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Insights into how phosphorylation of estrogen receptor at serine 305 modulates tamoxifen activity in breast cancer

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Abstract

Estrogen receptor (ER) is the most important factor in the pathophysiology of breast cancer. Consequently, modulation of ER activity has been exploited to develop drugs against ER+ breast cancer, such as tamoxifen, referred to as endocrine therapies. With deeper understanding of ER mechanism of action, posttranslational modifications (PTMs) are increasingly recognized as important in mediating ER activity. Some ER PTMs such as phosphorylation, are studied in the context of ligand-independent ER activity. However, they also play a pivotal role in defining the actions and outcome of the antiestrogen-bound ER. The complexity of these actions is increasing as new PTMs are identified, yet the functional consequences and clinical implications are not fully understood. This review will examine and summarize new emerging mechanistic knowledge and clinical data in breast cancer on how these PTMs affect antiestrogen-ER activity, with an emphasis on phosphorylation of serine 305 (S305). This phosphorylation site represents an integrated hub of oncogenic signaling to modulate ER conformation, dimerization, coregulators, and DNA binding to profoundly reduce sensitivity to endocrine therapy. Consequently, (i) S305 has the potential to become a useful marker of tamoxifen response, and (ii) blocking S305 phosphorylation defines a new therapeutic strategy to overcome tamoxifen resistance in breast cancer.

Keywords

estrogen receptor; posttranslational modification; breast cancer; tamoxifen; S305 phosphorylation; resistance

I. Estrogen receptor posttranslational modifications and endocrine therapy

Estrogen receptor (ER) remains the key factor in the development and progression of breast cancer. The two known receptors, ER α and ER β , belong to the nuclear receptor super family of steroid ligand-activated transcription factors. ERs contain several functional domains including, an activating N-terminal AF-1 domain, and a DNA binding domain that is connected through a hinge region to a C-terminal ligand binding domain, or AF-2¹. In breast

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tumors, ER α expression dominates, hence throughout the text ER refers to ER α . The endogenous agonistic ligand, estrogen, binds to ER to induce the transcriptional regulation of genes involved in cell proliferation and growth of breast cancer cells². Because the activity of a protein may be affected by posttranslational modifications (PTMs), significant effort on determining and quantifying such modifications in ER has been employed. The array of PTMs on ER constitutes a molecular code that dictates its conformation, dimerization, protein stability, subcellular localization, interacting partners or coregulators, and DNA binding, and therefore overall activity. In breast cancer cells, PTMs serve as triggers to mediate the coupling between the main genomic actions and extranuclear signaling cascades initiated by estrogen. PTMs including, phosphorylation, acetylation, methylation, sumoylation, palmitoylation, glycosylation, ubiquitination, nitrosylation, and thiol oxidation, have been reported and contribute to multifaceted mechanisms underlying ER action in breast cancer (Reviewed by Romancer et al.,³). By far, the most common and important PTM that modulates ER activity is phosphorylation of serine, threonine, and tyrosine residues - all implicated in endocrine therapy response.

Given ER's critical role in breast cancer, endocrine therapy, which blocks ER action, has been the standard of care for breast cancer treatment for decades. These drugs are: Selective Estrogen Receptor Modulators (SERMs), Selective Estrogen Receptor Downregulators (SERDs), and Aromatase Inhibitors. Tamoxifen, the archetype SERMs, remains to date the most successful targeted cancer therapy⁴. However, tamoxifen has substantial ER agonist activity in bone and in the uterus⁵. Other SERMs, such as raloxifene, toremifene, lasofoxifene, bazedoxifene and arzoxifene have been developed to attenuate the agonist effects of tamoxifen. SERDs are drugs that affect ER stability (degradation) and cause ER downregulation. The main drug in this class, fulvestrant (ICI 182,780) is referred to as a "pure" antiestrogen because it is a pharmacological antagonist in all tissues. Fulvestrant is the most effective treatment for patients with metastatic ER+ breast cancer, but tolerability and side effects may be limiting⁶. AIs, such as anastrozole, exemestane, and letrozole, are drugs that inhibit the aromatase, the enzyme required for the conversion of androgens to estrogens, hence indirectly affect ER activity⁷. Although AIs have improved efficacy over tamoxifen in postmenopausal patients at prolonging relapse, the extra benefit in overall survival is unclear.

