REVIEW

Endocrine-Related Cancer (2011) **18** R1–R14

Clinical significance of estrogen receptor phosphorylation

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Abstract

Multiple sites of phosphorylation on human estrogen receptor α (ERα) have been identified by a variety of methodologies. Now with the emerging availability of phospho-site-specific antibodies to ERα, the relevance of phosphorylation of ERα in human breast cancer *in vivo* is being explored. Multiple phosphorylated sites in ERα 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 ERα 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 ERα in human breast tumors to establish an 'ERα 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, ERα and ERβ, but the ER status of breast tumors and its clinical correlations are based on the measurement of generally only ERα (Harvey *et al*. 1999) and current clinical assays measure ERα immunohistochemically (IHC) with specific antibodies. The impact of ERβ 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 ERα 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 ERα (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+

DOI: 10.1677/ERC-10-0070
1351–0088/11/018–R1
Online version via http://www.endocrinology-journals.org

breast cancer. Such knowledge may also identify ways to circumvent resistance, as well as offering new biomarkers beyond ERα for the more precise prediction of therapy responses. Current downstream markers of ERα 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_2) 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 ERα 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 ERα (Cooper *et al*. 2004, Creighton *et al*. 2006, Lopez-Tarruella & Schiff 2007, West & Watson 2010).

Phosphorylation sites identified in ERα

ERα 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 ERα can potentially undergo (Lannigan 2003, Ward & Weigel 2009) and even less is known about their relevance *in vivo*. As shown in Fig. 1, ERα 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^{46}), serine 47 (S^{47}), tyrosine 52 (Y^{52}), serine 102 (S^{102}), serine 104 (S^{104}), serine 106 (S^{106}), serine 118 (S^{118}), serine 154 (S^{154}), and serine 167 (S^{167}); the DNA-binding or C domain, tyrosine 219 (Y^{219}), serine 236 (S^{236}; Chen *et al*. 1999); the hinge or D domain, serine 305 (S^{305}; Michalides *et al*. 2004), and the ligand-binding domain or E domain, threonine 311 (T^{311}; Lee & Bai 2002) and tyrosine 537 (Y^{537}; Arnold *et al*. 1995*b*). Recently, novel phosphorylation sites in ERα were identified (Britton *et al*. 2008, Williams *et al*. 2009). Table 1 lists the sites of phosphorylation in ERα, 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 ERα

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,

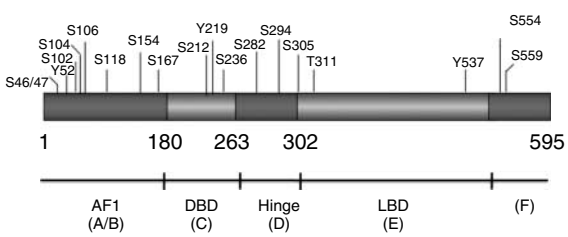

Figure 1 Multiple phosphorylated sites in ERα have been identified by a variety of approaches as listed in Table 1. These are shown schematically in the figure that depicts different structural (A, B, C, D, E) and functional domains: activation function 1 (AF1), DNA-binding domain (DBD), hinge region,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

Table 1 Phosphorylation sites identified experimentally in human estrogen receptor α

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

1, $[^{32}P]H_3PO_4$ 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 ERα 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 ERα is shown in Table 2.

Interestingly, within the A/B domain of ERα 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 ERα 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 ERα 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 ERα 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 ERα, as discussed below.

Phosphorylation, at least at S118, has been suggested to be involved in protein turnover via a proteosome-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, proteosome-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 ERα may cause tamoxifen resistance *in vivo*. For example, a well-studied p-ERα 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 α

