

# Clinical significance of estrogen receptor phosphorylation

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## Abstract

Multiple sites of phosphorylation on human estrogen receptor  $\alpha$  (ER $\alpha$ ) have been identified by a variety of methodologies. Now with the emerging availability of phospho-site-specific antibodies to ER $\alpha$ , the relevance of phosphorylation of ER $\alpha$  in human breast cancer *in vivo* is being explored. Multiple phosphorylated sites in ER $\alpha$  can be detected in multiple breast tumor biopsy samples, providing evidence of their relevance to human breast cancer *in vivo*. Published data suggest that the detection in primary breast tumors of phosphorylation at some sites in ER $\alpha$  is associated with a better clinical outcome while phosphorylation at other sites is associated with a poorer clinical outcome most often in patients who have been treated with tamoxifen. This suggests the hypothesis that phospho-profiling of ER $\alpha$  in human breast tumors to establish an 'ER $\alpha$  phosphorylation code', may be a more accurate marker of prognosis and/or response to endocrine therapy in human breast cancer.

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## Introduction

Targeting the estrogen receptor (ER) signaling pathway using the selective ER modulator (SERM), tamoxifen, is efficacious in both treating and preventing breast cancer (Jensen & Jordan 2003, Jordan 2003). Owing to the central role of ER in estrogen signaling, the ER status of breast tumors has long been used to successfully predict response to endocrine therapy (Osborne *et al.* 1996). There are two known ERs, ER $\alpha$  and ER $\beta$ , but the ER status of breast tumors and its clinical correlations are based on the measurement of generally only ER $\alpha$  (Harvey *et al.* 1999) and current clinical assays measure ER $\alpha$  immunohistochemically (IHC) with specific antibodies. The impact of ER $\beta$  remains unclear although roles in human breast cancer have been suggested (Leygue *et al.* 1998, Murphy & Watson 2006, Skliris *et al.* 2006, Gruvberger-Saal *et al.* 2007, Honma *et al.* 2008).

While ER $\alpha$  expression is the gold-standard biomarker for predicting response to endocrine therapy, it is imperfect, predicting treatment response in ~50% of ER+ tumors (Osborne 1998, Clarke *et al.* 2003).

Therefore, many ER+ tumors are *de novo* resistant to tamoxifen without any prior exposure. Furthermore, many of these tumors that initially respond to tamoxifen can acquire resistance during and after tamoxifen therapy. This so-called progression from hormone dependence to independence is an important clinical problem limiting the long-term usefulness of the relatively nontoxic endocrine therapies as well as possibly impacting the use of SERMs as preventative agents.

Most acquired tamoxifen resistance (70–80%) occurs despite continued expression of ER $\alpha$  (Robertson 1996). Newer therapies targeting ER via different mechanisms, such as aromatase inhibitors (AI; Goss *et al.* 2003) and selective ER downregulators (e.g. ICI182780; Robertson 2002), or potential new therapies, such as electrophilic modulators of ER zinc fingers (Wang *et al.* 2004), were all developed from basic research into molecular mechanisms of ER action, but development of therapy resistance is likely also to be a problem clinically. Understanding molecular mechanism(s) of ER action still holds promise for identifying complementary and/or alternative approaches targeting other levels of ER signaling to treat ER+

breast cancer. Such knowledge may also identify ways to circumvent resistance, as well as offering new biomarkers beyond ER $\alpha$  for the more precise prediction of therapy responses. Current downstream markers of ER $\alpha$  activity such as progesterone receptor (PR) improve prediction (Bardou *et al.* 2003), but remain imperfect, supporting the need for other biomarkers to assist in the accurate prediction of treatment response.

### Molecular mechanisms of estrogen action and possible mechanisms of tamoxifen resistance

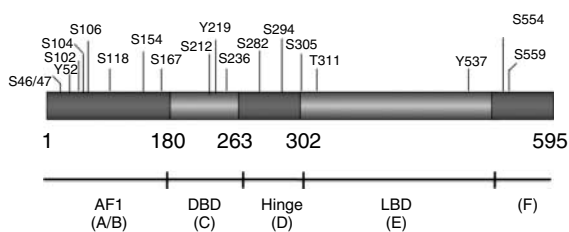
Basic research has significantly increased the knowledge of the molecular mechanisms of ER action (Hall *et al.* 2001, Nawaz & O'Malley 2004). Multifaceted mechanisms underlying estradiol (E<sub>2</sub>) action have been identified. These include multiple ERs and variants (Murphy *et al.* 2003); multiple subcellular localization sites (Murphy *et al.* 2003); multiple transcription coactivators and corepressors (McKenna *et al.* 1999); multiple posttranslational modifications (PTMs; Nawaz & O'Malley 2004); multiple levels of cross talk with other signaling pathways (Murphy *et al.* 2003); and multiple levels of control of ER expression, including proteasomal-mediated degradation (Reid *et al.* 2003). Alterations at any one of these levels could affect responsiveness to SERMs and/or AIs. There is evidence that multiple mechanisms are involved in altered SERM action during progression from hormone dependence to independence in breast cancer (Clarke *et al.* 2003, Murphy *et al.* 2003, Santen *et al.* 2004). In particular, growth factor receptor signaling pathways are frequently upregulated during tumorigenesis and cancer progression. The resulting increased cross talk with ER signaling is thought to be a mechanism of endocrine therapy resistance (Osborne *et al.* 2005). In part, this is due to kinases, activated by growth factor signaling, being able to phosphorylate and alter ER $\alpha$  activity in a ligand-independent manner (Kato *et al.* 1995). Effects on ER coactivator activity are also involved (Font de Mora & Brown 2000). It should also be noted that phosphorylation may influence ER protein levels through modulation of targeting ER for proteasomal degradation. ER can be lost during progression in 25–30% of ER+ tumors and it has been suggested that in some cases intra-tumoral factors such as hypoxia, growth factor, and cytokine signaling may act through phosphorylation of ER to cause reversible depression of ER $\alpha$  (Cooper *et al.* 2004, Creighton *et al.* 2006, Lopez-Tarruella & Schiff 2007, West & Watson 2010).