Despite the widely proven success of endocrine therapy in the clinic, unfortunately about 40-50% of patients treated endocrine therapy either fail to respond (*de novo* resistance) or become resistant (acquired resistance) to the treatment⁸. Interestingly, most of endocrine therapy resistance occurs despite continued expression of ER⁹, suggesting that ER PTMs may play a role. Therefore, understanding how these PTMs modulate endocrine therapy-ER actions is important in breast cancer. In general, phosphorylation is thought to be the main cause driving ligand-independent ER activity. In fact, most phosphorylation target residues are found within the AF-1 domain, which results in ligand-independent ER transactivation¹⁰. If ER is able to act on its own, employing drugs that lead to estrogen ablation, such as AIs, may not be sufficient to inhibit ER-driven activities. Three well-documented phosphorylation sites of ER (S118, S167, and S305) have been implicated in both ligand-independent activation of ER and resistance to antiestrogen therapies, particularly within the context of crosstalk with other growth factor and survival pathways. While S118 and S167

have generally been associated with good outcome, S305 is consistently associated with poor outcome^{3,11}. The aim of this review is to examine and summarize the mechanistic evidence and clinical data on how S305 impacts endocrine therapy-ER actions and its consequences in breast cancer.

II. Molecular events controlling phosphorylation of S305 and clinical implications

Several kinases have been reported to phosphorylate S305 leading to ER transcriptional activation. P21-activated kinase 1 (Pak1) was the first kinase shown to phosphorylate S305 in a murine model of mammary gland hyperplasia by Kumar lab¹². Pak1 is a protein involved in a number of cellular functions, including cell morphogenesis, motility, survival, angiogenesis, and mitosis, hence is implicated in both oncogenesis and cancer progression¹³. Pak1 directly phosphorylated S305, and mammary tissues with active Pak1 shows ER transactivation and elevated expression of endogenous ER target genes¹². Since then, additional *in vivo* and clinical data have emerged supporting the role of Pak1 and S305 in tamoxifen resistance^{14,15}. Michalides and colleagues showed that cAMP-dependent protein kinase A (PKA) can phosphorylate S305 *in vitro*¹⁶. They examined two consensus PKA phosphorylation sites in ER, S236 and S305. Only S305 was a bona fide site and the main target of PKA activity¹⁶. In the presence of tamoxifen, PKA led to ER transcriptional activity via S305 phosphorylation. This modification converted tamoxifen from an antagonist to an agonist, but did not change the actions of a SERD like fulvestrant. More importantly, in clinical samples, they found that downregulation of a negative regulator of PKA, PKA-R1 α , was associated with tamoxifen resistance prior to treatment¹⁶. Later, Zwart and colleagues showed that the PKA-anchoring protein, AKAP13, mediates the interaction between ER and PKA and is essential for the phosphorylation of S305 upon PKA activation¹⁷. In addition, AKAP13 mRNA expression levels correlated with poor outcome in patients who received tamoxifen treatment in the metastatic setting¹⁷.

A well-established ER crosstalk with the inflammatory NF κ B pathway has been shown to promote aggressiveness in breast cancer^{18,19}. Not surprisingly, at least two NF κ B pathway-related kinases have been shown to phosphorylate S305. Wei et al. reported that TANK binding kinase 1 (TBK1) regulates phosphorylation of S305 independent of its role in innate immune response²⁰. This is important because patients with tumors highly expressing TBK1 respond poorly to tamoxifen treatment and show high risk for relapse²⁰. Stender et al. demonstrated that inflammatory cytokine-induced phosphorylation of S305 is mediated by the inhibitor of NF κ B kinase 2 (IKK β)²¹. Frasar lab has shown that IKK β promotes the expansion of stem/basal-like cells and a dormant, metastatic phenotype in ER+ breast cancer²², suggesting that phosphorylation of S305 by IKK β may play a role.

