Mechanisms associated with resistance to tamoxifen in estrogen receptor-positive breast cancer (Review)

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Abstract. Anti-estrogens such as tamoxifen are widely used in the clinic to treat estrogen receptor-positive breast tumors. Patients with estrogen receptor-positive breast cancer initially respond to treatment with anti-hormonal agents such as tamoxifen, but remissions are often followed by the acquisition of resistance and, ultimately, disease relapse. The development of a rationale for the effective treatment of tamoxifen-resistant breast cancer requires an understanding of the complex signal transduction mechanisms. In the present study, we explored some mechanisms associated with resistance to tamoxifen, such as pharmacologic mechanisms, loss or modification in estrogen receptor expression, alterations in co-regulatory proteins and the regulation of the different signaling pathways that participate in different cellular processes such as survival, proliferation, stress, cell cycle, inhibition of apoptosis regulated by the Bcl-2 family, autophagy, altered expression of microRNA, and signaling pathways that regulate the epithelial-mesenchymal transition in the tumor microenvironment. Delineation of the molecular mechanisms underlying the development of resistance may aid in the development of treatment strategies to enhance response and compromise resistance.

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1. Introduction

Estrogens and their receptors (ERs) influence many physiological processes in mammals and are also implicated in the development or progression of numerous diseases, including various types of cancer (breast, ovarian, colorectal, prostate and endometrial), osteoporosis, neurodegenerative diseases, cardiovascular disease, insulin resistance, lupus erythematosus, endometriosis and obesity (1-3). Breast cancer is the most frequently detected female neoplasm worldwide. The ER is expressed by 60-70% of breast tumors (4,5) and the mechanism has been studied; binding of estrogens to ER stimulates proliferation of mammary cells, increasing the target cell number within the tissue, and the increase in cell division and DNA synthesis elevates the risk for replication errors, which may result in the acquisition of detrimental mutations that disrupt normal cellular processes such as apoptosis, cellular proliferation or DNA repair. A second mechanism of estrogen metabolism leads to the production of genotoxic by-products that could directly damage DNA, again resulting in point mutations. There is evidence that estrogen may act through both mechanisms to initiate and/or promote mammary cancer (2). In the treatment of breast cancer, tamoxifen is the most commonly used anti-estrogen, but resistance remains an obstacle in the treatment of hormone-dependent breast cancer. While up to one third of patients are resistant to tamoxifen at the beginning of treatment, the majority of patients who initially respond to tamoxifen will later also become resistant. Some mechanisms may include changes in the expression of *ERα* or *ERβ*, an alteration in co-regulatory proteins and an alteration in transduction pathways, altered expression of specific microRNA, and genetic polymorphisms involved in tamoxifen metabolic activity. Due to the clinical consequences of endocrine resistance, new treatment strategies are arising to render the cells sensitive to tamoxifen. In the present study,

we reviewed the current knowledge on the mechanisms of endocrine resistance in breast cancer cells.

2. Estrogen action and function

Estrogens perform their function by interacting with ER. Two ER genes have been identified in mammals: ERα and ERβ, which show similar DNA- and ligand-binding properties, but distinct tissue distributions and functions (6-8). The ER is a member of the nuclear receptor family of ligand-activated transcription factors. After entering the cell, estrogen binds the ER, which dissociates from heat shock proteins (HSPs) and undergoes conformational changes, phosphorylation and dimerization before binding to the estrogen response elements (ERE) upstream of estrogen-dependent genes. This is referred to as the classical mode of action. The majority of these genes in this model are involved in cell proliferation and survival or in maintaining tissular architecture (9,10). Ligand-bound ER can also modulate gene expression through interaction of the receptor with Fos and Jun at activator protein-1 (AP-1) binding sites (11) or with specificity protein 1 (SP-1) sites in DNA, thereby functioning as a co-regulator (12). Co-regulators serve as a fine tuning mechanism by increasing or reducing the receptor transcriptional activity (13). Several co-regulators have been implicated in cancer, most notably AIB1 (SRC-3), a gene that is amplified in a small percentage but overexpressed in two thirds of all breast cancers. Overexpression of this gene has been implicated in tamoxifen resistance (14,15).