### Phosphorylation sites identified in ER $\alpha$

ER $\alpha$  can undergo multiple PTMs, for example, phosphorylation, acetylation, ubiquitylation, and sumoylation (Lannigan 2003, Weigel & Moore 2007). However, relatively little is known about the function and regulation of any of the PTMs that ER $\alpha$  can potentially undergo (Lannigan 2003, Ward & Weigel 2009) and even less is known about their relevance *in vivo*. As shown in Fig. 1, ER $\alpha$  can be phosphorylated on multiple amino acid residues throughout the whole protein and within all major structural domains: the N-terminal A/B domain, i.e. serine 46 (S<sup>46</sup>), serine 47 (S<sup>47</sup>), tyrosine 52 (Y<sup>52</sup>), serine 102 (S<sup>102</sup>), serine 104 (S<sup>104</sup>), serine 106 (S<sup>106</sup>), serine 118 (S<sup>118</sup>), serine 154 (S<sup>154</sup>), and serine 167 (S<sup>167</sup>); the DNA-binding or C domain, tyrosine 219 (Y<sup>219</sup>), serine 236 (S<sup>236</sup>; Chen *et al.* 1999); the hinge or D domain, serine 305 (S<sup>305</sup>; Michalides *et al.* 2004), and the ligand-binding domain or E domain, threonine 311 (T<sup>311</sup>; Lee & Bai 2002) and tyrosine 537 (Y<sup>537</sup>; Arnold *et al.* 1995b). Recently, novel phosphorylation sites in ER $\alpha$  were identified (Britton *et al.* 2008, Williams *et al.* 2009). Table 1 lists the sites of phosphorylation in ER $\alpha$ , which have been identified experimentally, using different methodologies. Detection of phosphorylation at some but not all of these sites has been confirmed in human breast tumor biopsy samples (Table 1).

### Some potential functions of phosphorylation at different sites in ER $\alpha$

The exact role of phosphorylation at individual or multiple sites is underexplored although effects on transcription, nuclear localization, dimerization, DNA binding, coactivator recruitment, and ligand binding (Weis *et al.* 1996, Chen *et al.* 1999, Endoh *et al.* 1999, Likhite *et al.* 2006) have been demonstrated in cell culture models (Murphy *et al.* 2006). More recently,



**Figure 1** Multiple phosphorylated sites in ER $\alpha$  have been identified by a variety of approaches as listed in Table 1. These are shown schematically in the figure that depicts different structural (A, B, C, D, E) and functional domains: activation function 1 (AF1), DNA-binding domain (DBD), hinge region, and ligand-binding domain (LBD) of human ER $\alpha$ .

**Table 1** Phosphorylation sites identified experimentally in human estrogen receptor  $\alpha$ 

Site of phosphorylation	Domain	Method of identification	Substrate source	References	Breast tumors <i>in vivo</i>
Ser46/47	A/B	1	TT/COS1	Williams <i>et al.</i> (2009)	ND
Tyr52	A/B	3	TT/HEK	He <i>et al.</i> (2010)	ND
Ser102	A/B	2	MCF7	Atsriku <i>et al.</i> (2009)	ND
Ser104	A/B	2	MCF7	Atsriku <i>et al.</i> (2009)	Y
Ser106	A/B	2	MCF7	Atsriku <i>et al.</i> (2009)	Y
Ser118	A/B	2	MCF7	Atsriku <i>et al.</i> (2009)	Y
Ser154	A/B	2	MCF7	Atsriku <i>et al.</i> (2009)	ND
Ser167	A/B	2	MCF7	Atsriku <i>et al.</i> (2009)	Y
Ser212	C	2	MCF7	Atsriku <i>et al.</i> (2009)	ND
Tyr219	C	3	TT/HEK	He <i>et al.</i> (2010)	ND
Ser236	C	2	MCF7	Atsriku <i>et al.</i> (2009)	ND
Ser282	D	1	TT/COS1	Williams <i>et al.</i> (2009)	Y
Ser294	D	1, 2	MCF7	Atsriku <i>et al.</i> (2009) and Williams <i>et al.</i> (2009)	Y
Ser305	D	3	TT/HeLa	Wang <i>et al.</i> (2002)	Y
Thr311	E	1, 3	TT/Ishikawa	Lee & Bai (2002)	Y
Tyr537	E	1	Sf9rER/MCF7	Arnold <i>et al.</i> (1995a)	ND
Ser554	F	2	MCF7	Atsriku <i>et al.</i> (2009)	ND
Ser559	F	1, 2	MCF7	Atsriku <i>et al.</i> (2009) and Williams <i>et al.</i> (2009)	Y

