Phosphorylation of ERα at serine 118 in primary breast cancer and in tamoxifen-resistant tumours is indicative of a complex role for ERα phosphorylation in breast cancer progression

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Abstract

Oestrogen receptor-α (ERα) is an important prognostic marker in breast cancer and endocrine therapies are designed to inhibit or prevent ERα activity. *In vitro* studies have indicated that phosphorylation of ERα, in particular on serine 118 (S118), can result in activation in a ligand-independent manner, thereby potentially contributing to resistance to endocrine agents, such as tamoxifen and aromatase inhibitors. Here we report the immunohistochemistry (IHC) of S118 phosphorylation in 301 primary breast tumour biopsies. Surprisingly, this analysis shows that S118 phosphorylation is higher in more differentiated tumours, suggesting that phosphorylation at this site is associated with a good prognosis in patients not previously treated with endocrine agents. However, we also report that S118 phosphorylation was elevated in tumour biopsies taken from patients who had relapsed following tamoxifen treatment, when compared to pre-treatment biopsies. Taken together, these data are consistent with the view that S118 phosphorylation is a feature of normal ERα function and that increases in levels of phosphorylation at this site may play a key role in the emergence of endocrine resistance in breast cancer.

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Introduction

It is now clear that oestrogens play a central role in promoting breast cancer development and progression (Ali & Coombes 2002). In this respect, oestrogen action is mediated through the oestrogen receptors ERα and ERβ. Two-thirds of all primary breast cancers are known to express ERα, where its presence correlates with a better prognosis and likelihood of response to endocrine therapies. These findings have led to effective strategies aimed at preventing ER activation by reducing oestrogen levels using inhibitors of the oestrogen biosynthetic enzyme, aromatase (Johnston & Dowsett 2003) or, in the case of pre-menopausal women, using luteinizing hormone-releasing hormone (LHRH) agonists that act to inhibit oestrogen synthesis by suppression of ovarian function (Klijn *et al.* 2001).

In a second and highly effective approach in breast cancer treatment, tamoxifen is used as a selective oestrogen receptor modulator (SERM) that binds to ER, resulting in a conformational change, leading to inhibition of receptor activity. Thus, tamoxifen acts by competing with oestrogen for binding to ER, leading to inhibition of tumour growth. For many patients, tamoxifen is still the first line adjuvant agent in the treatment of ERα-positive breast cancer and provides

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response in pre-menopausal women, as well as in post-menopausal patients. Further, tamoxifen is an effective treatment for about half of all patients with ERα-positive metastatic disease and prolongs disease-free survival and overall survival in the adjuvant setting (EBCTC Group 1998, Osborne 1998, Ali & Coombes 2002). Additionally, tamoxifen treatment is protective for the incidence of contralateral invasive breast cancer, which has led to the proposal that it may be efficacious for breast cancer prevention. This hypothesis has been confirmed by the results of several clinical trials with tamoxifen and another SERM, raloxifene, which show reduction in early incidence of breast cancer in women in high-risk groups (Powles 2002, Cuzick *et al.* 2003).

A major complication in breast cancer treatment is the recognition that a substantial proportion of patients with ERα-positive breast cancer are *de novo* resistant to tamoxifen and many others who initially respond eventually acquire resistance (Ali & Coombes 2002, Ring & Dowsett 2004). Similar data have been obtained for other anti-oestrogens (Howell *et al.* 2004) and for aromatase inhibitors, although third-generation aromatase inhibitors may show delayed emergence of endocrine resistance, when compared with tamoxifen (Johnston & Dowsett 2003). The mechanisms underlying this resistance are still poorly understood and identification of the factors and pathways responsible for the development of resistance is therefore an important diagnostic and therapeutic challenge in breast cancer research.

It was originally proposed that resistance to endocrine agents involved the loss of ERα. However, it is now clear that the majority of tamoxifen-resistant tumours continue to express ERα (Robertson 1996, Ring & Dowsett 2004). Furthermore, tumours resistant to one form of endocrine therapy frequently respond to alternative endocrine treatment. For example, the aromatase inhibitor anastrozole has been shown to give response in around 30% of patients who had developed resistance to tamoxifen (Buzdar & Howell 2001). Similar responses have been achieved following relapse on tamoxifen, with the potent anti-oestrogen ICI 182780, also known as Fulvestrant (Howell *et al.* 1996). These findings provide *in vivo* evidence to indicate that ERα continues to play an important role in cancer cell growth in resistant tumours.