Bostner et al. observed that in primary tumors from postmenopausal patients, an overactive PI3K / Akt / mTOR pathway together with nuclear phosphorylated ER at S167 and S305 sites, was significantly associated with reduced response to tamoxifen²³. More recently, nuclear expression of raptor, which is also part of PI3K / mTOR signaling, was coupled to elevated ER phosphorylation at S167 and S305²⁴. The increase of nuclear raptor was

inversely correlated with tamoxifen benefit in clinical samples, and indicated worse prognosis on long-term follow-up²⁴. While mTOR signaling cascade is shown to phosphorylate ER at S104/106 and S167 residues, there is yet no evidence on direct phosphorylation of S305.

Additional molecular events that may impact S305 phosphorylation status include ER mutations and crosstalk with other PTM sites. Generally, these modifications can act sequentially and/or in concert to fine-tune the outcome of ER actions. ER mutations, although rare, have been documented to affect PTM target sites of the receptor. Fuqua lab identified an ER mutation that results in a lysine to arginine substitution at residue 303 (K303R) that promotes enhanced breast tumor cell growth under estrogen deprivation and resistance to both anastrozole²⁵ and tamoxifen²⁶. This mutation through enhanced crosstalk to growth factor pathways such as HER2 / IGF1R / IRS1 / PI3K / Akt results in S305 phosphorylation, which is then key in mediating the mutant actions, and failure to antiestrogen drugs^{25–27}.

While individual PTMs have been well-documented, interactions between two or more PTMs remain to be fully elucidated. An example of positive phosphorylation crosstalk has been reported for S305 and S118¹⁵. Using a S305E mutant, which mimics constitutive phosphorylation, Kumar group found that it triggers subsequent phosphorylation of S118 that is further potentiated by tamoxifen¹⁵. Furthermore, S305-induced ER transactivation requires a functional S118 and is mediated by Pak1¹⁵. This indicates a cooperative interaction between these two phosphorylation sites although located in different ER domains, yet are capable of coordinating overall transactivation activity of ER. More recently, Diakonova and colleagues have reported a positive feedback loop where estrogen activation of Pak1 requires tyrosyl phosphorylation of a complex that includes PKA²⁸. In turn, this complex potentiates PKA activity for maximal ER S305 phosphorylation²⁸. Once activated, S305 led to enhanced phosphorylation of S118 to promote cell proliferation and tumor growth²⁸.

An example of negative crosstalk was reported between S305 phosphorylation and K303 acetylation²⁹. Acetylation of K303 is constitutive and was shown to inhibit ER transcriptional activity. However, K303 resides adjacent to S305, and phosphorylation and acetylation in this region are inversely correlated. Fuqua and colleagues showed that phosphorylation of S305 by PKA blocks acetylation of K303²⁹. In addition, a S305D mutant that mimics S305 phosphorylation, also blocks acetylation of the K303 and enhances transcriptional response to that seen with the naturally occurring K303R mutant receptor²⁹. Hence, this is an example where upstream PKA signaling coordinates negative crosstalk between PTMs by coupling high S305 phosphorylation to low K303 acetylation to enhance ER hyperactivity²⁹. The full extent of crosstalk between phosphorylated S305 and additional ER mutations and/or other PTMs remains to be determined in clinical samples.

III. S305 phosphorylation modulates ER activity through multiple mechanisms

S305 phosphorylation has been demonstrated to affect ER conformation, dimerization, interacting partners or coregulators, and DNA binding, thus overall ER function. Such perturbation results in ligand-independent constitutive ER activity and failure to be inhibited by tamoxifen, both of which have major implications in breast cancer. S305 residue is located in the hinge region of ER, which is known to coordinate and determine the functional synergy between AF-1 and AF-2 in response to estrogen and tamoxifen³⁰. Mechanistically, Stender et al. showed that phosphorylated S305 forms a charge-linked bridge with the AF-2 domain of ER that enables inter-domain communication and constitutive activity from the coactivator-binding site²¹. Stable expression of wild type ER or S305E mutant in cervical cancer HeLa cells revealed that although a ligand was required for dimerization in both, the capability for dimerization was significantly higher in S305E mutant cells³¹. Furthermore, S305E mutants exhibited enhanced binding to target gene promoters, which may in part explain the increased ligand-independent cell growth observed in S305E mutant HeLa cells³¹. Kumar lab also reported that overexpression of S305E mutant in ER-negative MDA-MB-231 cells was sufficient to upregulate the expression of a few but not all ER-regulated genes, including zinc finger protein 147 and cyclin D1, a gene implicated in breast cancer progression³². These phenotypes of ligand-independent cell growth and regulation of bona fide ER-target genes should be validated in additional breast cancer cell lines and in clinically relevant animal models.