1, [ $^{32}$ P]H $_3$ PO $_4$  labeling; Edman degradation; phosphoamino acid analysis; phosphopeptide mapping. 2, mass spectroscopy. 3, site-directed mutagenesis; *in vitro* kinase assay; western blotting. ND, not determined; TT, transient transfection; Y, yes. Adapted from Murphy *et al.* (2006) with permission.

roles of ER $\alpha$  phosphorylation in RNA splicing (Auboeuf *et al.* 2002, 2007, Masuhiro *et al.* 2005) as well as in ER protein stability (Medunjanin *et al.* 2005, Grisouard *et al.* 2007) and regulation of other types of PTMs (Cui *et al.* 2004) have been suggested. A list of experimentally derived important functions of phosphorylation at different sites in ER $\alpha$  is shown in Table 2.

Interestingly, within the A/B domain of ER $\alpha$  often only small effects on transcriptional function were observed when any one site, e.g. S104, S106, and S118, was mutated to eliminate phosphorylation. While the effects of mutating all three sites appeared to be additive (Le Goff *et al.* 1994), giving ~50% reduction in transcriptional activity. Importantly, lack of phosphorylation of all of these sites does not eliminate estrogen-induced ER $\alpha$  transcriptional activity. Other data were reported showing that combinations of phosphorylation sites affected activity more than individual sites alone (Joel *et al.* 1998, Medunjanin *et al.* 2005). Furthermore, other data suggest that it is the combination of phosphorylation sites within ER $\alpha$  rather than any one individual site that may be important for mediating effects of any individual kinase (Rogatsky *et al.* 1999). The concept that combinations of PTMs of ER $\alpha$  rather than individual sites may be of primary importance in affecting function and response to endocrine therapies is

emerging (Barone *et al.* 2010, Skliris *et al.* 2010) and supports the hypothesis of a PTM code for ER $\alpha$ , as discussed below.

Phosphorylation, at least at S118, has been suggested to be involved in protein turnover via a proteasome-mediated mechanism (Valley *et al.* 2005, Grisouard *et al.* 2007). How other sites of phosphorylation may also affect receptor turnover is not clear and underexplored. However, proteasome-mediated turnover of steroid receptors has been shown to be essential for the dynamic and cyclical nature of receptor occupancy on target gene promoters, which is in turn critically important for transcriptional activity (Reid *et al.* 2003). Therefore, the further characterization of how phosphorylation at other sites may also affect receptor turnover and stability would be of interest.

An important hypothesis that has developed from laboratory models is that ligand-independent phosphorylation of ER $\alpha$  may cause tamoxifen resistance *in vivo*. For example, a well-studied p-ER $\alpha$  site (Lannigan 2003) is S118 (Fig. 1). Both E $_2$  and growth factors, e.g. epidermal growth factor (EGF) or insulin-like growth factor 1, stimulate phosphorylation of S118 (Joel *et al.* 1998, Chen *et al.* 2002, Lannigan 2003). Mitogen activated protein kinase (MAPK) (ERK1/2), an important enzyme activated by growth factor receptor pathways, can phosphorylate S118 in a ligand-independent manner *in vitro* (Kato *et al.* 1995)

**Table 2** Experimentally identified functional roles of site-specific phosphorylation in estrogen receptor  $\alpha$ 

Function	P-site	References
Ligand binding	Y537; general phosphorylation	Weis <i>et al.</i> (1996) and Arnold <i>et al.</i> (1997)
DNA binding	S167; Y219; S236; S305; Y537; general phosphorylation	Arnold <i>et al.</i> (1995a,b), Weis <i>et al.</i> (1996), Castana <i>et al.</i> (1997), Chen <i>et al.</i> (1999), Yudit <i>et al.</i> (1999), Shah & Rowan (2005), Tharakan <i>et al.</i> (2008) and He <i>et al.</i> (2010)
Dimerization	Y219; S236; Y537	Arnold <i>et al.</i> (1995b), Chen <i>et al.</i> (1999), Yudit <i>et al.</i> (1999) and He <i>et al.</i> (2010)
Transcription	S46/47; Y52; S104/106; S118; S167; Y219; S236; S282; S294; S305; T311; Y537; S559	Weis <i>et al.</i> (1996), Castana <i>et al.</i> (1997), Joel <i>et al.</i> (1998), Chen <i>et al.</i> (1999), Endoh <i>et al.</i> (1999), Rogatsky <i>et al.</i> (1999), Lee & Bai (2002), Tharakan <i>et al.</i> (2008), Williams <i>et al.</i> (2009) and He <i>et al.</i> (2010)
Coactivator binding	S104/106; S118; S305; T311; Y537	Weis <i>et al.</i> (1996), Endoh <i>et al.</i> (1999), Lee & Bai (2002), Dutertre & Smith (2003), Shah & Rowan (2005) and Tharakan <i>et al.</i> (2008)
Protein stability	Y52; S118; Y219	Valley <i>et al.</i> (2005), Murphy <i>et al.</i> (2006) and He <i>et al.</i> (2010)
Subcellular localization	T311	Lee & Bai (2002)
RNA splicing	S118	Masuhira <i>et al.</i> (2005)
Interaction with other PTMs	S305	Cui <i>et al.</i> (2004) and Rayala <i>et al.</i> (2006)
Cell growth/invasion	Y52; S118; Y219; S305	Murphy <i>et al.</i> (2006), Tharakan <i>et al.</i> (2008) and He <i>et al.</i> (2010)