ERα is a member of the nuclear receptor superfamily of ligand-activated transcription factors, which is activated upon binding oestrogen (Chawla *et al.* 2001). Oestrogen binding regulates ERα dimerization, intracellular localization and stability. Additionally, oestrogen binding stimulates ERα phosphorylation at several sites. The best studied of these is serine 118 (S118) (Ali *et al.* 1993, Le Goff *et al.* 1994). The oestrogen-stimulated phosphorylation of S118 is mediated by the TFIIH kinase cdk7 and phosphorylation by cdk7 stimulates ERα activity (Joel *et al.* 1998, Chen *et al.* 2000, Chen *et al.* 2002, Ito *et al.* 2004). Furthermore, S118 phosphorylation can be induced by growth factors such as epidermal growth factor (EGF) and insulin-like growth factor (IGF), likely acting through direct phosphorylation of S118 by extracellular signal regulated kinase (ERK)1/2 mitogen-activated protein kinase (MAPK) (Kato *et al.* 1995). The growth factor and MAPK-induced phosphorylation of S118 can result in ligand-independent activation of ERα (Bunone *et al.* 1996), which could be important in endocrine resistance, particularly given that MAPK activity is significantly increased in a large proportion of breast cancers (Sivaraman *et al.* 1997) and elevated MAPK levels correlate with a poor response to endocrine therapies (Gee *et al.* 2001).

These findings suggest that ligand-independent activation of ERα through phosphorylation of S118 may contribute to *de novo* and/or acquired resistance to endocrine therapies. In order to address this possibility we undertook a study to establish protocols for the immunohistochemical detection of ERα phosphorylated at S118 (P-S118) and to determine whether there is any relationship between levels of P-S118 and prognostic factors, survival and outcome of endocrine therapies.

Materials and methods

Human breast cancer samples

A total of 301 breast cancer cases were selected from the Charing Cross Hospital Breast Tumour Bank. All had been obtained from surgery carried out between 1981 and 2003. The patient's age at presentation, tumour grade, tumour size, ERα and progesterone receptor (PR) status were recorded, as were the dates of first relapse and of death. The majority of the tumours (94%) were ERα-positive at the time of initial surgery, as defined by ligand-binding assay or immunohistochemical staining. ERα and PR status were re-determined at the time of this study by immunohistochemical staining as described below. The clinico-pathological characteristics of the patient cohort are shown in Table 1. Response to endocrine therapy was determined by examining the clinical records of patients who had assessable disease and had received either neoadjuvant hormonal therapy or palliative hormonal treatments for metastatic disease. An additional 21 patients were identified and their

Table 1 Relationship between levels of ERα phosphorylated at serine 118 and clinical features

	P-S118-negative *n* (%)[b]	P-S118-positive *n* (%)[a]	Chi-squared[c]	*P* value[d]
Age (years)				
<50	23 (30)	15 (70)		
50–70	34 (18)	55 (82)	5.12	0.08
>70	6 (16)	13 (84)		
Grade				
I	4 (11)	32 (90)		
II	27 (15)	149 (85)	22.35	<0.001
III	31 (40)	46 (60)		
Unknown	1	11		
Tumour size				
<2 cm	23 (21)	87 (79)		
2–5 cm	29 (20)	113 (80)	2.70	0.26
>5 cm	9 (35)	17 (65)		
Unknown	2	21		
Nodal status				
Negative	19 (21)	73 (79)	0.20	0.66
Positive	39 (27)	108 (73)		
Unknown	5	57		
ERα				
Negative	15 (79)	4 (21)	41.20	<0.001
Positive	48 (17)	234 (83)		
PR				
Negative	26 (27)	71 (73)	2.91	0.09
Positive	37 (18)	166 (82)		
ND[e]	0	1		
c-erbB2				
Negative	17 (21)	66 (79)	1.02	0.31
Positive	7 (30)	16 (70)		
ND	39	156		
P-MAPK				
Negative	41 (44)	53 (56)	41.00	<0.001
Positive	22 (11)	180 (89)		
ND	0	5		

[a]P-S118-positive, H-score +, ++, +++.
[b]Percentage of cases in each horizontal group that were P-S118-negative.
[c]Pearson's chi-squared test. The analysis was carried out using known samples only.
[d]A value of $P<0.05$ denotes a statistically significant difference.
[e]ND denotes not determined.

evaluation of response to treatment was determined using response evaluation criteria in solid tumours (RECIST) (Therasse *et al.* 2000). Time to progression was defined as the time from initial diagnosis to documented date of first relapse, and the time to death was defined as the time from initial diagnosis to death.

Clinical records of patients who had become resistant to hormonal therapy were also examined, and where a repeat biopsy had been obtained at relapse, whilst the patient was taking tamoxifen, or for patients who relapsed 1–5 years following the completion of tamoxifen treatment. Immunostaining for ERα and P-S118 was performed in breast cancer samples obtained before and after becoming resistant to hormonal therapy. The mean time to relapse in this group of patients was 51 months ($n=21$; range 17–112 months following initial diagnosis). The study fulfilled the Institutional Ethics Review Board's guidelines for the use of stored tissues samples.

