. Charles Coombes,[1] and Simak Ali[1]

Abstract

Purpose: Ser^{167} was first identified as a major phosphorylation site of the estrogen receptor -α (ER) positive in the MCF7 breast cancer cell line. Subsequent studies have shown that Ser^{167} phosphorylation is important in the regulation of ER activity and have identified p90RSK and AKT as protein kinases that phosphorylate Ser^{167}. The purpose of this study was to determine the importance of Ser^{167} phosphorylation in breast cancer progression.

Experimental Design: Immunohistochemical staining of primary breast cancer biopsies (n = 290) was carried out using antibodies specific for ER phosphorylated at Ser^{167} and for phosphorylated p44/p42 mitogen-activated protein kinase (MAPK), phosphorylated p90RSK, and phosphorylated AKT.

Results: In ER-positive breast cancer patients, Ser^{167} phosphorylation was associated with low tumor grade (P = 0.011), lymph node negativity (P = 0.034), and relapse-free (P = 0.006) and overall (P = 0.023) survival. Further, Ser^{167} phosphorylation was strongly associated with phosphorylated p90RSK (P < 0.001), previously shown to phosphorylate Ser^{167} *in vitro*, as well as being associated with phosphorylated MAPK (P < 0.0005). The activities of both kinases also seemed to be indicative of better prognosis. There was, however, no association between HER2 positivity and Ser^{167} phosphorylation nor were the activities of MAPK or p90RSK associated with HER2 status, suggesting that other cell surface receptors may be important in regulating these activities in breast cancer.

Conclusions: These findings show that phosphorylation at Ser^{167} of ER predicts for likelihood of response of ER-positive breast cancer patients to endocrine therapies.

Estrogen is a major factor in the development and progression of breast cancer, and acts by binding to estrogen receptors, to regulate their activities (1). Two thirds of all primary breast cancers express estrogen receptor-α (ER) and the presence of ER is predictive for response to adjuvant endocrine agents. Indeed, endocrine therapies are designed to limit estrogen biosynthesis by suppression of ovarian function in premenopausal women (luteinizing hormone-releasing hormone agonists; ref. 2) or by inhibiting aromatase activity (3). Antiestrogens act by binding to the ER to inhibit its activity. Tamoxifen, an antiestrogen that has agonist activity in certain tissues, and is hence named a selective ER modulator, has become the standard endocrine agent for the treatment of ER-positive breast cancer, where it acts as an antagonist. However, only half of all patients respond to tamoxifen (*de novo* resistance), and of the patients who respond, a large proportion of the patients with primary breast cancer and most of those presenting with metastatic disease acquire resistance during the course of treatment, with tumor progression and eventual death (4). *De novo* and acquired resistance are not restricted to tamoxifen but are also observed for other antiestrogens and also for aromatase inhibitors (5).

ER is a member of the nuclear receptor superfamily of ligand-activated transcription factors (6), which regulates gene expression through direct binding to estrogen response elements in promoters of estrogen-regulated genes and indirectly through recruitment to gene promoters by interaction with other transcription factors (7). At least in the case of genes directly regulated by ER, estrogen-bound ER promotes gene expression by coordinating the recruitment of transcriptional coactivators and RNA polymerase II at gene promoters (8). Antiestrogens inhibit the expression of estrogen-regulated genes by inhibiting coactivator recruitment, stimulating corepressor recruitment, and/or promoting receptor degradation (9, 10). In addition to its activation by ligand binding, however, ER activity can be stimulated by phosphorylation at specific serine residues. Of particular importance are Ser^{118} and Ser^{167}, which map to the NH_2-terminal transcription activation function AF-1 (11–15). Phosphorylation at these sites stimulates ER activity in the absence of ligand, and at least in the case of Ser^{118}, to increase

Authors' Affiliations: [1]Cancer Research UK Laboratories, Department of Oncology, [2]Department of Histopathology, and [3]Statistical Advisory Service, Imperial College London, London, United Kingdom

Received 4/9/07; revised 6/10/07; accepted 7/3/07.

Grant support: Cancer Research UK and Breast Cancer Research Trust.

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Requests for reprints: Simak Ali, Department of Oncology, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, United Kingdom. E-mail: simak.ali@imperial.ac.uk.

doi:10.1158/1078-0432.CCR-07-0822

ER activity in the presence of tamoxifen (11, 12, 16–18). Ser^{118} is phosphorylated by extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK; refs. 17–19), whereas Ser^{167} is phosphorylated by AKT (20) and p90RSK (21, 22).