and *in vivo* (Joel *et al.* 1998). Interestingly, estrogen treatment is the most powerful stimulator of phosphorylation at S118 and it is independent of MAPK (ERK1/2; Joel *et al.* 1998). However, which kinase is responsible for estrogen-induced p-S118 is not clear. CDK7, IKK $\alpha$ , and GSK3 $\beta$  are the possible candidates (Chen *et al.* 2002, Medunjanin *et al.* 2005, Park *et al.* 2005). Since ligand-independent ER $\alpha$  activation may underlie tamoxifen resistance, and EGFR/HER2 upregulation is associated with clinical resistance to tamoxifen in breast cancer (Pietras *et al.* 1995, Dowsett *et al.* 2001, Knowlden *et al.* 2003, Schiff *et al.* 2004), a role of p-S118-ER $\alpha$  has been suggested. S167 is another site of ER $\alpha$  phosphorylation. AKT (Campbell *et al.* 2001) and pp90rsk (Joel *et al.* 1998) can phosphorylate ER $\alpha$  at S167 and increased p-AKT has been associated with poor clinical outcome in breast cancer patients treated with tamoxifen (Kirkegaard *et al.* 2005). Also, experimental data suggest that ligand-independent phosphorylation of S305 may have a role in tamoxifen resistance in breast cancer cells as well (Michalides *et al.* 2004, Holm *et al.* 2009). However, the relevance of p-ER $\alpha$  in breast cancer *in vivo* is unclear (Lannigan 2003).

## Regulation of ER $\alpha$ phosphorylation

Table 3 provides a list of several kinases that have been shown experimentally to have a role in regulation of ER $\alpha$  phosphorylation at various sites but the contributions of specific kinases *in vivo* are not known. Those studies providing evidence of a potentially

direct role of an individual kinase in phosphorylating ER $\alpha$  are also shown in Table 3.

Correlation of expression of kinases with individual p-ER $\alpha$  expression in human breast tumor samples (Murphy *et al.* 2004b, Sarwar *et al.* 2006, Jiang *et al.* 2007, Yamashita *et al.* 2008) is one approach to gain insight into the kinases involved in regulation *in vivo*. In this regard, even when p-S118 and/or p-S167 are found associated with the parameters of an intact estrogen-dependent signaling pathway and better clinical outcome on tamoxifen, they are also found to be positively associated with several activated kinases, i.e. MAPK/ERK1/2, p90RSK, and/or AKT in primary human breast tumor biopsy samples. This supports the possibility that they may be involved in phosphorylation of ER $\alpha$  in breast tumors *in vivo*, and/or that an intact estrogen-dependent signaling pathway is involved in regulation of pathways involving activation of MAPK/ERK1/2, p90RSK, and/or AKT (Cheskis *et al.* 2008, Santen *et al.* 2009). In addition, when only PAK1-positive tumors, independent of location, were considered, a positive correlation of p-S305 with nuclear PAK1 expression was found (Bostner *et al.* 2010), suggesting a role of PAK1 in nuclear ER $\alpha$  phosphorylation at S305.

Interestingly, in primary human breast tumor samples there is generally a lack of correlation of overexpression of EGFR or HER2 with p-ER $\alpha$  (Weitsman *et al.* 2006, Jiang *et al.* 2007, Murphy *et al.* 2009). Although some studies found a weak positive association of HER2 expression and p-S118 (Jiang *et al.* 2007, Yamashita *et al.* 2008, Zoubir *et al.* 2008), overall most studies suggest that overexpression