In the ligand-independent mechanism, ER is phosphorylated by membrane receptor tyrosine kinases including the epidermal growth factor receptor (EGFR) (16), ErbB receptor 2 (ERBB2/HER2), and the insulin-like growth factor receptor (IGF1-R) (17) or signaling molecules leading to dimerization, DNA binding, and the activation of transcription (18). Crosstalk between the growth factor receptors (GFR) and ER pathways has been established through several other mechanisms. Estrogen can increase the expression of ligands such as transforming growth factor-α (TGFα) and IGF1 (12,19,20) which can then activate the GFR pathway (17,20). On the other hand, estrogen signaling downregulates the expression of EGFR and ERBB2/HER2 while it induces the expression of IGF1-R (21). Activation of the phosphoinositide 3 kinase (PI3K)-kinase AKT (AKT) and the p42/44 mitogen-activated protein kinase (MAPK) pathways by these receptors, in turn, downregulates the expression of ER and progesterone receptors (PR) (22,23). Thus, while receptor tyrosine kinases can activate the transcriptional function of ER, they can also reduce estrogen dependence by downregulating the expression of ER, perhaps contributing to the relative resistance to endocrine therapies in tumors amplified for ERBB2/HER2 (24). Studies also suggest that ER may work by non-transcriptional mechanisms. Low levels of ER have been found outside of the nucleus, in the membrane, cytoplasm or even in the mitochondria, although the exact location for this receptor remains controversial (25). Some of the non-genomic action of estrogen appears to be too rapid for a transcriptional effect to active GFR signaling, including the PI3K/AKT and Ras/p42, 44 MAPK pathways. Thus, ER, through this non-genomic activity, can alter the expression of genes normally regulated by growth factors (25,26). Finally, the stress kinase pathway via p38 and JNK can also modulate ER function by phosphorylation of ER and its co-regulators (27). The microenvironment and its associated integrin signaling may exert similar activity (28). Thus, ER activity and signaling is modulated by a variety of pathways, which could also contribute to resistance to ER-targeted therapies, especially when the pathways display aberrant activity in a cancer cell.

3. Mechanism of tamoxifen resistance

Several mechanisms of resistance to tamoxifen have been studied and include the following: pharmacologic mechanisms; loss or modification in ER expression; alterations in co-regulatory proteins and regulation of different signaling pathways that participate in the cellular process, such as survival, proliferation, stress response, cell cycle, inhibition of apoptosis regulated by the Bcl-2 family, autophagy, altered expression of microRNAs and signaling pathways that regulate epithelial-mesenchymal transition (EMT) in the tumor microenvironment. Some proposed mechanisms responsible for tamoxifen resistance are described below (Fig. 1).

Pharmacologic mechanisms. Cytochrome P450 2D6 (CYP2D6) is crucial in the metabolism of tamoxifen to its active metabolite, endoxifen (29). Retrospective clinical data suggest that specific single nucleotide polymorphisms (SNPs) of CYP2D6 can lead to null or reduced enzyme activity resulting in poorer outcomes for patients with these when they are treated with tamoxifen for hormone receptor (HR)-positive breast cancer (30). Polymorphic CYP2D6 is the key enzyme in this biotransformation, and recent mechanistic, pharmacologic and clinical evidence suggests that genetic variants and drug interaction with CYP2D6 inhibitors exert an influence on the plasma concentrations of active tamoxifen metabolites and the outcomes of tamoxifen-treated patients. In particular, non-functional (poor metabolizer) and severely impaired (intermediate metabolizer) CYP2D6 alleles are associated with higher recurrence rates (31).

Loss or modification in ER expression. Expression of ERα has long been considered a determinant of a clinical response to endocrine or anti-estrogen therapy. The ERα status of breast tumors provides prognostic information and is the primary target for endocrine therapy. Effective strategies to treat ER-positive breast cancer include endocrine agents that compete with estrogen for binding to its receptor, such as selective estrogen-receptor modulators (SERM) and anti-estrogens or reducing the levels of circulating estrogens by the administration of agents such as third-generation aromatase inhibitors (32), which have been shown to be more effective than tamoxifen in postmenopausal women in neoadjuvant and adjuvant settings (33). Patients with tumors lacking ERα generally do not benefit from tamoxifen therapy, although a fraction of ERα-negative tumors appear to be sensitive to tamoxifen (34). Several studies have measured ERβ in breast cancer tumors and have sought to clarify the relationship between ERβ and its role in response to endocrine treatment; however, some of the results have been conflicting and the majority have focused on ERβ as a resistance marker in ERα-positive tumors (35,36). ERβ has been shown to bind tamoxifen (37), and it has been