Using fluorescence resonance energy transfer (FRET), a technique that allows monitoring of conformational changes, Michalides and colleagues showed that upon S305 phosphorylation, tamoxifen bound to ER, but could not induce the inactive conformation, instead it led to ER-dependent transcriptional transactivation¹⁶. PKA activity thus converts tamoxifen from an antagonist to an agonist¹⁶. Interestingly, this activation does not modify the response to SERDs such as fulvestrant¹⁶. In a comprehensive *in vitro* study using the same FRET technique, various SERMs and SERDs were screened for inducing rapid ER conformational changes upon binding in the context of S305, S236, and S118 phosphorylation³³. Tamoxifen and EM-652 were the most susceptible to kinase activities, whereas fulvestrant and ICI-164,384 were the most stringent³³. The different responses of antiestrogens to the various combinations of phospho-modifications in ER elucidate as to why certain antiestrogens are more inclined than others to develop resistance.

Phosphorylation of S305 is reported to modulate the binding of coactivators to ER. Zwart et al. showed that S305 phosphorylation did not influence overall binding of coactivator SRC-1, but rather changed the orientation between ER and SRC-1³⁴. This altered orientation renders ER transcriptionally active in the presence of tamoxifen, also corroborated in additional biological assays³⁴. This finding was extended beyond just SRC-1 in the study by Houtman et al³⁵. A high throughput peptide array, which allowed assessment of multiple coregulators at once, revealed that in the presence of tamoxifen, wild type ER adopted a conformation that reduced binding of coactivators³⁵. However, phosphorylated S305 had enhanced binding to coregulators in a ligand-independent manner, further providing

evidence of ER's ability to circumvent the suppressive effects of tamoxifen. This was tested also in clinical samples where two ER+ tumors were selected, one of which was phosphorylated at S305. In the patient with phosphorylated S305, the ER lost substantial binding capacity when the sample was dephosphorylated, suggesting phosphorylation was essential for ER activation. This patient also experienced recurrence after tamoxifen treatment, further providing a correlation between S305 phosphorylation of ER and tamoxifen resistance³⁵.

Zwart and colleagues were the first to characterize the phosphorylated S305-ER transcriptome in breast cancer cells³⁶. They found that S305 modification upon activation of PKA signaling redirects ER to new transcriptional start sites. By altering the chromatin-binding pattern, S305 phosphorylation of ER translates into a 26-gene expression classifier that identifies breast cancer patients with a poor disease outcome after tamoxifen treatment³⁶. Stender et al., showed that inflammatory cytokine-induced S305 phosphorylation establishes an ER cistrome that substantially overlaps with the estrogen-dependent ER cistrome, supporting a cytokine-induced constitutive activation of ER²¹. This activation requires and is mediated by IKK β ²¹. Cytokine treatment prevented the effects of tamoxifen in MCF-7 breast cancer cells with wild-type ER, while not affecting the anti-proliferative effects of tamoxifen in MCF7 cells expressing the S305A ER mutant unable to be phosphorylated²¹. Cytokine treatment of MCF-7 cells also increased extravasation and invasion. Most importantly, inhibition of IKK β restores tamoxifen sensitivity in the presence of inflammatory cytokines²¹.

Overall these findings demonstrate how, upon binding of tamoxifen, subtle changes in ER caused by phosphorylation of S305 confer resistance to tamoxifen in this context. Additional cellular consequences S305 phosphorylation include ligand-independent cell growth, regulation of some bona-fide ER-target genes and others, and invasiveness as depicted in Figure 1.