Function	P-site	References
Ligand binding	Y537; general phosphorylation	Weis *et al.* (1996) and Arnold *et al.* (1997)
DNA binding	S167; Y219; S236; S305; Y537; general phosphorylation	Arnold *et al.* (1995*a*,*b*), Weis *et al.* (1996), Castana *et al.* (1997), Chen *et al.* (1999), Yudt *et al.* (1999), Shah & Rowan (2005), Tharakan *et al.* (2008) and He *et al.* (2010)
Dimerization	Y219; S236; Y537	Arnold *et al.* (1995*b*), Chen *et al.* (1999), Yudt *et al.* (1999) and He *et al.* (2010)
Transcription	S46/47; Y52; S104/106; S118; S167; Y219; S236; S282; S294; S305; T311; Y537; S559	Weis *et al.* (1996), Castana *et al.* (1997), Joel *et al.* (1998), Chen *et al.* (1999), Endoh *et al.* (1999), Rogatsky *et al.* (1999), Lee & Bai (2002), Tharakan *et al.* (2008), Williams *et al.* (2009) and He *et al.* (2010)
Coactivator binding	S104/106; S118; S305; T311; Y537	Weis *et al.* (1996), Endoh *et al.* (1999), Lee & Bai (2002), Dutertre & Smith (2003), Shah & Rowan (2005) and Tharakan *et al.* (2008)
Protein stability	Y52; S118; Y219	Valley *et al.* (2005), Murphy *et al.* (2006) and He *et al.* (2010)
Subcellular localization	T311	Lee & Bai (2002)
RNA splicing	S118	Masuhiro *et al.* (2005)
Interaction with other PTMs	S305	Cui *et al.* (2004) and Rayala *et al.* (2006)
Cell growth/invasion	Y52; S118; Y219; S305	Murphy *et al.* (2006), Tharakan *et al.* (2008) and He *et al.* (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, IKKα, and GSK3β are the possible candidates (Chen *et al*. 2002, Medunjanin *et al*. 2005, Park *et al*. 2005). Since ligand-independent ERα 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-ERα has been suggested. S167 is another site of ERα phosphorylation. AKT (Campbell *et al*. 2001) and pp90rsk (Joel *et al*. 1998) can phosphorylate ERα 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-ERα in breast cancer *in vivo* is unclear (Lannigan 2003).

Regulation of ERα phosphorylation

Table 3 provides a list of several kinases that have been shown experimentally to have a role in regulation of ERα 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 ERα are also shown in Table 3.

Correlation of expression of kinases with individual p-ERα expression in human breast tumor samples (Murphy *et al*. 2004*b*, 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 ERα 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 ERα 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-ERα (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 α phosphorylation

Site of phosphorylation	Domain	Kinase (putative)	Direct or indirect	References	Kinase expressed *in vivo*
Ser46/47	A/B	PKC	?	Williams *et al.* (2009)	Y (Lahn *et al.* 2004, Assender *et al.* 2007)
Tyr52	A/B	cABL	Direct	He *et al.* (2010)	Y (Zhao *et al.* 2010)
Ser102	A/B	GSK3β	?	Atsriku *et al.* (2009)	Y (Plotnikov *et al.* 2008)
Ser104	A/B	Cyclin A/CDK2	Direct	Rogatsky *et al.* (1999)	Y (Wakasugi *et al.* 1997)
		ERK1/2	?	Thomas *et al.* (2008)	Y (Adeyinka *et al.* 2002)
		GSK3β	?	Atsriku *et al.* (2009)	Y (Plotnikov *et al.* 2008)
Ser106	A/B	Cyclin A/CDK2	Direct	Rogatsky *et al.* (1999)	Y (Wakasugi *et al.* 1997)
		ERK1/2	?	Thomas *et al.* (2008)	Y (Adeyinka *et al.* 2002)
		GSK3β	?	Atsriku *et al.* (2009)	Y (Plotnikov *et al.* 2008)
Ser118	A/B	CDK7	?	Chen *et al.* (2002)	
		ERK1/2	Direct/ indirect	Park *et al.* (2005)	Y (Adeyinka *et al.* 2002)
		IKKα	Direct	Park *et al.* (2005)	
		GSK3β	Direct	Medunjanin *et al.* (2005)	
		ILK	?	Acconcia *et al.* (2006)	Y (Plotnikov *et al.* 2008)
		EGFR	Indirect	Santen *et al.* (2009)	Y (Morena *et al.* 2004)
		IGF1R	Indirect	Santen *et al.* (2009)	Y (Gee *et al.* 2005)
		DNA-PK	Direct	Medunjanin *et al.* (2010)	Y (Gee *et al.* 2005)
		RET	Indirect	Plaza-Menacho *et al.* (2010)	Y (Someya *et al.* 2007)
Ser154	A/B	AKT	?	Britton *et al.* (2008)	Y (Kirkegaard *et al.* 2005)
Ser167	A/B	p90 RSK1	Direct	Joel *et al.* (1998)	Y (Jiang *et al.* 2007)
		S6 K1	Direct	Yamnik *et al.* (2009)	Y (Barlund *et al.* 2000)
		AKT	Direct ?	Campbell *et al.* (2001)	
		IKKε	Direct	Guo *et al.* (2010)	Y (Kirkegaard *et al.* 2005)
		CK2	Direct	Arnold *et al.* (1994)	
		RET	Indirect	Plaza-Menacho *et al.* (2010)	Y (Landesman-Bollag *et al.* 2001)
Ser212	C	?	?	Atsriku *et al.* (2009)	?
Tyr219	C	cABL	Direct	He *et al.* (2010)	Y (Zhao *et al.* 2010)
Ser236	C	PKA	Direct	Chen *et al.* (1999)	Y (Miller *et al.* 1993)
Ser282	D	CK2	?	Williams *et al.* (2009)	Y (Landesman-Bollag *et al.* 2001)
Ser294	D	Proline-directed kinase	?	Atsriku *et al.* (2009)	?
Ser305	D	PAK1	Direct	Wang *et al.* (2002)	Y (Bostner *et al.* 2010)
		PKA	?	Michalides *et al.* (2004)	Y (Kok *et al.* 2010)
Thr311	E	p38 SAPK	Indirect	Lee & Bai (2002)	Y (Gutierrez *et al.* 2005)
Tyr537	E	c-SRC	Direct	Arnold *et al.* (1995*a*)	Y (Elsberger *et al.* 2009)
Ser554	F	?	?	Atsriku *et al.* (2009)	?
Ser559	F	CK2	?	Williams *et al.* (2009)	Y (Landesman-Bollag *et al.* 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-ERα in human breast cancer biopsy samples