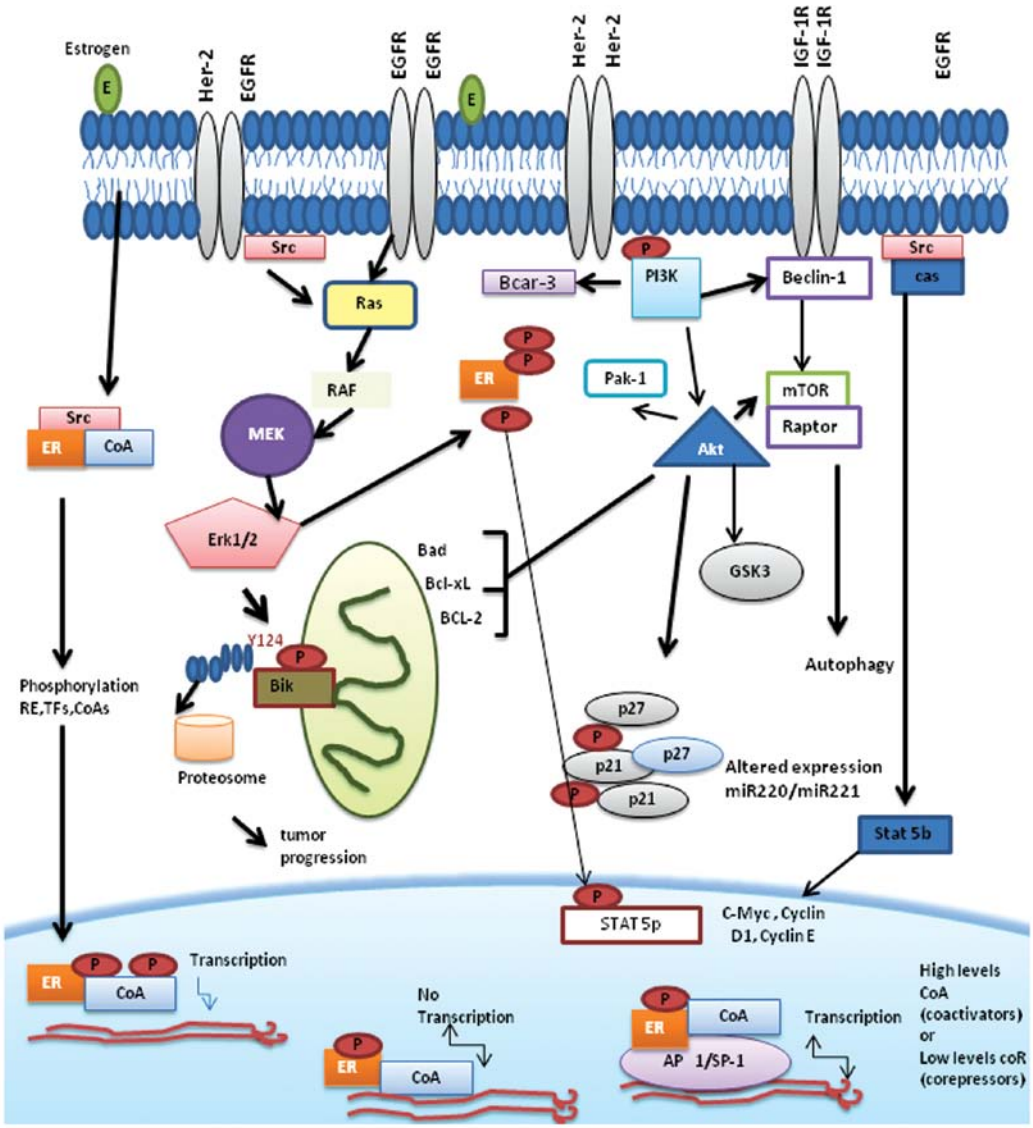

Figure 1. Molecular mechanism of tamoxifen resistance. Model of molecules implicated in anti-estrogen resistance and discussed in this review.

suggested that low levels of ERβ are associated with tamoxifen resistance (35). Conversely, some studies have shown that the expression of ERβ had a beneficial effect on disease-free and overall survival in a group of 186 tamoxifen-treated tumors; however, the authors found no such association in their set of 119 untreated patients, suggesting a role for *ERβ* as a predictive marker for tamoxifen sensitivity, but not as a prognostic marker (36).

Several mechanisms have been proposed to explain the absence of ER expression. These mechanisms comprise epigenetic changes, such as aberrant CpG island methylation of the ER promoter and histone deacetylation, resulting in a compact nucleosome structure that limits transcription (38-40). Co-treatment with inhibitors of DNA methyltransferase-1 (DNMT-1), such as 5-Aza-2-deoxycytidine (AZA), or inhibitors of histone deacetylase (HDAC), like trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), induce ER gene expression in ER(-) breast cancer cells and restore sensitivity to anti-estrogen (38,41,42). In ER(-) MDA-MB-231 cells, which overexpress EGFR, SAHA may not only reactivate silenced ER, but it also simultaneously depletes EGFR expression and abolishes EGF-initiated signaling pathways including phosphorylated p21-activated kinase-1 (PAK1), p38MAPK and AKT (43). *In vitro* and *in vivo* studies showed that treatment with the histone deacetylase inhibitor entinostat (ENT) increased the expression of *ERα* and aromatase. Notably, *ERα* and aromatase upregulation resulted in sensitization of breast cancer cells to estrogen and letrozole (44).

Another mechanism that has been studied is mutations in ER genes that may lead to a functionally negative ER phenotype without the loss of ER expression as determined by protein-based immunohistochemical assays. Using site-directed mutagenesis in the AF-2 region of mouse ER, it is possible to reduce estrogen-dependent transcriptional activation without significantly affecting hormone and DNA binding (45). A mutation substituting aspartate for tyrosine at position 351 has been identified in a tamoxifen-stimulated cell line (46). More recently, substitution of aspartate with glycine at amino acid 351 in the ER has been shown in an experimental system to silence the agonist activity of 4-hydroxytamoxifen (47).