Epidermal growth factor (EGF) can mimic the uterotropic effects of estrogen in ovariectomized mice in an ER-dependent manner, the rescue of uterine growth being prevented by the antiestrogen ICI164, 384 (23), whereas such a rescue does not occur in ER knockout mice (24), suggesting that EGF can activate ER in a ligand-independent manner. Moreover, EGF, as well as other growth factors that activate ERK1/2 MAPK and/or AKT signaling pathways, can activate ER in the absence of ligand and stimulate the activity of the estrogen- or tamoxifen-bound ER. Further, breast cancer models of acquired tamoxifen resistance have elevated levels of EGF receptor (EGFR) and HER2 as well as elevated ERK1/2 and AKT activities (25, 26), whereas HER2 overexpression in the ER-positive and estrogen-sensitive MCF7 cell line yields cell lines that are resistant to tamoxifen (27, 28). Elevated HER2 expression as well as ERK1/2 activity and phosphorylated Ser^{118} (P-S118) levels are also observed in MCF7 cells that become hypersensitive to estrogen, following long-term culturing in estrogen-depleted medium (29–31). In the clinical setting, ER-positive patients with ER-positive disease and elevated expression of EGFR and/or HER2 respond poorly to tamoxifen (reviewed in refs. 25, 26, 32). Further, ERK1/2 MAPK levels and/or levels of phosphorylated (i.e., activated) MAPK (P-MAPK) are frequently elevated in breast cancer (33). However, in primary breast cancer, P-MAPK is a good prognostic marker and is associated with longer disease-free interval (34, 35), although in ER-positive breast cancer high levels of P-MAPK have been correlated with poor response to hormonal therapy and shorter patient survival period in some studies (36). Levels of AKT phosphorylated at Ser^{473} (P-AKT), as well as levels of PDK-I, a kinase that activates AKT, are also often elevated in breast cancer (37), with P-AKT in ER-positive breast cancer being associated with decreased overall survival for patients receiving hormone therapy (38–40).

Collectively, these findings suggest the possibility that increased HER2/EGFR expression, ERK1/2, and/or AKT activities could contribute to endocrine resistance on stimulation of ER activity following Ser^{118} and/or Ser^{167} phosphorylation. In agreement with the association of high-level ERK1/2 activity being a good prognostic marker, however, recent studies using immunostaining to detect Ser^{118} phosphorylation in 117 primary breast tumors from node-negative patients showed that P-S118 is a good prognostic marker, P-S118 levels being correlated with progesterone receptor (PR) levels and longer disease-free survival (41, 42). Another study also found P-S118 to be associated with good prognosis but did not find a correlation with relapse-free or overall survival (35). In a larger study of ~300 ER-positive primary breast tumors, we also observed a trend toward an association between P-S118 levels and PR, which did not, however, reach statistical significance ($P = 0.09$; ref. 43). Further, there was a strong association between Ser^{118} phosphorylation and low histologic grade ($P < 0.001$), also indicating that P-S118 is a good prognostic marker.

Given the strong association between AKT and poor prognosis, we have carried out immunostaining for phosphorylated Ser^{167} (P-S167) and P-AKT using 290 primary breast cancer biopsies. Because Ser^{167} is also phosphorylated by p90RSK, we determined phosphorylated p90RSK (P-p90RSK) levels in this series. Ser^{167} phosphorylation was also compared with HER2 and phosphorylated ERK1/2 and P-S118 so as to more fully evaluate these signaling pathways in mediating Ser^{167} phosphorylation in breast tumors.

Materials and Methods

Breast cancer samples. Two hundred and ninety patients with primary invasive breast cancer, who had undergone surgery at Charing Cross Hospital between 1981 and 2003, were selected based on the availability of clinical details at presentation and follow-up, including time to relapse, time to death, and ER and PR status (43). ER and PR status was confirmed using ER (Vector Laboratories) and PR (Biogenex) antibodies as described (43). The clinicopathologic characteristics of the patient cohort are shown in Table 1. Of the 290 patients, 247 (85%) received tamoxifen (20 mg/d), with 70 of these also receiving chemotherapy. Twenty-six patients (9%) received other endocrine agents, whereas 9 patients (3%) received only chemotherapy.