**Table 3** Candidate kinases involved in site-specific estrogen receptor  $\alpha$  phosphorylation

Site of phosphorylation	Domain	Kinase (putative)	Direct or indirect	References	Kinase expressed <i>in vivo</i>
Ser46/47	A/B	PKC	?	Williams <i>et al.</i> (2009)	Y (Lahn <i>et al.</i> 2004, Assender <i>et al.</i> 2007)
Tyr52	A/B	cABL	Direct	He <i>et al.</i> (2010)	Y (Zhao <i>et al.</i> 2010)
Ser102	A/B	GSK3 $\beta$	?	Atsriku <i>et al.</i> (2009)	Y (Plotnikov <i>et al.</i> 2008)
Ser104	A/B	Cyclin A/CDK2	Direct	Rogatsky <i>et al.</i> (1999)	Y (Wakasugi <i>et al.</i> 1997)
		ERK1/2	?	Thomas <i>et al.</i> (2008)	Y (Adeyinka <i>et al.</i> 2002)
		GSK3 $\beta$	?	Atsriku <i>et al.</i> (2009)	Y (Plotnikov <i>et al.</i> 2008)
Ser106	A/B	Cyclin A/CDK2	Direct	Rogatsky <i>et al.</i> (1999)	Y (Wakasugi <i>et al.</i> 1997)
		ERK1/2	?	Thomas <i>et al.</i> (2008)	Y (Adeyinka <i>et al.</i> 2002)
		GSK3 $\beta$	?	Atsriku <i>et al.</i> (2009)	Y (Plotnikov <i>et al.</i> 2008)
Ser118	A/B	CDK7	?	Chen <i>et al.</i> (2002)	
		ERK1/2	Direct/ indirect	Park <i>et al.</i> (2005)	Y (Adeyinka <i>et al.</i> 2002)
		IKK $\alpha$	Direct	Park <i>et al.</i> (2005)	
		GSK3 $\beta$	Direct	Medunjanin <i>et al.</i> (2005)	
		ILK	?	Acconcia <i>et al.</i> (2006)	Y (Plotnikov <i>et al.</i> 2008)
		EGFR	Indirect	Santen <i>et al.</i> (2009)	Y (Morena <i>et al.</i> 2004)
		IGF1R	Indirect	Santen <i>et al.</i> (2009)	Y (Gee <i>et al.</i> 2005)
		DNA-PK	Direct	Medunjanin <i>et al.</i> (2010)	Y (Gee <i>et al.</i> 2005)
		RET	Indirect	Plaza-Menacho <i>et al.</i> (2010)	Y (Someya <i>et al.</i> 2007)
Ser154	A/B	AKT	?	Britton <i>et al.</i> (2008)	Y (Kirkegaard <i>et al.</i> 2005)
Ser167	A/B	p90 RSK1	Direct	Joel <i>et al.</i> (1998)	Y (Jiang <i>et al.</i> 2007)
		S6 K1	Direct	Yamnik <i>et al.</i> (2009)	Y (Barlund <i>et al.</i> 2000)
		AKT	Direct ?	Campbell <i>et al.</i> (2001)	
		IKK $\epsilon$	Direct	Guo <i>et al.</i> (2010)	Y (Kirkegaard <i>et al.</i> 2005)
		CK2	Direct	Arnold <i>et al.</i> (1994)	
		RET	Indirect	Plaza-Menacho <i>et al.</i> (2010)	Y (Landesman-Bollag <i>et al.</i> 2001)
Ser212	C	?	?	Atsriku <i>et al.</i> (2009)	?
Tyr219	C	cABL	Direct	He <i>et al.</i> (2010)	Y (Zhao <i>et al.</i> 2010)
Ser236	C	PKA	Direct	Chen <i>et al.</i> (1999)	Y (Miller <i>et al.</i> 1993)
Ser282	D	CK2	?	Williams <i>et al.</i> (2009)	Y (Landesman-Bollag <i>et al.</i> 2001)
Ser294	D	Proline-directed kinase	?	Atsriku <i>et al.</i> (2009)	?
Ser305	D	PAK1	Direct	Wang <i>et al.</i> (2002)	Y (Bostner <i>et al.</i> 2010)
		PKA	?	Michalides <i>et al.</i> (2004)	Y (Kok <i>et al.</i> 2010)
Thr311	E	p38 SAPK	Indirect	Lee & Bai (2002)	Y (Gutierrez <i>et al.</i> 2005)
Tyr537	E	c-SRC	Direct	Arnold <i>et al.</i> (1995a)	Y (Elsberger <i>et al.</i> 2009)
Ser554	F	?	?	Atsriku <i>et al.</i> (2009)	?
Ser559	F	CK2	?	Williams <i>et al.</i> (2009)	Y (Landesman-Bollag <i>et al.</i> 2001)

of EGFR and HER2 signaling pathways, at least in primary breast tumors, is unlikely to be involved in estrogen independence and tamoxifen resistance *de novo*.

### Studies of p-ER $\alpha$ in human breast cancer biopsy samples

Over the past 5 years or so antibodies to specific phosphorylated sites in ER $\alpha$  have become available, enabling the determination of the relevance of these PTMs in human breast tissues *in vivo*. Validation of such antibodies for IHC is extremely important and has been reported in some cases (Holm *et al.* 2009). There are also some reports concerning the effect of

breast biopsy collection and processing times on phospho-epitope detection (Skliiris *et al.* 2009), however, such studies are limited in scope (Barnes *et al.* 2008).