However, it has been reported that only 17-28% of patients with acquired resistance to tamoxifen lose the expression of ERα (47).

Several kinase pathways have been associated with tamoxifen resistance, including activation of the protein kinase A (PKA) (17), MAPK (18) and PAK-1 signaling pathways (19). These kinases induce phosphorylation of ERα or of its co-regulators. Some phospho-modification sites have been studied in ERα that could contribute to an altered response to tamoxifen and in which kinase pathways and upstream activators are involved.

Serine residues S102, S104 and S106 at the N-terminal AF-1 region of ERα are phosphorylated by glycogen synthase kinase-3 (GSK-3) and by extracellular signal-regulated kinases 1 and 2 (ERK1/2) and MEK1/2 pathways; these modifications lead to ligand-independent transcription of ERα and to an agonistic activity of tamoxifen (48,49). S102, a phosphorylation site discovered by mass spectrometry, requires concurrent phosphorylation of S104 (50). ERα phosphorylation by GSK-3, which also targets S118, stabilizes ERα without ligand and modulates ERα transcriptional activity upon ligand binding. S104 and S106 can also be phosphorylated by the CDK2/cyclin A complex (51). Cyclin A has been reported as a predictive marker for tamoxifen resistance in patients with breast cancer (52).

Serine 118 is one of the most reported phosphorylation sites of ERα. It is targeted by a number of kinase pathways: MAPK, GSK-3, IKKα, CDK7 and the mammalian target of rapamycin (mTOR)-p70 S6 ribosomal kinase (p70S6K) and S118 phosphorylation by MAPK increases the binding of co-activator SRC3 and renders ERα hypersensitive to estradiol (53). Phosphorylated S118 decreases ERα affinity for tamoxifen and reduces binding to DNA, when ERα is tamoxifen bound (54). Upstream, the RAS/MAPK pathway can be activated by IGF stimulation inducing phosphorylation of ERα S118 and resulting in ERα activation and enhanced response to estradiol (55).

Serine 282 resides in the hinge region and, similar to S167, can be phosphorylated by CK2. Estradiol increases phosphorylation of S282, stabilizes ER, and induces transcriptional activity (50).

Serine 305 resides at the C-terminus of the hinge region, which provides a center of rotation to the total *ERα*. The region around Ser305 is a multifunctional domain that binds to many co-regulatory proteins and is involved in the regulation of the activity and stability of *ERα* (56,57).

Phosphorylation of Ser305 occurs by means of protein kinase A (PKA) and is associated with resistance to tamoxifen in patients.

Alterations in co-regulatory proteins. The transcriptional regulatory activity of ER is mainly mediated by the formation of complexes with co-activator or co-repressor proteins. In general, co-activators bind the ER when it is bound by estrogen, enhancing target gene transcription. When an antagonist such as tamoxifen is bound to ER, co-repressors are typically recruited, which results in repression of target gene transcription. Under specific conditions, such as high ERBB2/HER2 activity, a tamoxifen-ER complex may also recruit co-activator proteins, causing agonistic effects.

Altered expression of co-regulators may therefore play a role in tamoxifen resistance (58,59). It has been demonstrated *in vitro* that co-activator proteins AIB1, PGC-1β and SRC1 enhance the agonistic activity of tamoxifen (60,61). In patients receiving adjuvant tamoxifen therapy, high levels of AIB1 alone or in combination with high levels of ERBB2/HER2 are associated with shorter disease-free survival in patients (62).

These findings support a role for the overexpression of co-activators in tamoxifen resistance. However, high levels of SRC1 were associated with favorable response to tamoxifen in patients (61), which is not in line with this hypothesis. The presence of other factors, such as ERBB2/HER2, may play a role in this outcome (59). In addition, two studies showed that low levels of the co-repressor protein NCOR1 predict poor response to tamoxifen (63). These results support the possibility that reductions in co-repressor activity may also contribute to tamoxifen resistance.

Growth factor receptor signaling pathways. Estrogen receptor may initiate rapid cellular signaling via direct interaction with components of growth factor signaling pathways. Several studies have found that overexpression of EGFR or ERBB2/HER2 in ER-positive, anti-estrogen-sensitive breast cancer confers resistance to this drug (3,64). EGFR and HER2 are associated with reduced response to tamoxifen that, in experimental systems, can actually stimulate their growth (15,16).