Table 1. Characteristics of ER-positive breast cancer patients ($n = 290$) and their primary tumors

	No. patients (%)
Age (y)	
<50	74 (25)
≥50	216 (75)
Tumor size (cm)	
<2	109 (38)
2-5	134 (46)
>5	26 (9)
Unknown	21 (7)
Histologic grade	
1	40 (14)
2	176 (61)
3	62 (21)
Unknown	11 (4)
Lymph node status	
Negative	87 (30)
Positive	139 (48)
Unknown	64 (22)
PR	
Negative	83 (29)
Positive	207 (71)
HER2/c-erbB2	
Negative	225 (77)
Positive	57 (20)
Not determined	8 (3)
P-MAPK (Thr^{202}/Tyr^{204})	
Negative	90 (31)
Positive	200 (69)
P-AKT (Ser^{473})	
Negative	84 (29)
Positive	190 (66)
Not determined	16 (5)
P-p90RSK (Thr^{359}/Ser^{363})	
Negative	40 (14)
Positive	234 (81)
Not determined	16 (5)
P-S118	
Negative	50 (17)
Positive	240 (77)
P-S167	
Negative	56 (19)
Positive	229 (79)
Not determined	5 (2)

Immunohistochemistry. Sections (4 μm thick) were cut from formalin-fixed, paraffin-embedded archival tissue blocks and processed as described (43). Antigen retrieval was carried out by pressure cooking using 10 mmol/L citrate buffer (pH 6.0) and immunohistochemistry was done as described (43) using antibodies for ER, PR, P-S118 (Cell Signaling Technology), P-MAPK (Thr^{202}/Tyr^{204}; Cell Signaling Technology), and P-p90RSK (Thr^{359}/Ser^{363}; Cell Signaling Technology). Immunohistochemistry for c-erbB2 (Dako) was carried out using the manufacturer's recommended methodology. P-AKT (Ser^{473}; Cell Signaling Technology) was used as described (38). P-S167 (Cell Signaling Technology) was used following determination of optimal conditions as described in Results. Immunostaining levels were scored using the modified McCarty's H-scoring system based on percentage of positive cells and the intensity of staining to provide a total score varying from 0 to 300. The staining was designated as negative (H-score of <50), weakly positive (+; H-score of 51-100), moderately positive (++; H-score of 101-200), or strongly positive (+++, H-score of 201-300; ref. 44). For determining the specificity of immunostaining for P-S167, the antibody was preincubated with a 100-fold molar excess of a peptide having the sequence 157-RRQGGRERLA(P)STNDKGSM-174, in which phosphoserine was present at the position corresponding to Ser^{167}.

Statistical analyses. Statistical comparisons were carried out using the Pearson's χ^2 test and McNemar test for categorical variables and the *t* and Mann-Whitney tests for continuous data. Exact distributions were used whenever needed. Association between Ser^{167} status and several clinicopathologic features was investigated using χ^2 tests. The McNemar test was used to determine whether there was a significant difference in the distribution of Ser^{167} phosphorylation before and after treatment failure. The Kaplan-Meier method was used to estimate survival functions. Survival analysis (Kaplan-Meier method and Cox regression) was used for analysis of overall and relapse-free survival, where relapse-free interval was defined as the time from initial diagnosis to documented date of first relapse and overall survival was defined as the time from initial diagnosis to death. Survival was compared in each of the clinicopathologic features using log-rank tests. Cox proportional hazards model was used to model survival in the presence of several clinicopathologic features simultaneously.

Results

Immunohistochemical staining of breast tumors. We determined the specificity of immunohistochemical staining for ER phosphorylated at Ser^{167} in several ways. First, immunostaining of 12 ER-negative and 4 ER-positive breast tumors showed no staining of the ER-negative cases (an example is shown in Fig. 1M and N), whereas nuclear staining was observed for some of the ER-positive cases. Further, immunostaining using P-S167 antibody that had been preincubated with a 100-fold excess of a peptide corresponding to amino acids 157 to 174, and containing phosphoserine at the position corresponding to residue 167 of ER, resulted in loss of immunostaining, whereas a peptide corresponding to serve 102 did not compete (Fig. 1A-D). Together, these data indicate that the P-S167 antibody specifically recognizes ER phosphorylated at Ser^{167}. Immunostaining for P-S118 and P-MAPK (Thr^{202}/Tyr^{204}) has previously been described (43). Immunostaining using antibodies for P-AKT and P-p90RSK was done using commercially available antibodies according to the manufacturers' protocols.

Relationship of ER phosphorylation at Ser^{167} with PR, HER2, and active protein kinases in primary breast cancer. Tissue blocks from 290 patients with ER-positive breast tumors were immunostained for P-S167. Of these, 229 (79%) were positive for P-S167, the remainder being negative (Table 1). The immunostaining was scored according to the H-scoring method (44). Of the P-S167–positive cases, 142 (50%) tumors received an H-score of 1, 66 (23%) tumors were scored as 2, and 21 (7%) received an H-score of 3 (see Fig. 1E-L). H-scores were similarly obtained following immunostaining for ER, PR, P-MAPK, P-AKT, and P-p90RSK. HER2 staining was scored as either positive or negative.