Immunohistochemistry

Sections of 4 μm were cut from formalin-fixed, paraffin-embedded archival tissue blocks and mounted on 3-aminopropyltriethoxysilane (APES; Sigma) treated slides and dried overnight at 37 °C. Sections were placed in a 60 °C oven for 1 h prior to being dewaxed in xylene and rehydrated through graded alcohols. Endogenous peroxidase was blocked by immersing in 2% hydrogen peroxide in methanol for 10 min. Antigen retrieval was carried out by pressure cooking, using 10 mM citrate buffer pH 6.0 and immunohistochemistry (IHC) was performed as previously described (Taylor *et al.* 1998), using mouse monoclonal antibodies for P-S118 (cat. no. 2511; New England Biolabs, UK), activated mitogen-activated protein kinase (MAPK, cat. no. 4376; New England Biolabs, Herts, UK), ERα (cat. no. VP-E613; Vector Laboratories, Peterborough, UK), progesterone receptor (PR, cat. no. MU328-UC; Biogenex, USA) and a rabbit polyclonal antibody for c-erbB2 (cat. no. A 0485; Dako). P-S118 levels were scored by a trained histopathologist, using the modified McCarty's H-scoring system, which was based on the 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 <50), weakly positive (+; H-score of 51–100), moderately positive (++; 101–200) or strongly positive (+++, 201–300) (McCarty *et al.* 1985). For determining the specificity of immunostaining for P-S118, the antibody was pre-incubated with a 100-fold molar excess of a peptide having the sequence 112-HPPPQLSPFLQPH-124 or the same peptide in which the serine residue was substituted by phosphoserine.

Statistical analyses

All statistical analyses were carried out using Stata 7 software (http://www.stata.com/). P-S118 scores were compared with different clinico-pathological features using the Pearson chi-squared test (χ tests) for categorical variables and the *t* and Mann–Whitney tests for continuous data. Association between S118 status and a number of clinico-pathological

features were investigated using chi-squared tests (where applicable Fisher's exact tests were used). The Wilcoxon matched pairs signed rank test was used to determine whether there was a significant difference in the distribution of S118 phosphorylation before and after treatment failure. Survival was compared in each of the clinico–pathological features using log rank tests for equality of survivor function.

Cell culture

All cells were cultured at 37 °C in a humidified 5% v/v CO_2 incubator. MCF7 cells (ATCC, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS). For preparation of whole cell lysates MCF7 cells plated on 9-cm dishes were transferred to DMEM lacking phenol red and containing 5% dextran-coated charcoal-stripped fetal calf serum (DSS) for 72 h, prior to the addition of oestradiol-17β (E_2; 10 nM), 4-hydroxytamoxifen (OHT; 100 nM), ICI 182780 (ICI; 100 nM), epidermal growth factor (EGF; 100 ng/ml) or phorbol myristate acetate (PMA; 100 ng/ml) for 15 min, as appropriate. The MAPK/ERK-kinase (MEK) inhibitor, U0126 (25 μM) was added 1 h prior to the addition of E_2, EGF or PMA. An equal volume of the solvent in which the different reagents were prepared was added to the appropriate samples to control for solvent effects. MCF7 Tam-R and matched MCF7 cells were maintained in RPMI 1640 lacking phenol red, supplemented with 5% DSS and containing OHT (100 nM), as described (Knowlden *et al.* 2003). MCF7/TAMR-4 have previously been described (Madsen *et al.* 1997). MCF7/TAMR-4 and matched MCF7 parental cells were maintained in DMEM/F12 lacking phenol red, supplemented with 1% FCS and containing 10^{-6} M tamoxifen, as described (Lykkesfeldt *et al.* 1994, Madsen *et al.* 1997), but may also be maintained with the addition of 100 nM of the more potent OHT, as used in this study. For preparing lysates from the Tam-R, MCF7/TAMR-4 and their respective matched parental MCF7 cells, the media were replaced by medium lacking FCS and in the absence of tamoxifen for 24 h prior to harvesting.

Cell lysate preparation and immunoblotting

Lysates were prepared essentially as previously described (Joel *et al.* 1998), by washing the cells with pre-warmed (37 °C) PBS. Then 0.5 ml of 2× sample buffer (0.12 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 0.2 M dithiothreitol, 0.008% bromophenol blue), heated to 100 °C, was added to each 9-cm dish. The cells were scraped into 1.5 ml Eppendorf tubes in a heat block at 100 °C. The extracts were heated for 10 min, cooled on ice, aliquots obtained and then stored at −80 °C prior to use. Cell lysates were resolved using 10% SDS-PAGE and immunoblotting was carried out as previously described (Chen *et al.* 2002) using monoclonal antibodies for ERα (NCL-L-ER-6F11; Novocastra Laboratories, UK), P-S118 (cat. no. 2511; New England Biolabs, UK), ERK1, C-16 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated ERK1/2 MAPK (cat. no. 9106; New England Biolabs) and β-actin (C-2; Santa Cruz).