Published studies to date in which phospho-specific sites in ER $\alpha$  have been determined using IHC in human breast tumor biopsy samples are listed in Table 4. The majority of these studies have focused on p-S118, p-S167, and p-S305 although more recently other novel sites have been determined as antibodies become available or have been custom generated (Skliiris *et al.* 2009). However, more effort is required to generate reliable, high-quality antibodies suitable for IHC, western blotting, immunoprecipitation, and chromatin immunoprecipitation not only for phospho-specific

**Table 4** Published studies of the determination of p-estrogen receptor  $\alpha$  (ER $\alpha$ ) expression in human breast cancer biopsy samples

p-ER $\alpha$	Number of cases	References	Biomarker association
p-S104/106	301	Skiris <i>et al.</i> (2009)	Positive with PR
p-S118	45	Murphy <i>et al.</i> (2004b)	Negative with grade
p-S118	113	Murphy <i>et al.</i> (2004a)	Positive with PR
p-S118	?	Gee <i>et al.</i> (2005)	Positive with PR
p-S118	75	Yamashita <i>et al.</i> (2005)	Positive with PRA
p-S118	370	Skiris <i>et al.</i> (2009)	Positive with PR
p-S118	301	Sarwar <i>et al.</i> (2006)	Negative with grade
p-S118	279	Bergqvist <i>et al.</i> (2006)	Positive with PR
p-S118	290	Jiang <i>et al.</i> (2007)	Negative with grade
p-S118	16	Yamashita <i>et al.</i> (2009)	Decreased expression with neoadjuvant AI treatment ( $P < 0.0001$ )
p-S118	80	Zoubir <i>et al.</i> (2008)	Decreased expression with neoadjuvant Tam and AI treatment ( $P = 0.0001$ )
p-S167	290	Jiang <i>et al.</i> (2007)	Negative with size
p-S167	75	Yamashita <i>et al.</i> (2005)	Positive with PRA
p-S167	16	Yamashita <i>et al.</i> (2009)	Decreased expression with neoadjuvant AI treatment ( $P = 0.0005$ )
p-S305	377	Holm <i>et al.</i> (2009)	Positive with grade Positive with MI
p-S305	841	Bostner <i>et al.</i> (2010)	Positive with small tumor size
p-T311	406	Skiris <i>et al.</i> (2009)	Positive with PR

PR, progesterone receptor (ligand binding or IHC); MI, mitotic index; AI, aromatase inhibitor. Adapted from Murphy *et al.* (2009a) with permission.

sites but also for other posttranslationally modified sites in ER $\alpha$ .

In some of these studies, associations with known histopathological markers were found and these are listed in Table 4. Although contradictory results are sometimes found, possibly due to small numbers of cases and different patient characteristics in the study cohorts, differences in scoring and quantification methods, as well as different definitions of positivity and negativity, common themes have emerged. First, in contrast to what was expected, either p-S118 or p-S167 was found associated with the parameters of less aggressive and more differentiated tumors as well as an intact estrogen-responsive signaling pathway (Murphy *et al.* 2004b, Jiang *et al.* 2007).

In addition, recently p-S305 has been a focus; however, in contrast to p-S118 and p-S167, detection of p-S305 is more likely to be associated with features of more aggressive tumors (Holm *et al.* 2009; Table 4). In apparent contrast to this latter finding, p-S305 has also been found to be associated with smaller size (Bostner *et al.* 2010). One study also compared the level of p-S118 and p-S167 expression in primary breast tumors compared to secondary tumors from 10 patients after relapse (Yamashita *et al.* 2005) and found that there was increased levels of both p-S118 and p-S167 in the secondary versus the primary tumors, although this was statistically significant only for p-S118. These

observations suggest the possibility that p-ER $\alpha$  may be a useful biomarker in metastatic breast cancer as well.

From the above studies it seems that some phosphorylation sites in ER $\alpha$  such as p-S118 may be associated with better clinical outcome and others such as p-S305 may be associated with poor clinical outcome. Published studies reporting relationships of p-ER $\alpha$  with clinical outcome in breast cancer are listed in Table 5.

### Association of p-ER $\alpha$ with clinical outcome in breast cancer

Several studies have now been published where p-ER $\alpha$  expression has been explored with respect to clinical outcome in breast cancer patients, most often focusing on patients treated with tamoxifen. In contrast to what would have been expected from laboratory model systems, higher expression of either p-S167 and/or p-S118 is most often but not always associated with a better clinical outcome in patients on tamoxifen therapy (Table 5; Murphy *et al.* 2004a, Yamashita *et al.* 2005, 2008, Jiang *et al.* 2007). Most recently, the predictive and prognostic value of p-S118 was assessed in a randomized controlled trial of no systemic treatment versus 2 years of adjuvant tamoxifen therapy (Kok *et al.* 2009). Improved recurrence-free survival was found in those patients whose tumors expressed high levels of p-S118. This study is consistent with our