Analysis of the immunohistochemical scores using the Pearson's χ^2 test showed that P-S167 was positively associated with P-MAPK ($P < 0.0005$), P-p90RSK ($P < 0.0005$), and P-AKT ($P = 0.001$), but there was no association with HER2 status ($P = 0.265$) or PR ($P = 0.646$; Table 2). There was also a positive association between P-S167 staining and P-S118 levels ($P < 0.0005$). Further, P-MAPK and P-p90RSK levels were also not significantly associated with HER2, although there was a positive relationship between P-AKT levels and HER2 status ($P = 0.004$). There was no difference between levels of phosphorylated ER, P-MAPK, P-p90RSK, or P-AKT and ER or PR expression.

Phosphorylation of ER at Ser^{167} is a predictor of better prognosis in primary breast cancer. Comparison of the immunohistochemical scores using the Pearson's χ^2 test showed a negative association between P-S167 staining and tumor size ($P = 0.002$), but there was no relationship with age, histologic grade, or lymph node status. There was no association between P-S118 and tumor size or node status, but there was a negative association with tumor grade ($P = 0.009$) as described before (43). There was also a strong negative association of grade with P-MAPK ($P < 0.0005$), whereas HER2 positivity ($P = 0.002$) was associated with high grade. The only other relationships that reached statistical significance were that lymph node positivity was associated with greater tumor size ($P < 0.0005$) and with lower ER expression ($P = 0.004$) and a negative association between tumor size and P-p90RSK levels ($P = 0.031$).

P-S167–positive patients were less likely to relapse than P-S167–negative patients ($P = 0.001$), and there were fewer cancer deaths in the P-S167–positive group than in the P-S167–negative group ($P = 0.01$; Table 3). Of the protein kinases, only P-AKT showed an association. Here, high levels of P-AKT were observed in patients who relapsed ($P = 0.004$) and P-AKT positivity was associated with a greater likelihood of cancer death ($P < 0.0005$). Additionally, the expected relationships between disease-free interval and time to death with lymph node status, tumor size, tumor grade, and HER2 were observed, whereas high H-scores for ER were associated with longer disease-free interval ($P < 0.0005$) and time to death ($P < 0.0005$).

Multivariate Cox regression analysis that included all of the clinical features described above (Table 1), as well as P-S167, P-S118, P-MAPK, P-p90RSK, and P-AKT, was used to determine their association with disease-free interval and time to death. In this analysis, P-S167 positivity was significantly associated with longer disease-free interval ($P = 0.006$; Fig. 2A; Table 4) along with lymph node positivity ($P < 0.0005$) and HER2 ($P = 0.012$). No other variables were statistically significant predictors of disease-free interval. P-S167 positivity was also associated with better overall survival in multivariate Cox regression analysis ($P = 0.023$; Fig. 2B; Table 5) together with lymph node positivity (hazard ratio, 4.319; 95% confidence interval, 2.053-9.088; $P < 0.0005$), AKT (both levels 1 and 2 significantly differ from 0), and tumor grade (grade 3 versus 1: hazard ratio, 6.027; 95% confidence interval, 1.413-25.702; $P = 0.015$).