Gefitinib, which inhibits ERBB2/HER2, improved the antitumor effect of tamoxifen and delayed the acquisition of resistance, but had no effect on estrogen-stimulated growth. Phosphorylated levels of p42/44 and p38 MAPK (both downstream of EGFR/HER2) were increased in tamoxifen-resistant tumors and were suppressed by gefitinib. There was no apparent increase in phosphorylated AKT, (also downstream of EGFR/HER in resistant tumors, but it was nonetheless suppressed by gefitinib. Phosphorylated IGF-1R, which can interact with both EGFR and membrane ER, was elevated in the tamoxifen-resistant tumors compared with the sensitive group. However, ER-regulated gene products, including total IGF-IR itself and the PR, remained suppressed even at the time of acquiring resistance. Tamoxifen's antagonism of classic ER genomic function was retained in these resistant tumors and even in tumors that overexpress ERBB2/HER2 (MCF-7 HER2/18). In conclusion, EGFR/HER2 may mediate tamoxifen resistance in ER-positive breast cancer despite continued suppression of ER genomic function by tamoxifen. IGF-IR expression remains dependent on ER but is activated in tamoxifen-resistant tumors (65).

IGF-1R. The IGF-1R signaling pathway has important roles in regulating energy metabolism, cellular proliferation and apoptosis. IGF-1R is a receptor tyrosine kinase that exerts its biologic effects through binding of the ligands IGF-I and IGF-II. Following ligand binding and receptor activation, adaptor molecules are recruited, leading to activation of downstream pathways, including the RAS/MAPK and PI3K pathways (66,67). The expression of ER is controlled by IGF in breast cancer cells (68). Conversely, genomic and non-genomic actions of ER can activate the mitogen-inducing signals of the IGF pathway (69). Estrogen signaling can also enhance

IGF-1R signaling through transcriptional upregulation of IGF1R, IRS-1 and IGF-II (70-72). Reciprocally, IGF1R has been shown to phosphorylate and activate ER in serine-167 through an S6-kinase mechanism (73). One mechanism by which IGF-1-treated breast cancer cells may escape from tamoxifen-induced apoptosis is via IGF-mediated activation of AKT and subsequent phosphorylation of ER, leading to the ligand-independent activation of ER (74). Previous *in vitro* studies have aimed to elucidate the role of IGF-1R in breast cancer resistance. A gene that confers resistance to tamoxifen is for IGF binding protein 5 (IGFBP5) (75). IGFBP5 is a secreted protein that inhibits growth factor binding to IGF-1R (76). In addition, IGFBP5 has recently been shown to play a critical role in breast cancer progression and metastasis (77). Studies have demonstrated that knockdown of IGFBP5 resulted in resistance to tamoxifen treatment in MCF-7 cells and in mice tumor xenografts. IGFBP5 knockdown-induced resistance to tamoxifen occurred potentially via altered IGF signaling and loss of ER expression (55,68,78). It has also been shown that inhibition of IGF-1R (by an anti-IGF-1R antibody) (79) or by an IGF-1R tyrosine kinase inhibitor (80) reduces the growth of tamoxifen-resistant MCF-7 breast cancer cells. Furthermore, it has been demonstrated in an *in vivo* model that an IGF-1R monoclonal antibody enhances the antitumor activity of tamoxifen in ER-receptor-positive breast cancer xenografts (81).

In vitro study has shown that increased IGF-IR signaling interferes with the action of the HER-2 antibody trastuzumab in HER2 positive breast cancer cells (82). Unlike EGFR, several studies have reported that tamoxifen continues to suppress the IGF-1R level, even during the development of resistance (65).

Mechanism of resistance to tamoxifen associated with signaling proteins p130Cas and c-Src. The focal adhesion adapter protein p130 (p130Cas) also known as breast cancer anti-estrogen resistance 1, is involved in different cellular process, including proliferation, survival, cell adhesion and migration as an adapter or scaffolding protein (83). High expression of p130Cas is associated with resistance to tamoxifen and promotes the proliferation and inhibition of apoptosis in MCF-7 cells and in human tumors (84). Interactions between c-Src and p130Cas result in activation of c-Src activity, promotion of serum- and anchorage-independent growth and enhancement of cellular migration; concomitantly Cas is phosphorylated and activated by c-Src (83).

Two substrates of c-Src, tyrosine 845 (Tyr-845) on the EGFR and signal transducer and activator of transcription 5b (STAT 5b) (85), have been implicated in p130Cas-dependent tamoxifen resistance (86). Pharmacologic inhibition of c-Src in MCF-7 cells enhances the inhibitory effects of tamoxifen on cell growth (86). It is clear that p130Cas overexpression diminishes the antiproliferative and pro-apoptotic effects of tamoxifen on breast cancer cells *in vitro*, and data suggest that it accomplishes this via activation of the p130Cas/c-Src/EGFR/STAT5 signaling pathway. Whether these pathways are also clinically significant in resistance to tamoxifen has not yet been established; however, a large percentage of human breast tumors expresses high levels of both c-Src and p130Cas, as well as elevated c-Src kinase activity, and c-Src/Cas complexes can be isolated from a majority of breast cancer cell lines (3). Taken together, these data suggest that tamoxifen resistance may be mediated in part through this mechanism involving c-Src and p130Cas.