**Table 5** Clinical outcome studies of p-estrogen receptor  $\alpha$  (ER $\alpha$ ) expression in human breast cancer

p-ER $\alpha$	Number of cases	References	Type of hormonal therapy	Outcome	Significance
p-S118	113	Murphy <i>et al.</i> (2004a)	Tamoxifen	Positive longer RFS	$P=0.0018$ univariate
p-S118	?	Gee <i>et al.</i> (2005)	Tamoxifen	Positive longer TTP	$P<0.009$ univariate
p-S118	75	Yamashita <i>et al.</i> (2005)	Tamoxifen	No effect	
p-S118	301	Sarwar <i>et al.</i> (2006)	Tamoxifen	No effect	
p-S118	108/279	Bergqvist <i>et al.</i> (2006)	Tamoxifen	No effect	
p-S118	290	Jiang <i>et al.</i> (2007)	Tamoxifen	No effect	
p-S118	278	Yamashita <i>et al.</i> (2008)		Low longer RFS	$P=0.0003$ multivariate
p-S118	114	Generali <i>et al.</i> (2009)	Letrozole	Positive better clinical response	$P=0.004$
			Neoadjuvant 6 months		
p-S118 <sup>a</sup>	239	Kok <i>et al.</i> (2009)	Tamoxifen	Positive better RFS	$P=0.037$ multivariate
p-S118	80	Zoubir <i>et al.</i> (2008)	AI and Tam neoadjuvant	Larger decreased expression associated with better outcome	$P=0.017$
p-S167	290	Jiang <i>et al.</i> (2007)	Tamoxifen	Positive longer RFS Positive longer OS	$P=0.006$ univariate $P=0.023$ multivariate
p-S167	75	Yamashita <i>et al.</i> (2005)	Tamoxifen	Positive longer RFS	$P=0.033$ univariate
p-S167	278	Yamashita <i>et al.</i> (2008)		High longer RFS	$P=0.0002$ multivariate
p-S305	377	Holm <i>et al.</i> (2009)	Tamoxifen	Negative longer RFS	$P=0.01$ multivariate
p-S305	334	Kok <i>et al.</i> (2010)	Tamoxifen	No effect alone <sup>b</sup>	
p-S305	841	Bostner <i>et al.</i> (2010)	Tamoxifen	No effect alone <sup>b</sup>	

RFS, relapse free survival; TTP, time to progression; OS, overall survival.

<sup>a</sup>Clinical trial material.

<sup>b</sup>Significant when interactions with PKA and/or PAK1 expression considered.

previously published retrospective analysis (Murphy *et al.* 2004a), which we have also recently confirmed in a larger cohort of patients representing over 300 cases (Skliris *et al.* 2010). In addition, there are data to support the view that combinations of p-S118 with known biologically relevant biomarkers such as PR may further improve the prediction of prognosis and response to endocrine therapy (Murphy *et al.* 2004a). Such data support the combined use of biologically relevant markers for the improved prediction of therapy response.

Interestingly, the results published by Jiang *et al.* (2007) and Yamashita *et al.* (2008) show that high levels of p-S167 expression are the better predictor of benefit from tamoxifen and also suggest that both of these phosphorylation sites either alone or in combination in primary breast tumors may be useful biomarkers of endocrine therapy response.

These data strongly support undertaking further studies, potentially using the tissue microarrays generated from the collected tissue samples of large endocrine therapy clinical trials, such as Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial (Forbes *et al.* 2008), to determine the value of measuring p-S118 and/or p-S167 as more precise

biomarkers of endocrine therapy response in human breast cancer. However, standardization of antibodies and methodologies for such analyses should be decided upon and used such that the protocols can be more easily transferred and reproduced in a clinical laboratory environment. Furthermore, there may be other novel sites of phosphorylation in ER $\alpha$  that may be more tightly associated with prognosis and clinical benefit from endocrine therapies. Supporting such speculation are recently published data focusing on some novel phosphorylation sites (Murphy *et al.* 2009a, Williams *et al.* 2009, Skliris *et al.* 2010).

Since the majority of studies so far reported have only determined p-ER $\alpha$  in primary breast tumors and therefore only address associations with *de novo* intrinsic endocrine resistance, an important gap in our knowledge is the relationships of phosphorylated forms of ER $\alpha$  with acquired resistance *in vivo*.

### Multiple phosphorylated forms of ER $\alpha$

Detection in any one tumor of multiple phosphorylated forms of ER $\alpha$  is another emerging theme (Jiang *et al.* 2007, Yamashita *et al.* 2008, Skliris *et al.* 2009, 2010). In some cases, one p-ER $\alpha$  isoform was positively

correlated with one or more other p-ER $\alpha$  isoforms (Yamashita et al. 2005, 2008, Jiang et al. 2007, Skliris et al. 2009). Furthermore, mass spectroscopy data (Atsriku et al. 2009) and co-immunoprecipitation data (Murphy et al. 2009) support the idea that there is a population of ER $\alpha$  molecules phosphorylated at multiple sites at least in MCF7 human breast cancer cells, which endogenously express ER $\alpha$ .