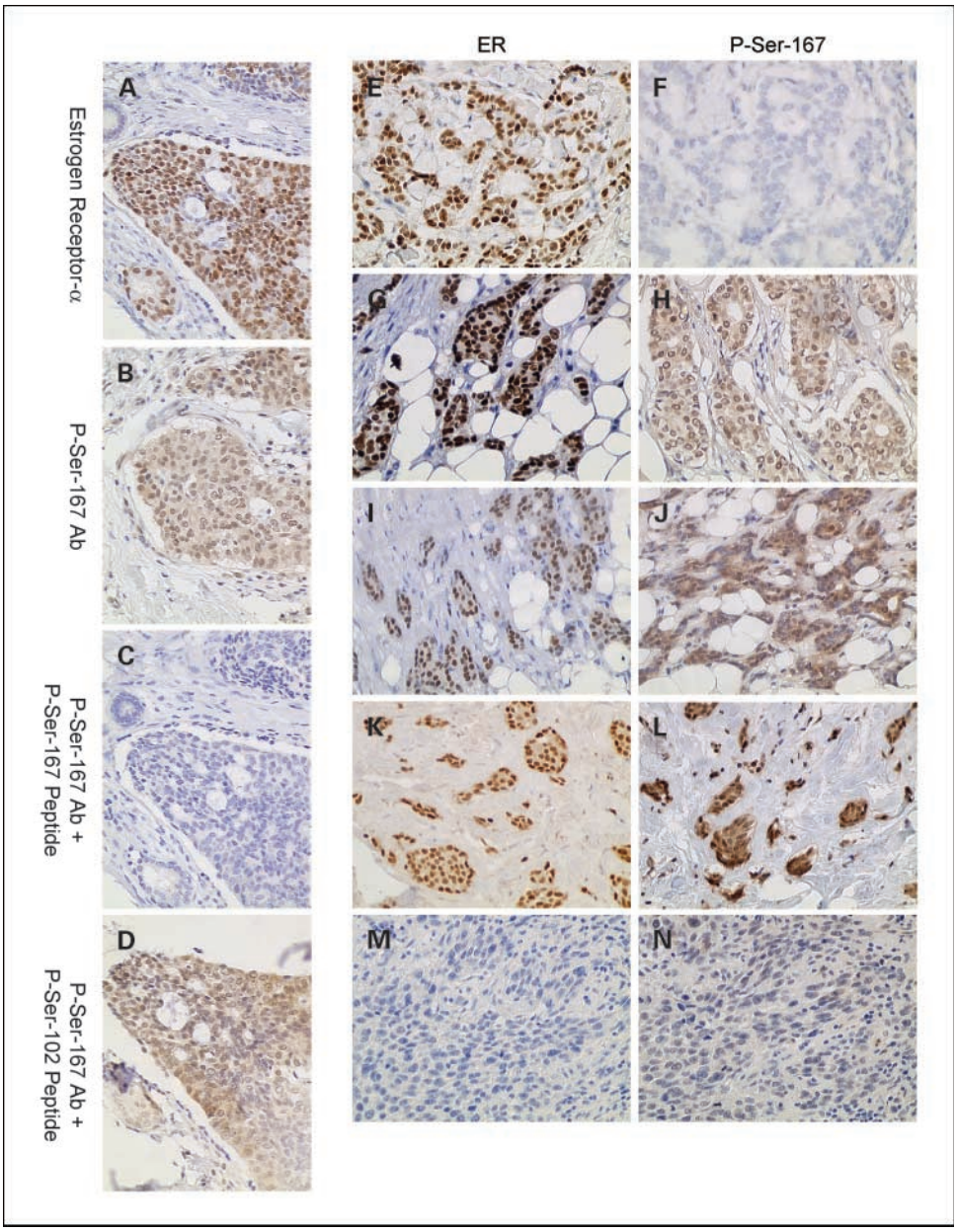

Fig. 1. Immunohistochemical detection of ER phosphorylated at Ser^{167} in human breast cancer sections. Magnification, ×200. Serial sections from a breast cancer biopsy were immunostained with antibodies for ER or P-S167. *A* to *C,* ER-positive breast carcinoma immunostained for ER (*A*) or with the P-S167 antibody in the absence (*B*) or presence (*C*) of a peptide containing phosphoserine at position 167 or a peptide containing phosphoserine at position 102 (*D*). *E* to *L,* serial sections from four breast tumors that were strongly ER positive (H-score: +++; *E, G, I,* and *K*) or shown alongside staining of a serial section for P-S167 scored as -(*F*), +(*H*), ++(*J*), and +++(*L*), respectively *M* and *N,* serial sections of an ER-negative breast carcinoma immunostained for ER (*M*) or P-S167 (*N*).

Discussion

The importance of phosphorylation of ER for its activity is indicated by studies using phosphorylation site mutants of ER, and ER activity is stimulated by its phosphorylation through activation of peptide growth factor–regulated signal transduction pathways (13, 14). Several phosphorylation sites have been identified, two of which, Ser^{118} and Ser^{167}, have received

Table 2. Relationships between the molecular markers P-S167, P-S118, P-AKT, P-p90RSK, and P-MAPK (*P* values from the χ^2 test for association)

	P-S118	ER	PR	HER2	P-AKT	P-MAPK	P-p90RSK
P-S167	<0.0005	0.513	0.646	0.265	0.001	<0.0005	<0.0005
P-S118		0.130	0.562	0.014	0.035	<0.0005	<0.0005
ER			0.115	0.002	0.683	0.603	0.509
PR				0.452	0.164	0.252	0.535
HER2					0.004	0.136	0.132
P-AKT						0.002	<0.0005
P-MAPK							0.001

Table 3. Pearson's χ^2 test for determining the relationships of clinicopathologic features with disease-free interval and overall survival (*P* values from the χ^2 test for association)