Breast cancer anti-estrogen resistance 3 (BCAR3) protein. BCAR3 is a member of the novel *S*rc homology 2 (SH2)-containing protein family (87) that includes two other members, Chat/SHEP1 and NSP1. These proteins share a common domain structure consisting of an amino-terminal SH2 domain and a carboxyl-terminal domain with sequence homology to the Cdc25-family of guanine nucleotide exchange factors (GEF). Several studies have shown that BCAR3 expression results in the activation of numerous small GTPases, including Rap1, R-Ras, RalA, Cdc42 and Rac1 (88,89). The carboxyl-terminal domain of BCAR3 has been shown to bind to the carboxyl terminus of p130Cas, providing additional support for a functional relationship between these proteins (88).

BCAR3 was discovered in a genetic screening for genes associated with anti-estrogen resistance and cell motility (90). It encodes a component of intracellular signal transduction and interacts directly with p130Cas. It has been demonstrated that breast cancer cells transfected with BCAR3 to induce anti-estrogen resistance, suggesting that upregulation of BCAR3 stimulates an alternative growth path independent of hormone, both in the presence and absence of anti-estrogens by activating PI3K, which induced Rac activation (91).

PI3K and AKT pathway. The PI3K pathway is a key signal transduction system that links oncogenes and multiple receptor classes to many essential cellular functions, promotes anti-estrogen resistance and is perhaps the most commonly activated signaling pathway in human cancer (92-94). Studies have found that overexpression of HER2 or FGF-1R, or loss of the inositol polyphosphate phosphatase 4B (INPP4B), activate the PI3K pathway and also confer anti-estrogen resistance in patients with ER^+ breast cancer (95).

PI3K is commonly activated in breast cancer cells by growth factor receptor tyrosine kinases or G-protein-coupled receptors. The signaling cascades triggered by PI3K, including PDK1, AKT and SGK among others, promote cell growth and survival (94). PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (96). In turn, PIP_2 recruits several pleckstrin homology (PH) domain-containing proteins to the plasma membrane such as the serine/threonine-protein kinase PDK1 and AKT, which on activation drive cell-cycle progression and survival. Negative regulation of this pathway is conferred by the tumor suppressors phosphatase and tensin homolog (PTEN) and INPP4B, which are lipid phosphatases and dephosphorylate PIP_3 and PIP_2, respectively (32,97).

AKT activates mTOR-containing complex 1 (TORC1), which regulates protein synthesis. mTOR is also part of another complex, TORC2, which lies upstream of AKT. In addition to its pro-survival and growth-promoting roles, the PI3K pathway interacts with ER directly and indirectly. ER phosphorylation at Ser_{167} by AKT or p70S6K increases estrogen-induced, tamoxifen-induced, and ligand-independent ER transcriptional activity (74). Additionally, PI3K and Ras promote c-Jun phosphorylation. c-Jun complexes with c-Fos to form the AP-1 complex, which cooperates with ER tran-

scription. Other oncogenic kinase pathways (MAPK, protein kinase C) also contribute to the modulation of ER and transcription cofactors (98).

Stress-activated protein kinase/c-Jun kinase pathway. Estrogens induce apoptosis by regulating the c-Jun NH2-terminal kinases (JNKs). JNKs are stimulated by multiple factors, including cytokines, DNA-damaging agents, and environment stress, and are important in controlling apoptosis in the process of cellular stress, increasing AP-1 transcriptional activity via phosphorylation. AP-1 is a transcription factor heterodimer composed of Jun and Fos family members and binds to DNA at AP-1 response elements. It has been reported that the development of tamoxifen resistance in MCF-7 cells is accompanied by increased AP-1 DNA binding (99).

These findings have been examined in a panel of 30 ER-positive primary human breast tumors with acquired tamoxifen resistance, and these tumors also displayed a significant increase in AP-1 DNA binding activity when compared with untreated control tumor. In a tamoxifen-resistant xenograft model, increased phosphorylated c-Jun and JNK levels accompanied the increase in AP-1-dependent transcription following tamoxifen treatment, and the conversion to a resistant phenotype was associated with an increase in oxidative stress (OS) (100).

Cell cycle regulators associated with resistance to tamoxifen. Cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors are the major regulators of cell cycle progression (3). Estrogen accelerates progression from G1 to S phase, while tamoxifen inhibits cell cycle progression by affecting these key cell cycle proteins (101). The expression and activity of these proteins have been demonstrated to have the potential to significantly impact tamoxifen sensitivity and resistance.

