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.

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, Homma 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+
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 (E2) action have been identified. These include multiple ERs and variants (Murphy et al. 2003); multiple subcellular localization sites (Murphy et al. 2003); multiple 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. 2001). 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 (S46), serine 47 (S47), tyrosine 52 (Y52), serine 102 (S102), serine 104 (S104), serine 106 (S106), serine 118 (S118), serine 154 (S154), and serine 167 (S167); the DNA-binding or C domain, tyrosine 219 (Y219), serine 236 (S236, Chen et al. 1999); the hinge or D domain, serine 305 (S305; Michalides et al. 2004), and the ligand-binding domain or E domain, threonine 311 (T311; Lee & Bai 2002) and tyrosine 537 (Y537, Arnold et al. 1995b). 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,
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 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 E2 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)

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**Table 1** Phosphorylation sites identified experimentally in human estrogen receptor α.

<table>
<thead>
<tr>
<th>Site of phosphorylation</th>
<th>Domain</th>
<th>Method of identification</th>
<th>Substrate source</th>
<th>References</th>
<th>Breast tumors in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser46/47</td>
<td>A/B</td>
<td>1</td>
<td>TT/COS1</td>
<td>Williams et al. (2009)</td>
<td>ND</td>
</tr>
<tr>
<td>Tyr52</td>
<td>A/B</td>
<td>3</td>
<td>TT/HEK</td>
<td>He et al. (2010)</td>
<td>ND</td>
</tr>
<tr>
<td>Ser102</td>
<td>A/B</td>
<td>2</td>
<td>MCF7</td>
<td>Atsriku et al. (2009)</td>
<td>ND</td>
</tr>
<tr>
<td>Ser104</td>
<td>A/B</td>
<td>2</td>
<td>MCF7</td>
<td>Atsriku et al. (2009)</td>
<td>Y</td>
</tr>
<tr>
<td>Ser106</td>
<td>A/B</td>
<td>2</td>
<td>MCF7</td>
<td>Atsriku et al. (2009)</td>
<td>Y</td>
</tr>
<tr>
<td>Ser118</td>
<td>A/B</td>
<td>2</td>
<td>MCF7</td>
<td>Atsriku et al. (2009)</td>
<td>Y</td>
</tr>
<tr>
<td>Ser154</td>
<td>A/B</td>
<td>2</td>
<td>MCF7</td>
<td>Atsriku et al. (2009)</td>
<td>ND</td>
</tr>
<tr>
<td>Ser167</td>
<td>A/B</td>
<td>2</td>
<td>MCF7</td>
<td>Atsriku et al. (2009)</td>
<td>Y</td>
</tr>
<tr>
<td>Ser212</td>
<td>C</td>
<td>2</td>
<td>TT/HEK</td>
<td>He et al. (2010)</td>
<td>ND</td>
</tr>
<tr>
<td>Tyr219</td>
<td>C</td>
<td>3</td>
<td>TT/HEK</td>
<td>He et al. (2010)</td>
<td>ND</td>
</tr>
<tr>
<td>Ser236</td>
<td>C</td>
<td>2</td>
<td>MCF7</td>
<td>Atsriku et al. (2009)</td>
<td>ND</td>
</tr>
<tr>
<td>Ser282</td>
<td>D</td>
<td>1</td>
<td>TT/COS1</td>
<td>Williams et al. (2009)</td>
<td>Y</td>
</tr>
<tr>
<td>Ser294</td>
<td>D</td>
<td>1, 2</td>
<td>MCF7</td>
<td>Atsriku et al. (2009) and Williams et al. (2009)</td>
<td>Y</td>
</tr>
<tr>
<td>Ser305</td>
<td>D</td>
<td>3</td>
<td>TT/HeLa</td>
<td>Wang et al. (2002)</td>
<td>Y</td>
</tr>
<tr>
<td>Thr311</td>
<td>E</td>
<td>1, 3</td>
<td>TT/Ishikawa</td>
<td>Lee &amp; Bai (2002)</td>
<td>Y</td>
</tr>
<tr>
<td>Tyr537</td>
<td>E</td>
<td>1</td>
<td>Sf9ER/MCF7</td>
<td>Arnold et al. (1995a)</td>
<td>ND</td>
</tr>
<tr>
<td>Ser554</td>
<td>F</td>
<td>2</td>
<td>MCF7</td>
<td>Atsriku et al. (2009)</td>
<td>ND</td>
</tr>
<tr>
<td>Ser559</td>
<td>F</td>
<td>1, 2</td>
<td>MCF7</td>
<td>Atsriku et al. (2009) and Williams et al. (2009)</td>
<td>Y</td>
</tr>
</tbody>
</table>