Results

ERα phosphorylation in MCF7 breast cancer cells

We have previously used antibodies specific to ERα phosphorylated at S118 and transient transfection of COS-1 cells to demonstrate that ERα is phosphorylated by ERK1/2 MAPK in a ligand-independent manner, but that the E_2-stimulated phosphorylation of S118 is not mediated by MAPK. Rather, the E_2-stimulated phosphorylation of S118 was shown to be mediated by the TFIIH protein kinase, Cdk7 (Chen *et al.* 2000, 2002). In order to confirm that two distinct signalling pathways in breast cancer cells also mediate S118 phosphorylation, total lysates were prepared from MCF7 cells treated with E_2, PMA or EGF. In the case of E_2, P-S118 levels were raised within 5 min, peaked at 30 min and started to fall after 45 min (data not shown). With PMA or EGF, P-S118 levels rose within 5 min, peaked at 15 min and started to fall thereafter (data not shown). Figure 1A shows the levels of P-S118 at 15 min following the addition of E_2, EGF or PMA. As expected, pre-treatment with the MEK inhibitor, U0126, prevented S118 phosphorylation upon addition of EGF or PMA (Fig. 1B), but did not have any inhibitory effect on the stimulation of S118 phosphorylation by E_2 (Fig. 1C). Interestingly, the anti-oestrogens OHT and ICI 182780 also stimulated S118 phosphorylation (Fig. 1C). U0126 also failed to inhibit S118 phosphorylation stimulated by OHT and ICI.

The steady-state levels of P-S118 were next investigated in several MCF7 sub-lines derived by long-term culturing in the presence of tamoxifen in the culture medium (Lykkesfeldt *et al.* 1994, Knowlden *et al.* 2003). In the tamoxifen-resistant MCF7 sub-lines investigated the steady-state levels of P-S118 were elevated in the absence of E_2, OHT or serum, when compared with the parental MCF7 cells, as were levels of activated (phosphorylated) MAPK (Fig. 2). These data suggest that increased MAPK activity, as well as ERα phosphorylation and consequently increased ERα activity may feature in tamoxifen resistance in breast cancer.

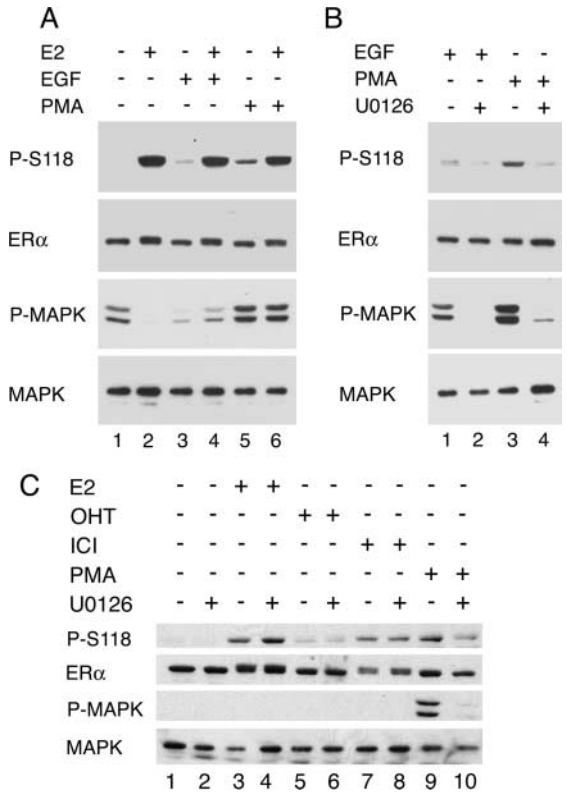

Figure 1 Immunoblotting of MCF7 human breast cancer cells for ERα phosphorylation at serine 118. MCF7 cells were harvested 15 min following the addition of E_2, OHT, ICI, EGF or PMA, as indicated. U0126 was added 1 h prior to the addition of ligands or growth factors. An equal volume of the solvent used to prepare the different ligands was added to the no-ligand controls. MCF7 lysates were resolved by 10% SDS-PAGE and immunoblotting was carried out using antibodies for P-S118, ERα, P-MAPK and MAPK, as shown.

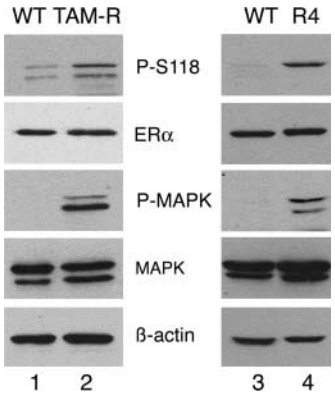

Figure 2 Immunoblotting of tamoxifen-resistant MCF7-derived sub-lines for P-S118. The tamoxifen-resistant sub-lines, together with parental MCF7 cells from which the lines were derived, were cultured in medium lacking FCS for 24 h prior to harvesting. The lysates were resolved by 10% SDS-PAGE and immunoblotting was carried out using antibodies for P-S118, ERα, P-MAPK, MAPK and β-actin, as shown. TAM-R and matched unselected (wild-type) MCF7 lines have been described (Knowlden *et al.* 2003) (lanes 1, 2). TAM-R4 and the matched unselected (wild-type) MCF7 have also been described (Madsen *et al.* 1997) (lanes 3, 4).