Since estrogen treatment has been shown to increase phosphorylation at several sites in ER $\alpha$  it is possible that all of these sites similarly represent a functional ligand-dependent pathway in human breast tumors (Lannigan 2003, Murphy et al. 2006, Weitsman et al. 2006, Williams et al. 2007, 2009). Further support for this conclusion is the observation that both p-S118 and p-S167 are decreased by neoadjuvant treatment with AIs (Zoubir et al. 2008, Yamashita et al. 2009) and that several p-ER $\alpha$  are correlated with the PR status (Murphy et al. 2004a, Yamashita et al. 2005, Bergqvist et al. 2006, Sarwar et al. 2006). Another possibility is that phosphorylation or other PTMs (Cui et al. 2004) at one site increases the possibility of phosphorylation at another site (Yang et al. 2007).

It is interesting that the phospho-epitopes predicting for good clinical outcome are clustered in the N-terminus of ER $\alpha$  and that the one p-ER $\alpha$  consistently associated with a poor clinical outcome *in vivo*, p-S305, is more C-terminal. Our recent studies have identified two other sites, p-T311 and p-S559 (Murphy et al. 2009a), that seem to be associated with a poorer clinical outcome (Skliris et al. 2010), interestingly, however, T311 and S559 are also located more C-terminally in the ER $\alpha$  protein. The significance of this is unclear at the moment, however, taking into consideration these latter data together with the p-S305 published data, it would seem that not all types of p-ER $\alpha$  necessarily predict good prognosis or outcome to endocrine therapies.

The presence of multiple phosphorylation sites in ER $\alpha$  (Britton et al. 2008, Murphy et al. 2009a) that may have differential effects on activity suggests that it may be necessary to consider the balance of multiple phosphorylation sites *in vivo* in terms of predicting clinical outcome with respect to endocrine treatment responsiveness. Recently, data have been published where up to seven different phosphorylation sites in ER $\alpha$  in any individual tumor were taken into account by developing a mathematical model that balances the presence of phosphorylation sites associated with good clinical benefit and those associated with poor clinical benefit. The resulting score generated from this analysis (called the  $P^7$ -score) was found using multivariate analysis to be independently associated with

overall survival and relapse-free survival in patients treated with tamoxifen (Skliris et al. 2010), raising the possibility that phospho-profiling of ER $\alpha$  may provide more precise prediction of prognosis and potentially treatment response to endocrine therapies. These interesting results require replication in other cohorts. Furthermore, since large clinical sample numbers are required to achieve the statistical power needed when multiple sites of phosphorylation are to be determined, the development and use of tissue microarray methods will facilitate this process.

Most recently the detection of ER $\beta$  phosphorylated on S105 in human breast tumor samples was reported (Hamilton-Burke et al. 2010), and high levels of nuclear staining for p-S105-ER $\beta$  were found associated with good prognosis in breast cancer patients. Similar to ER $\alpha$ , there are likely to be more sites in ER $\beta$  which can be phosphorylated (Sanchez et al. 2010) and can affect function. These data establish the relevance of p-ER $\beta$  in breast cancer *in vivo* and lead to the speculation that a phosphorylation and/or PTM code for ER $\beta$  in breast cancer exists. Therefore, phosphorylation profiling of both ERs may be more informative than either alone (Murphy & Watson 2006). This possibility remains to be explored when more tools, e.g. phospho-site-specific antibodies, become more widely available.

The concept of PTM codes or profiles is best studied and functionally relevant for histones (Sims & Reinberg 2008). But recently, the relevance of a PTM code for nonhistone proteins, of significance to steroid receptors, has been underscored using a knockin allele of an *SRC3/AIB1/NCOA3* gene mutated in four conserved phosphorylation sites that resulted in marked changes in systemic function, which were distinct from overexpressing or knocking out the whole gene (York et al. 2010).

## Summary and speculation

Retrospective clinical outcome studies and, more recently, a randomized clinical trial (Kok et al. 2009) strongly support a positive association of p-S118 and/or p-S167-ER $\alpha$  with better clinical outcome to tamoxifen. Therefore, these two phosphorylation sites, in contrast to what was predicted from laboratory-based models, are unlikely to be a mechanism of *de novo* resistance to tamoxifen. Furthermore, data have been published suggesting that p-S118 may also predict response to AIs (Generali et al. 2009). Their role in acquired resistance remains to be determined. In contrast, the results, where p-S305 has been determined in human breast cancer biopsy samples,



support its association with lack of benefit from tamoxifen treatment. These data, together with the detection of multiple different phosphorylation sites in any one human breast tumor, support the hypothesis that phospho-profiling of ER $\alpha$  in human breast tumors, to establish an ER $\alpha$  phosphorylation code, may be a more accurate biomarker of prognosis and/or response to endocrine therapy.

Furthermore, since other PTMs such as acetylation, can occur in ER $\alpha$  (Faus & Haendler 2006) by analogy with the ‘histone code’ (Fischle *et al.* 2003), an ER $\alpha$  PTM code may exist, which more accurately reflects the balance of ligand-mediated and cross talk signal transduction (ligand-independent) pathways affecting the breast tumor cells. ER $\alpha$  is pivotal in breast cancer biology, and it is likely to be an important site, where integration of diverse signals occurs, to regulate breast cancer cell growth and survival. This, we suggest, will be reflected in a PTM code.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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