IV. The relevance of S305 phosphorylation in a clinical setting

The experimental data summarized above suggests that phosphorylation of S305 has an important role in tamoxifen resistance in breast cancer. However, there is no evidence linking S305 to SERD resistance¹⁶, and only limited *in vitro* data connecting S305 to AI sensitivity²⁷. In clinical studies, inconsistent results are sometimes observed possibly due to small numbers of cases, different patient characteristics, type of antibodies used, differences in scoring and quantification, as well as varying definitions of positivity and negativity. However, the common theme that has emerged is that S305 phosphorylation is associated with poor response to tamoxifen. This is important because tamoxifen for decades was the first-in-line drug to treat breast cancer, and is expected to continue to be in wide use for some time. As more specific antibodies become available, this will enable better determination the S305 relevance in breast cancer and in predicting response to tamoxifen. Validation of such antibodies for immunohistochemistry (IHC) is extremely important, and more effort is required to generate reliable, high-quality antibodies suitable for IHC in larger patient cohorts. Additionally, incorporating other techniques to detect S305 phosphorylation

that are amendable to clinical samples, such as mass spectrometry, may prove useful in the future.

Numerous clinical studies show that tamoxifen resistance occurred in endocrine-treated patients with detectable phosphorylated S305^{14,23,37,38}. S305 phosphorylation has no effect on patients that received no endocrine treatment, but was a predictive marker for treatment outcome in patients that received it³⁸. This suggest that S305 is a marker of therapy benefit, but does not predict general ER+ tumor progression. In the study by Holm et al. S305 phosphorylation alone was negatively correlated with tamoxifen response³⁸. However, in two later studies, phosphorylated S305 failed to predict tamoxifen sensitivity by itself, but rather in combination with other markers^{14,23,37}. These findings strongly imply that S305 phosphorylation should be examined in the context of other ER phosphorylation PTMs and overactivated oncogenic signaling pathways that may control S305 phosphorylation status.

As previously noted, in contrast to S305, phosphorylation of S118 and S167 are associated with good prognosis^{3,11}. Bostner et al. reported that coexpression of S305 with S167 showed borderline statistical significance in terms of tamoxifen resistance²³. Detection of multiple phosphorylation sites and well-documented crosstalk between them supports the notion that phospho-profiling of ER in breast tumors to establish an ER phosphorylation score may be a more precise marker of prognosis and/or response to endocrine therapy. Numerous studies emphasize the interplay between a kinase activity or an oncogenic pathway with S305 phosphorylation status in predicting tamoxifen effectiveness. Therefore, therapeutic interventions aimed at inhibiting these kinases or pathways would be desirable, as they would simultaneously achieve blocking of the target and S305 phosphorylation. Bostner et al. showed that in samples from postmenopausal patients nuclear localization of Pak1 together with phosphorylated S305 decrease benefit from tamoxifen treatment¹⁴. Kok et al. also confirmed the correlation between Pak1 and tamoxifen resistance³⁷. Additionally, they showed that coexpression of phosphorylated PKA and S305 predicts tamoxifen response³⁷. A later study by Bostner et al. showed that a combination score of phosphorylated ER at S167 and S305 together with phosphorylated Akt and mTOR predicts further reduced benefit from tamoxifen treatment²³. Hence, inhibiting these kinases or pathways may provide a personalized breast cancer treatment for patients unlikely to respond to tamoxifen alone.

In conclusion, phosphorylation of ER at S305 site represents an integrated hub of oncogenic signaling to modulate ER conformation, dimerization, coregulatory binding, and DNA binding to profoundly reduce sensitivity to endocrine therapy. Consequently, (i) S305 has the potential to become a useful marker of tamoxifen therapy benefit, and (ii) blocking S305 phosphorylation defines a new therapeutic strategy to overcome tamoxifen resistance.

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Highlights:

- PTMs and especially phosphorylation are critical to modulate ER activity in breast cancer
- Numerous kinases and crosstalk to other PTMs control phosphorylation of S305
- S305 phosphorylation of ER affects its conformation, dimerization, coregulator and DNA binding
- Important cellular consequences of phosphorylated S305 include, tamoxifen resistance, ligand-independent cell growth, gene regulation and invasion
- Phosphorylated S305 predicts poor outcome to tamoxifen therapy

Figure 1.

A schematic representation of ER S305 phosphorylation and its molecular and cellular consequences in breast cancer.