Immunohistochemical detection of ERα phosphorylated at serine 118 in human breast tumours

The utility of the P-S118 antibody for IHC was explored by developing protocols using formalin-fixed, paraffin-embedded MCF7 tumours generated in nude mice (data not shown) and subsequently optimized using primary breast tumour blocks from several ERα-positive and ERα-negative tumours. In establishing the IHC methodology, there was no detectable immunostaining of ERα-negative cases with the P-S118 antibody (data not shown), whereas nuclear staining was detectable in many of the ERα-positive cases (Fig. 3A, B). The specificity of staining was determined by pre-incubating the P-S118 antibody with a 100-fold excess of peptides encoding amino acids 112–124 of human ERα, in which a serine or a phosphoserine was present at position 118. Pre-incubation of the P-S118 antibody with the phosphorylated peptide resulted in loss of immunostaining (Fig. 3C), whereas the unphosphorylated peptide did not affect immunostaining (Fig. 3D). Further, competition was also performed using a peptide corresponding to amino acids 97–112 of human ERα and containing a phosphoserine at position 106 that is followed by a proline (as is S118), a previously described ERα phosphorylation site (Le Goff *et al.* 1994). Pre-incubation of the P-S118 antibody with this peptide, did not inhibit P-S118 staining (data not shown). Collectively, these data indicate that the P-S118 antibody specifically recognizes ERα phosphorylated at S118.

Tissue blocks from our cohort of 301 patients were sectioned for IHC to detect P-S118. In total, 282 (94%) of the tumours were ERα-positive, as determined by IHC. Of the 282 ERα-positive patients, 234 (83%) had detectable nuclear staining for P-S118.

Relationship between P-S118 staining and known prognostic features of breast tumours

Analysis of P-S118 positive cases by comparing the number of positive specimens in any given year showed that there was no statistical relationship between P-S118 staining and archival age, indicating that it is possible to evaluate relationships between P-S118 staining and prognostic features in this patient group. However, statistical analyses showed that there was no relationship between P-S118 staining (two

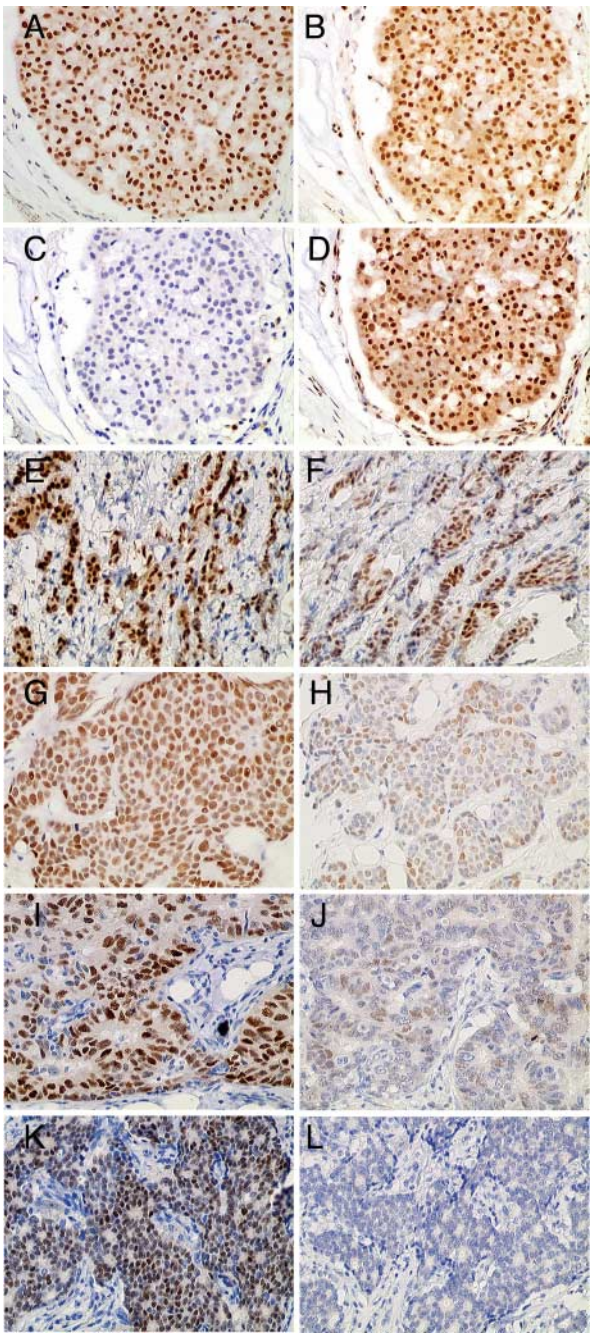

Figure 3 Immunohistochemical detection of ERα phosphorylated at S118 in human breast cancer sections (magnification ×200). Serial sections from a breast cancer biopsy were immunostained using antibodies for ERα (A) or P-S118 (B–D), in the presence or absence of a peptide-containing phosphoserine (C) or serine (D) at position 118. Serial sections from four breast tumours that were strongly ERα-positive (H-score: +++) (E, G, I, K) are shown alongside staining of a serial section for P-S118, scored as +++ (F), ++ (H), + (J) and – (L), respectively.