Cyclin D1 plays an important role in the regulation of cell cycle, promoting progression through to G1-S phase. In mammary epithelial cells, the expression of cyclin D1 is regulated through the ER signaling (102). Cyclin D1 is a direct transcriptional target of estrogen signaling; thus, tamoxifen reduces cyclin D1 expression (103). *In vitro* sustained expression of cyclin D1 is evident in breast cancer cells during their acquisition of tamoxifen resistance, and downregulation of cyclin D1 with siRNA restored the sensitivity of these cells to tamoxifen (103). Cyclin D1 is one co-regulator, known to interact with ERα and can potentiate its transcriptional activity independently of estrogen and may not be inhibited by tamoxifen (104). Overexpression of cyclin D1 has been reported to result in a conformational change in ERα that induces receptor activation in the presence of tamoxifen, which in turn promotes growth of MCF-7 cells-indicating a change from antagonist to agonist (105,106). Patients with cyclin D1 negative tumors show better relapse-free survival when they are treated with tamoxifen (107), whereas multiple clinical studies have demonstrated that overexpression of cyclin D1 is correlated with poor outcome on tamoxifen treatment (107,108).

Cyclin D1 inhibits Rb early in G1 phase, and the transcription factor E2F strongly induces the expression of cyclin E, which is associated with CDK2 to form an active complex that promotes entry into S phase (108). Cyclin E2 expression has been associated with poor outcome in ER-positive breast cancer (109), and cyclin E2 is included in genes that predict disease progression in either tamoxifen-resistant breast cancer or metastatic breast cancer, whereas cyclin E1 is absent (110,111). Cyclin E1 overexpression can reduce anti-estrogen sensitivity *in vitro* (112,113), but cyclin E2 has not been studied in this context, although it is strongly estrogen regulated (114). In addition, CDK2 activation is a possible mechanism of resistance to a CDK4 inhibitor that preferentially inhibits ER-positive breast cancer cell lines and can overcome acquired resistance to tamoxifen (115,116). We therefore considered cyclin E1 and E2 to be most important in anti-estrogen resistance in ER-positive breast cancer and potential therapeutic targets in endocrine-resistant disease.

Cell cycle progression upon ligand binding of ER has been shown to be mediated by cyclin/cyclin-dependent kinase (CDK) complexes and CDK inhibitors (117,118). p21 is a member of the Cip/Kip family of CDK inhibitors and acts as a G_1 checkpoint protein, preventing cell cycle progression into S phase. p21 functions as a downstream effector of p53, and loss of p21 expression is seen in a high percentage of human breast cancers (117-119). p21 and p27 are CDK inhibitors and are negative regulators of cell cycle progression. These proteins counteract the activities of cyclin D1 and E. Expression of the ER, a good prognostic factor in breast cancer, is associated with higher levels of both p21 and p27 proteins (120,121).

p27 induction in breast cancer cells by tamoxifen induces quiescence and clinical data also support a role of these CDK inhibitors in response to tamoxifen treatment. In premenopausal women with early breast cancer, an increase in p27/KIP1 expression was able to predict better relapse-free survival upon tamoxifen combination treatment (122).

Studies using immortalized human breast epithelial cells with somatic deletion of the p21 gene showed a growth proliferative response to tamoxifen as absence of p21 enabled cyclin-CDK complexes to aberrantly phosphorylate ER when bound to tamoxifen, resulting in a growth-stimulatory phenotype. On the other hand, p21 wild-type cells demonstrated growth inhibition upon tamoxifen exposure (123).

Bcl-2 family of proteins and their effect in resistance to tamoxifen. Deregulation of anti-apoptotic Bcl-2 family members, including Bcl-2, Bcl-xL and MCL-1, has been implicated in the progression of several different types of cancer. Studies have shown that initial expression of Bcl-2 correlates with ER expression, responsiveness to adjuvant hormonal therapy and ultimately a favorable prognosis (124). Several studies have also suggested that estrogen promotes resistance to chemotherapeutic drugs tamoxifen and cisplatin by increasing the Bcl-2: Bax ratio (196,214). Also, *in vitro* studies in MCF-7 cells have shown that overexpression of HER-2 increases anti-apoptotic Bcl-2 and Bcl-xL proteins, leading to suppression of tamoxifen-induced apoptosis and ultimately tamoxifen resistance (50). Activation of the PI3K/Akt pathway causes phosphorylation of Bad leading to modulation of cellular apoptosis (125). Clinical studies have shown that high levels of Bad expression, not phosho-Bad levels, are associated with improved disease-free survival when compared to tumors with low levels of Bad expression (125).

Another important member of the Bcl2 family is BIK, which is inducible by estrogen-starvation and anti-estrogen

treatment and plays an important role in anti-estrogen-induced apoptosis of breast cancer cells (126-128).