Variable	Relapse, yes/no (*P*)	Dead of cancer, yes/no (*P*)
P-S167	0.001	0.01
P-S118	0.331	0.106
ER	<0.0005	<0.0005
PR	0.036	0.005
HER2	<0.0005	0.001
P-AKT	0.004	<0.0005
P-MAPK	0.301	0.092
P-p90RSK	0.372	0.953
Age	0.481	0.521
Tumor size	<0.0005	<0.0005
Tumor grade	0.064	<0.0005
Lymph node status	<0.0005	<0.0005

particular attention, especially as both have been implicated in ligand-independent activation of ER. Much of this work has been carried out *in vitro* and it is less clear as to which sites are used *in vivo* and in particular how they feature in breast cancer. The availability of phosphorylation site-specific antibodies has allowed immunohistochemical investigation of the phosphorylation status of many proteins. Using antibodies for P-S118, it has been shown that ER is phosphorylated at Ser^{118} *in vivo* and seems to be an indicator of better prognosis.

Here, we present the results of immunohistochemical staining of 290 ER-positive primary breast cancer biopsies. In a large proportion of cases, ER was phosphorylated at one or both of the major phosphorylation sites located in the NH_2-terminal region of ER at positions Ser^{167} (79%) or Ser^{118} (77%). Although there was an association between P-S167 positivity and P-S118 ($P < 0.0005$), there was no association between ER levels and levels of P-S167 or P-S118, indicating that ER phosphorylation shows differential regulation in breast cancer and is not simply a reflection of levels of ER protein. Ser^{167} is phosphorylated by p90RSK (21) and AKT (20). In agreement, P-S167 levels were strongly associated with P-p90RSK ($P < 0.0005$) and also associated with P-AKT ($P = 0.001$; Fig. 3), although the association of P-AKT with poor survival suggests that p90RSK may be more important than AKT for Ser^{167} phosphorylation (see below). However, there was also a statistically significant association of P-S118 with P-AKT, this perhaps being a reflection of the strong association between P-AKT and P-MAPK ($P = 0.002$).

The association between levels of active AKT and active MAPK suggests that both signaling pathways are activated in a proportion of ER-positive breast cancers. Although HER2 status was associated with P-AKT ($P = 0.004$), a similar relationship with P-MAPK was not seen, suggesting that other tyrosine kinases, such as HER1/EGFR and/or insulin-like growth factor receptors, are important for the elevated MAPK activity observed in some breast cancers. Indeed, increased EGFR levels have been reported in breast cancer cell lines that "acquire" tamoxifen resistance following continuous culturing in the presence of tamoxifen (45, 46), whereas response to tamoxifen is considerably lower in patients expressing EGFR and/or HER2 than in patients negative for EGFR and HER2 (47). Increased insulin-like growth factor-I receptor activity has also been linked to tamoxifen resistance in breast cancer cell lines, whereas elevated insulin-like growth factor-I receptor expression is common in breast tumors and insulin-like growth factor-I receptor and downstream signaling components have been linked to disease progression and recurrence (48, 49).

Earlier studies using cytosols prepared from mammary carcinomas indicated that ERK1/2 MAPK activity is elevated in breast cancer (33) and MAPK activity correlated with node-positive disease, with a higher risk of relapse (50), although the correlation did not reach statistical significance in multivariate analysis. Whereas one immunohistochemical study of 90 patients with locally advanced or metastatic breast cancer showed that high levels of phosphorylated ERK1/2 correlated with decreased survival and poor response to tamoxifen in ER-positive cases (36), another study in which immunostaining for P-MAPK was carried out for 120 patients presenting with primary breast cancers showed that high P-MAPK levels were