Over the past 5 years or so antibodies to specific phosphorylated sites in ERα 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 (Skliris *et al.* 2009), however, such studies are limited in scope (Barnes *et al.* 2008).

Published studies to date in which phospho-specific sites in ERα 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 (Skliris *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 α (ERα) expression in human breast cancer biopsy samples

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

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

sites but also for other posttranslationally modified sites in ERα.

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*. 2004*b*, 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-ERα may be a useful biomarker in metastatic breast cancer as well.

From the above studies it seems that some phosphorylation sites in ERα 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-ERα with clinical outcome in breast cancer are listed in Table 5.

Association of p-ERα with clinical outcome in breast cancer

Several studies have now been published where p-ERα 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*. 2004*a*, 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 α (ERα) expression in human breast cancer

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

RFS, relapse free survival; TTP, time to progression; OS, overall survival.
[a]Clinical trial material.
[b]Significant when interactions with PKA and/or PAK1 expression considered.

previously published retrospective analysis (Murphy *et al.* 2004*a*), 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.* 2004*a*). 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 ERα 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.* 2009*a*, Williams *et al.* 2009, Skliris *et al.* 2010).

Since the majority of studies so far reported have only determined p-ERα 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 ERα with acquired resistance *in vivo*.

Multiple phosphorylated forms of ERα

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

correlated with one or more other p-ERα 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 ERα molecules phosphorylated at multiple sites at least in MCF7 human breast cancer cells, which endogenously express ERα.

Since estrogen treatment has been shown to increase phosphorylation at several sites in ERα 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-ERα are correlated with the PR status (Murphy *et al*. 2004*a*, 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 ERα and that the one p-ERα 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*. 2009*a*), 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 ERα 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-ERα necessarily predict good prognosis or outcome to endocrine therapies.

The presence of multiple phosphorylation sites in ERα (Britton *et al*. 2008, Murphy *et al*. 2009*a*) 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 ERα 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 ERα 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 ERβ phosphorylated on S105 in human breast tumor samples was reported (Hamilton-Burke *et al*. 2010), and high levels of nuclear staining for p-S105-ERβ were found associated with good prognosis in breast cancer patients. Similar to ERα, there are likely to be more sites in ERβ which can be phosphorylated (Sanchez *et al*. 2010) and can affect function. These data establish the relevance of p-ERβ in breast cancer *in vivo* and lead to the speculation that a phosphorylation and/or PTM code for ERβ 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-ERα 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 ERα in human breast tumors, to establish an ERα 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 ERα (Faus & Haendler 2006) by analogy with the 'histone code' (Fischle *et al*. 2003), an ERα 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. ERα 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.

Funding

This work was supported by the Canadian Institutes of Health Research (CIHR), the CancerCare Manitoba Foundation (CCMF), and the Canadian Breast Cancer Foundation (CBCF). This work was also supported by the Manitoba Breast Tumor Bank, a member of the Canadian Tumor Repository Network and was funded in part by CCMF and CIHR.

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Received in final form 20 October 2010
Accepted 30 November 2010
Made available online as an Accepted Preprint
13 December 2010