levels negative and positive) and tumour size, nodal status, PR and c-erbB2 status (Table 1). By contrast, there was a positive correlation of P-S118 with ERα ($P<0.001$). Furthermore, there was a clear inverse relationship between P-S118 staining and tumour grade ($P<0.001$), with lower grade tumours associated with higher H-scores for P-S118 staining ($P<0.001$). There was also some evidence of an association between P-S118 and age ($P=0.08$). However, P-S118 was not associated with survival, i.e. there was no statistically significant difference in survival between those positive and negative for P-S118 (log rank test for equality of survivor function χ^2, 1 degree of freedom = 1.43, $P=0.23$). Similarly there was no evidence of a statistically significant difference in survival for ERα (log rank test for equality of survivor function χ^2, 1 degree of freedom = 2.07, $P=0.15$), and c-erbB2 (log rank test for equality of survivor function χ^2, 1 degree of freedom = 1.59, $P=0.21$) in this cohort of patients. With respect to the other factors, there was a statistically significant difference in survival with regard to nodal status (log rank test for equality of survivor function χ^2, 1 degree of freedom = 14.82, $P<0.001$), grade (log rank test for equality of survivor function χ^2, 2 degrees of freedom = 13.46, $P=0.001$), tumour size (log rank test for equality of survivor function χ^2, 2 degrees of freedom = 16.22, $P<0.001$), age (log rank test for equality of survivor function χ^2, 2 degrees of freedom = 7.45, $P=0.02$) and PR (log rank test for equality of survivor function χ^2, 1 degree of freedom = 5.41, $P=0.02$).

IHC was also carried out using antibodies specific for activated MAPK. In total, 202 (68%) of the 296 cases immunostained scored positive for nuclear, phosphorylated (activated) MAPK. There was a positive correlation between phospho-MAPK staining and P-S118 staining ($P<0.001$), as might have been expected, given that ERK1/2 MAPK phosphorylates ERα at S118. There was no evidence of a correlation between P-MAPK and survival (log rank test for equality of survivor function χ^2, 1 degree of freedom = 0.46, $P=0.50$).

P-S118 levels do not predict for response to endocrine treatments following relapse on tamoxifen

As described above, immunostaining of 301 primary breast cancer biopsies showed a positive correlation of P-S118 staining with ERα, as well as a negative correlation with tumour grade, and evidence for an association with PR status ($P=0.09$). These data suggest that phosphorylation of S118 is a marker of better

prognosis and likelihood of response to endocrine treatment. We identified a group of patients who presented with ERα-positive primary breast cancer, were given adjuvant tamoxifen following surgical resection of the primary tumour and who then relapsed, at which point they received second-line endocrine agents. The patients in this group were subsequently scored as responders and non-responders according to RECIST criteria (Therasse *et al.* 2000). IHC of the primary breast cancer biopsies from these patients for P-S118 revealed that levels of P-S118 immunostaining were variable in both groups and there was no correlation between P-S118 staining and response.

Comparison of S118 phosphorylation in breast cancer pre- and post-tamoxifen treatment

The above data indicate that P-S118 levels in primary breast tumours are not predictive of response to endocrine therapies. However, for patients who initially respond and then relapse, it is possible that P-S118 levels increase during acquisition of resistance. Indeed, in MCF7-derived models of tamoxifen resistance P-S118 levels are elevated, as are levels of activated MAPK (Fig. 2). IHC was performed on biopsies from 21 patients who relapsed, either whilst receiving tamoxifen or after they had completed 5 years on tamoxifen, and for whom biopsy material taken prior to the initiation of tamoxifen treatment, was also available. Comparison of H-scores showed that P-S118 levels were increased following tumour regrowth (Table 2). Overall, there was a statistically significant difference in the distribution of S118 phosphorylation before and after treatment ($z=-2.357$, $P=0.02$). There were 2 comparisons in which S118 phosphorylation was higher pre-treatment, 11 where phosphorylation was higher post-treatment and 8 comparisons in which P-S118 levels were the same. The difference in P-S118 levels in pre- and post-treatment samples was not due to differences in total ERα levels, with no significant difference in the distribution of ERα before and after treatment ($z=-0.815$, $P=0.42$), whilst PR levels were significantly lower in the post-tamoxifen series, compared to the pre-treatment samples ($z=2.350$, $P=0.02$).

Table 2 Levels of serine 118 phosphorylation in primary tumours and biopsies taken following relapse after tamoxifen adjuvant therapy[a]

	P-S118	ERα	PR
Increased	11	7	3
Decreased	2	3	11
No change	8	10	7
Total	21	21	21
z[b]	−2.357	−0.815	2.350
P[c]	0.02	0.42	0.02

[a]Immunostaining was scored as −, +, ++, +++, using the H-scoring method. Changes in H-score for matched pre- and post-treatment biopsies are shown.
[b]The Wilcoxon matched pairs signed rank test was used to determine statistically significant differences.
[c]A value of $P<0.05$ denotes a statistically significant difference.