1. $[^{32}P]$H$_2$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.
Table 2 Experimentally identified functional roles of site-specific phosphorylation in estrogen receptor α

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

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, Knowelden 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. 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 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...
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
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. 2004b, Jiang et al. 2007).

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.

### Table 4 Published studies of the determination of p-estrogen receptor α (ERα) expression in human breast cancer biopsy samples

<table>
<thead>
<tr>
<th>p-ERα</th>
<th>Number of cases</th>
<th>References</th>
<th>Biomarker association</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-S104/106</td>
<td>301</td>
<td>Skliris et al. (2009)</td>
<td>Positive with PR</td>
</tr>
<tr>
<td>p-S118</td>
<td>45</td>
<td>Murphy et al. (2004b)</td>
<td>Negative with grade</td>
</tr>
<tr>
<td>p-S118</td>
<td>113</td>
<td>Murphy et al. (2004a)</td>
<td>Positive with PR</td>
</tr>
<tr>
<td>p-S118</td>
<td>?</td>
<td>Gee et al. (2005)</td>
<td>Positive with PR</td>
</tr>
<tr>
<td>p-S118</td>
<td>75</td>
<td>Yamashita et al. (2005)</td>
<td>Positive with PRA</td>
</tr>
<tr>
<td>p-S118</td>
<td>370</td>
<td>Skliris et al. (2009)</td>
<td>Positive with PR</td>
</tr>
<tr>
<td>p-S118</td>
<td>201</td>
<td>Sarwar et al. (2006)</td>
<td>Negative with grade</td>
</tr>
<tr>
<td>p-S118</td>
<td>279</td>
<td>Berqvist et al. (2006)</td>
<td>Positive with PR</td>
</tr>
<tr>
<td>p-S118</td>
<td>290</td>
<td>Jiang et al. (2007)</td>
<td>Negative with grade</td>
</tr>
<tr>
<td>p-S118</td>
<td>16</td>
<td>Yamashita et al. (2009)</td>
<td>Decreased expression with neoadjuvant AI treatment (P&lt;0.0001)</td>
</tr>
<tr>
<td>p-S118</td>
<td>80</td>
<td>Zoubir et al. (2008)</td>
<td>Decreased expression with neoadjuvant Tam and AI treatment (P=0.0001)</td>
</tr>
<tr>
<td>p-S167</td>
<td>290</td>
<td>Jiang et al. (2007)</td>
<td>Negative with size</td>
</tr>
<tr>
<td>p-S167</td>
<td>75</td>
<td>Yamashita et al. (2005)</td>
<td>Positive with PRA</td>
</tr>
<tr>
<td>p-S167</td>
<td>16</td>
<td>Yamashita et al. (2009)</td>
<td>Decreased expression with neoadjuvant AI treatment (P=0.0005)</td>
</tr>
<tr>
<td>p-S305</td>
<td>377</td>
<td>Holm et al. (2009)</td>
<td>Positive with grade</td>
</tr>
<tr>
<td>p-S305</td>
<td>841</td>
<td>Bostner et al. (2010)</td>
<td>Positive with small tumor size</td>
</tr>
<tr>
<td>p-T311</td>
<td>406</td>
<td>Skliris et al. (2009)</td>
<td>Positive with PR</td>
</tr>
</tbody>
</table>

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

### 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. 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

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

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. 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-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. 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 phosho-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. 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 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. 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 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 P7-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 ERα 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 ERz in human breast tumors, to establish an ERz 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 ERz (Faus & Haendler 2006) by analogy with the ‘histone code’ (Fischle et al. 2003), an ERz 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. ERz 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, 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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