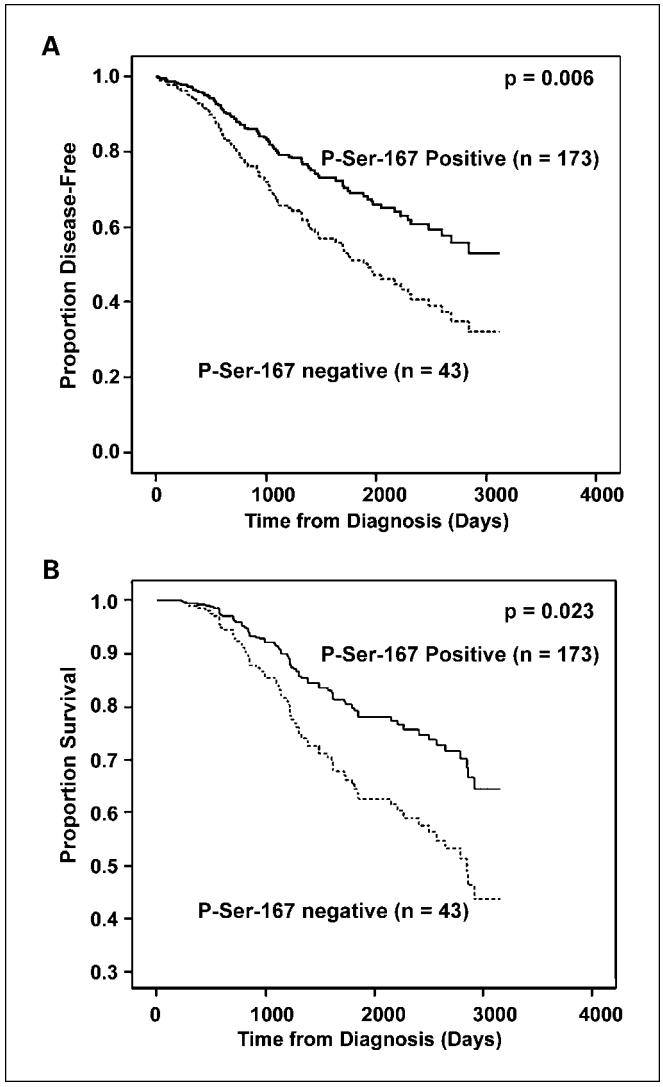

Fig. 2. Kaplan-Meier survival curves for disease-free and overall survival. Disease-free (*A*) and overall (*B*) survival according to P-S167 positivity (+, ++, and +++), with P-S167 negativity being defined as an H-score of <50. The hazard ratios are given in Tables 4 and 5.

associated with a better prognosis (34). In the series of primary breast tumors described here, high levels of P-MAPK were associated with low grade ($P = 0.009$), suggesting that P-MAPK is an indicator of better prognosis, in agreement with the latter study. Further, it was recently shown that P-S118 is negatively associated with tumor grade (42, 43), a finding confirmed here. Additionally, P-S118 has been found in one study to be associated with better disease outcome in patients treated with tamoxifen (41). In agreement with the above-described studies, there was a positive association of P-S118 with P-MAPK in our patient group ($P < 0.0005$), which in light of the positive prognostic association for P-S118 provides further support for P-MAPK being a marker of better prognosis in ER-positive breast cancer.

A recent study of 75 women with metastatic breast cancer indicated that Ser^{167} phosphorylation is correlated with increased postrelapse survival (51). Evaluation of our series of 290 primary breast cancer cases using Pearson's χ^2 test revealed that P-S167 staining was associated with a lower likelihood of patient relapse ($P = 0.006$) when the comparison was carried out using the H-scores for P-S167 levels, suggesting that high levels of P-S167 are predictive of better outcome. Further, P-S167 positivity was also associated with reduced relapse ($P = 0.001$). P-S167 positivity was also associated with better overall survival ($P = 0.01$). This was confirmed with Cox regression multivariate analysis, which showed that P-S167–positive patients were almost half as likely to relapse as P-S167–negative patients. A similar survival advantage was observed for overall survival. Whereas active MAPK and p90RSK, which phosphorylates Ser^{167}, were associated with markers of good prognosis, immunostaining data for the active form of AKT, which also phosphorylates Ser^{167}, showed that P-AKT was associated with HER2, and with a greater likelihood of relapse ($P = 0.004$) and death due to cancer ($P < 0.0005$), and although P-AKT was not associated with disease-free survival, P-AKT positivity was associated with a 2- to 3-fold reduction in overall survival. This suggests that p90RSK, rather than AKT, may mediate Ser^{167} phosphorylation in breast cancer cells and that AKT is instead involved in regulating other cellular processes, leading to reduced patient survival. However, *in vitro* studies have indicated that Ser^{167} phosphorylation is principally mediated by AKT in the MCF7 breast cancer cell line and that AKT overexpression in these cells led to tamoxifen resistance (52) and indicates that AKT-mediated Ser^{167} phosphorylation may be important in nonresponse to tamoxifen in breast cancer patients.

Table 4. Cox multivariate analysis of disease-free survival in ER-positive breast cancer

Variable	HR (95% CI)	P*
Lymph node status	4.047 (2.137-7.663)	<0.0005
P-S167	0.506 (0.313-0.819)	0.006
HER2	1.833 (1.145-2.935)	0.012

NOTE: Multivariate analysis was done using stepwise Cox proportional hazards regression with forward selection. Candidate exploratory variables included age, tumor size, tumor grade, lymph nodes, ER, PR, HER2, P-MAPK, P-AKT, P-p90RSK, P-S118, and P-S167.
Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.
*Two-sided Wald test was used.