In 11 cases, the patients relapsed whilst receiving tamoxifen. Since tamoxifen stimulates S118 phosphorylation, it is possible that the elevated P-S118 levels are due to the continued presence of tamoxifen in the blood and tissues. It has been estimated that significant levels of tamoxifen may remain in the blood for up to 6 weeks following discontinuation of administration. In the case of six of these patients, there was no difference in levels of P-S118 in the pre- and post-tamoxifen biopsies. For the other three cases, levels of P-S118 were increased in the resistant specimens. These data indicate that P-S118 levels do increase in a proportion of patients who have previously received tamoxifen and that the increase observed is not due solely to the presence of tamoxifen in the blood and tissues.

Discussion

We have previously used immunoblotting of extracts prepared from breast tumours to demonstrate the presence of ERα phosphorylated at S118 (Chen *et al.* 2002). In that study, S118 immunoblotting was carried out using an antibody specific for ERα phosphorylated at S118. Here we have employed a P-S118 antibody to develop an immunohistochemical protocol. Immunostaining of 301 breast cancer biopsies showed that a large proportion (84%) of ERα-positive breast tumours contain ERα phosphorylated at S118. As expected, statistical analysis demonstrated a positive correlation between P-S118 staining and ERα status. There was also a correlation between levels of P-S118 and tumour grade, with P-S118 levels being higher in lower grade tumours than in high-grade tumours. These findings indicate that phosphorylation of ERα at S118 is associated with a better prognosis, perhaps indicative of functional ERα, as suggested by previous reports showing that S118 phosphorylation is induced by oestrogen binding to ERα (Ali *et al.* 1993, Joel *et al.* 1995, 1998, Chen *et al.* 2000). Despite the correlation with tumour grade, there was no significant association between S118 phosphorylation and disease-free survival or overall survival in our data. Whilst these data were being analysed, another study examining P-S118 in 113 ERα-positive breast tumours reported similar

findings, namely a negative association of P-S118 with tumour grade (Murphy *et al.* 2004*b*). Additionally, the latter study reports that phosphorylation at S118 correlates with longer disease-free survival and a trend towards better overall survival, a finding recently confirmed in another small study (Gee *et al.* 2005). This correlation is not held up in our considerably larger examination of 282 ERα-positive primary breast cancers.

The above studies have not reported on ERα-negative breast tumours immunostained for P-S118. Unexpectedly, in our study a small number of ERα-negative tumours stained positive for P-S118. Previous studies have shown that breast cancer cell lines that are ERα-negative by immunostaining and biochemically, can express ERα mRNA (Castles *et al.* 1993, 1995, Daffada *et al.* 1994, Poola *et al.* 2000). Similarly, breast tumours negative for ERα by IHC have been shown to express ERα mRNA (Shaw *et al.* 1996, Jarzabek *et al.* 2005). It is possible that ERα mRNA detected in ERα-negative breast cancer cells are alternative mRNAs, such as alternative splice variants, which may generate truncated ERα proteins some of which are not detected by IHC, such as the truncated ERα polypeptide generated by skipping of ERα exon 5 sequences that can be detected in the ERα-negative BT20 cell line (Castles *et al.* 1993) and in breast tumours (Desai *et al.* 1997). Some of these variant ERα proteins may be phosphorylated at S118, but not detected by IHC for ERα. It should be noted, however, that three out of the four P-S118-positive tumours had low levels of nuclear ERα immunostaining, with H-scores of 10–20. Breast tumours are scored ERα-negative for H-scores less than 50 (McCarty *et al.* 1985, Goulding *et al.* 1995), the method also used for this study. Therefore, the P-S118 positivity in these cases may be due to high-level phosphorylation of the small amounts of ERα in these cells.

Although our study and the publication by Murphy *et al.* (2004*b*) indicate that S118 phosphorylation is a predictor of positive response to endocrine therapies, IHC for phosphorylated (activated) MAPK (P-MAPK) demonstrated a highly significant correlation between levels of P-S118 and P-MAPK, as has also been described for a study involving 45 breast tumours (Murphy *et al.* 2004*a*). Breast cancer cell lines that show regrowth following long-term oestrogen deprivation and show increased ER activity, demonstrate increased levels of activated ERK1/2 MAPK and some dependence on MAPK activity for proliferation in cell culture, as well as in xenografts (Jeng *et al.* 1998, Santen *et al.* 2002). P-S118 levels are also higher in these cells (Martin *et al.* 2003). Levels of activated MAPK are also higher in tamoxifen-resistant MCF7 cells, as shown here. Moreover, ERK1/2 MAPK is overexpressed in some breast tumours (Sivaraman *et al.* 1997) and elevated MAPK activity has been correlated with lymph-node positivity (Adeyinka *et al.* 2002), decreased disease-free survival (Mueller *et al.* 2000, Gee *et al.* 2001) and poor response to endocrine therapy (Gee *et al.* 2001). In light of our finding of S118 phosphorylation being a good prognostic marker, the strong association of P-S118 with P-MAPK is surprising, since MAPK phosphorylation of S118 has been shown to result in ligand-independent activation of ERα (Bunone *et al.* 1996), which would indicate that S118 phosphorylation should correlate with reduced likelihood of response to endocrine therapies and hence poor prognosis. By contrast, although levels of P-S118 and P-MAPK were elevated in post-tamoxifen treatment biopsies compared to the pre-treatment biopsies, the change in P-S118 levels between pre- and post-tamoxifen biopsies was not associated with changes in P-MAPK levels in the same samples (data not shown). This indicates that other signalling pathways are involved in the increase in S118 phosphorylation, perhaps involving S118 phosphorylation by Cdk7 (Chen *et al.* 2000, 2002, Ito *et al.* 2004).

Collectively, the IHC data presented here for 301 unselected primary breast cancer biopsies show that ERα is differentially phosphorylated at S118 in breast tumours. However, the data presented here do not support the contention arising from *in vitro* studies, which indicate that S118 phosphorylation may be predictive of failure to respond to endocrine therapies. This is confirmed by IHC performed on primary tumours from a group of patients who were initially treated with tamoxifen, relapsed and subsequently either did or did not respond to further endocrine therapy. Indeed, our findings suggest that phosphorylation of S118 is a marker of functional oestrogen signalling in breast cancer, which is amenable to inhibition by use of endocrine therapies.

We also carried out IHC for ERα, PR, P-MAPK and P-S118 in biopsies from patients who subsequently received tamoxifen and for whom biopsies at relapse were identified. Changes in ERα and PR have previously been reported to be reduced at progression or relapse in patients receiving tamoxifen (Johnston *et al.* 1995). In the current study, PR levels were significantly lower in the post-tamoxifen treatment compared to the pre-treatment specimens, in agreement with the above report, although we did not observe a significant reduction in ERα expression in our series, as judged by changes in H-score. Furthermore, it has been shown that levels of phosphorylated ERK1/2

(P-MAPK) and phosphorylated p38 MAPK were elevated in biopsies taken from patients who relapsed whilst receiving adjuvant tamoxifen, with a small proportion of cases showing amplification and/or overexpression of HER2 (Gutierrez *et al.* 2005), suggesting that cross-talk between ERα, HER2 and downstream protein kinases may contribute to tamoxifen resistance. In agreement with this idea the pre- and post-tamoxifen treatment specimens analysed here also demonstrated a significant increase in phosphorylated ERK1/2 levels.

Given these findings, the elevation in S118 phosphorylation in tamoxifen-resistant specimens was expected, especially as P-S118 levels in primary breast tumours were associated with levels of P-MAPK. However, there was no correlation between the increase in P-S118 levels and the increases in P-MAPK in the emergence of tamoxifen resistance, indicating that other signalling pathways are required for the increase in P-S118 levels, perhaps due to S118 phosphorylation by cdk7, although levels of cdk7 were not different in pre- and post-tamoxifen treatment biopsies (data not shown). However, cdk7-mediated phosphorylation of S118 is dependent on its recruitment to ERα by the association of the TFIIH complex, the recruitment of TFIIH being mediated by the XPD and p62 subunits of TFIIH (Chen *et al.* 2000), the levels of which were not determined. Furthermore, a recent report indicates that S118 may additionally be phosphorylated by glycogen synthase kinase-3 (GSK-3) (Medunjanin *et al.* 2005). Altered regulation of TFIIH subunits, including the activity of cdk7 and/or GSK-3 activity could contribute to the elevated S118 phosphorylation observed here. Additionally, an interaction between ERα and the catalytic subunit of protein phosphatase 2A or with protein phosphatase 5 results in dephosphorylation at S118 (Lu *et al.* 2003, Ikeda *et al.* 2004). Finally, results from studies in MCF7 cells indicate that ERα turnover, localization and activity are influenced by the activities of different protein kinases, in particular MAPK (Marsaud *et al.* 2003). Deregulation of any of these activities may contribute to the increased levels of P-S118 in tamoxifen-resistant cases.

We conclude that measurement of P-S118 levels is not indicative of likelihood of failure on endocrine therapies, although changes in P-S118 levels during the course of tamoxifen treatment described here raise the possibility that phosphorylation of ERα at S118 is important in the emergence of resistance. Further, S118 phosphorylation in primary breast tumours is likely to be mediated by MAPK, although MAPK may not be important for S118 phosphorylation following emergence of tamoxifen resistance. Indeed, in a randomized trial using the epidermal growth factor receptor (EGFR) inhibitor Gefitinib (ZD1839, Iressa) given one month prior to surgery, P-S118 levels were reduced in 42 (79%) of 53 tumours, with complete loss of P-S118 staining in 34% of these tumours. There was no significant loss in total ERα following treatment with Gefitinib (Polychronis *et al.* 2005). Thus, at least for EGFR-positive, ERα-positive cases, S118 phosphorylation is likely to be mediated largely by EGFR-mediated activation of MAPK.

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