Table 5. Cox multivariate analysis of overall survival

Variable	HR (95% CI)	P*
Lymph node status	4.319 (2.053-9.088)	<0.0005
P-S167	0.529 (0.305-0.917)	0.023
P-AKT (1) †	2.154 (1.066-4.351)	0.033
P-AKT (2)	3.132 (1.419-6.913)	0.005
Grade 1 vs 2	2.310 (0.556-9.599)	0.249
Grade 1 vs 3	6.027 (1.413-25. 702)	0.015

NOTE: Multivariate analysis was done using stepwise Cox proportional hazards regression with forward selection. Candidate exploratory variables included age, tumor size, tumor grade, lymph nodes, ER, PR, HER2, P-MAPK, P-AKT, P-p90RSK, P-S118, and P-S167, where ER and PR were continuous.
*Two-sided Wald test was used.
†P-AKT (1) is the comparison of cases scored negative for P-AKT compared with cases receiving a score of 1. P-AKT (2) is the comparison of cases scored negative for P-AKT compared with cases receiving a score of 2.

What are the clinical implications of our findings? To date, there is no accepted method of predicting which patients with ER-positive, node-negative disease that also has other poor prognostic factors require cytotoxic chemotherapy in addition to tamoxifen. Tests such as the Oncotype DX (53) require

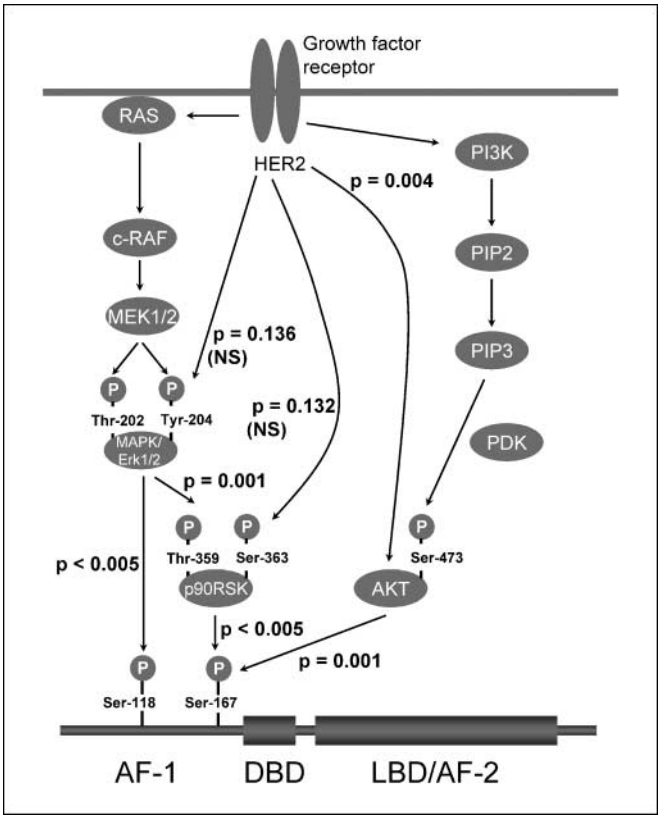

Fig. 3. The signal transduction pathways leading to Ser^{167} phosphorylation showing the associations observed in this study. The P values represent Pearson's χ^2 testing for correlation between the groups. PI3K, phosphatidylinositol 3-kinase; MEK, MAPK/ERK kinase; NS, not significant.

real-time assay of many transcripts, and a simple immunostaining protocol such as described here would be desirable. If our findings are confirmed in a prospective study, those patients whose tumors stain for P-S167/P-S118 ER may well be the patients who do not require chemotherapy unless there is evidence of lymph node involvement. One other area for future investigation is whether immunostaining for P-S167/P-S118 ER also predicts for patients who do not require sequential aromatase inhibitor following tamoxifen, such as advocated in the Intergroup Exemestane Study trial (54). Future probing of these samples will give us this information.

In summary, immunohistochemical determination of P-S167 in 290 ER-positive primary breast cancers shows that P-S167 positivity is a good prognostic marker, being associated with longer disease-free interval and overall survival. As the majority of the patients in this series went on to receive tamoxifen as the adjuvant agent, this would suggest that P-S167 positivity is associated with better response to antiestrogens. It would be interesting to determine whether a similar beneficial association would be observed for patients who become resistant to aromatase inhibitors.

Acknowledgments

We thank the members of the laboratory for helpful discussions and Drs. Laki Buluwela and Ross Thomas for their help with the manuscript.

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