Our group has observed that Bik is a critical factor for resistance to tamoxifen, and utilizing MCF-7 cells has demonstrated that BIK mRNA and protein were strongly induced by estrogen-starvation or anti-estrogen treatment (126,128). Conversely, knockdown of BIK by siRNA significantly inhibited the apoptosis caused by tamoxifen treatment, finding low expression of BAX, BAK and PUMA pro-apoptotic proteins and high expression of some anti-apoptotic proteins, such as BCL-2 and MCL-1 in BIK siRNA-transfected cells after treatment with TAM (26,27). These data demonstrated that Bik is an important factor in the TAM-induced apoptosis process, which may regulate mitochondrial integrity by modulation of pro- and anti-apoptotic proteins. Our results showed that suppression of the *BIK* gene exhibited anti-apoptotic effects in TAM-treated MCF-7 cells. These data may be useful for future studies to establish the mechanisms of regulation of TAM resistance in breast cancer. In women with this neoplasm and with positive ER, it may be important to determine BIK protein levels to define whether or not TAM is the appropriate treatment (128). A possible mechanism of resistance to apoptosis has been established and has been described using mouse fibroblasts transformed with v-src as a model. This group demonstrated that Src-dependent resistance to cell death relies on Src ability to inhibit the mitochondrial pathway of apoptosis by specifically increasing the degradation rate of the BH3-only protein Bik by proteasome due to the phosphorylation of Bik. This effect relies on the activation of the Ras-Raf-Mek1/2-Erk1/2 pathway and on the phosphorylation of Bik on Thr124, driving Bik ubiquitylation on Lys33 and subsequent degradation by the proteasome. These results suggest that Bik could be a rate-limiting factor for apoptosis induction of tumor cells exhibiting deregulated Erk1/2 signaling, which may provide new opportunities for cancer therapies (129).

Role for autophagy in anti-estrogen resistance. Recent studies have demonstrated that autophagy (also referred to as macroautophagy) is critical to the development of anti-estrogen resistance (130). This pro-survival role for autophagy in anti-estrogen-treated breast cancer cells is consistent with one function of the autophagosomes, which is to recycle metabolites from degraded cellular constituents to support a basal level of cell maintenance under starvation conditions and OS, thus rendering it essential for cellular viability.

The mammalian target of rapamycin (mTOR) kinase represents the major negative regulator of autophagy in human cells. Under physiological conditions, mTOR prevents autophagy by maintaining the hyperphosphorylation of the proteins required for the initiation of the autophagic cascade. Conversely, mTOR activity is rapidly shut down under conditions of stress, which allows for the rapid upregulation of autophagy (131). mTOR participates in multiple signaling cascades that regulate cell growth, and especially in those emanating from receptor tyrosine kinases (RTKs). In cancer cells, constitutively active RTK and/or the production of autocrine growth factors lead to the hyperphosphorylation of mTOR substrates. Moreover, several tumors are characterized by activating mutations of the key signal transducers connecting RTK to mTOR, including the small GTPase Ras, phosphatidylinositol 3-kinase (PI3K), as well as the Akt1 kinases and 3-phosphoinositide dependent protein kinase 1 (PDPK1) (132,133). The essential autophagy regulator beclin 1 interacts with several cofactors (including Ambra1, Bif-1 and UVRAG) to activate the lipid kinase Vps34, which is required for the initiation of the autophagic pathway (134).

Beclin 1 is a haploin-sufficient tumor suppressor containing a BH3 domain that contributes to its interaction with BCL-2, but the avidity of this interaction is quite weak compared with the BH3 domains present in the BH3-only proteins typically associated with apoptosis regulation (135). Moreover, BH3-containing Beclin 1 does not appear to antagonize the anti-apoptotic function of BCL-2 at either the ER or mitochondria (136). Thus, canonical BH3-only proteins such as BAD have been shown to displace Beclin 1 from BCL-2 (137), leading to the proposal that an analogous signaling cascade to that associated with the release of BCL-2 antagonists by BH3-only proteins in the apoptosis pathway also extends to autophagy (138). BIK is the founding member of the BH3-only family proteins (139). A number of reports have shown that ectopic overexpression of BIK results in apoptotic cell death. However, BIK has also been reported to cause non-apoptotic cell death in human malignant glioma (140), melanoma cells (141) and in samples of human breast cancer (142), which could be associated with a mechanism of autophagy.

Another important member in autophagy is Vps34, which has a pro-survival function and increased Vps34 expression levels, and tyrosine phosphorylation by pp60c-Scr contributes to enhanced tumorigenic activity in breast cancer cells (143). In MCF-7 cells, knockdown of Vps34 by RNA interference reduced cytoprotective autophagy mediated by BH3 domain and potentiated apoptosis induction (144). Lastly, Vps34 possesses a tumor suppressor function in MCF-7 mouse xenograft tumors mediated through distinct Beclin-1 binding and enhancement of starvation-induced autophagy (145). These studies corroborate lines of evidence demonstrating that the PI3K/AKT/mTOR pathway, implicated in cell survival, contributes to anti-estrogen resistance (146). In contrast, our studies have identified loss of Vps15 and Raptor as the candidate gene target for mediating breast cancer tamoxifen resistance *in vitro*, suggesting that Vps15- or Raptor mediated autophagy promotes cell death induced by tamoxifen. In support of this suggestion, inhibitors of PI3K, Akt and mTOR are in clinical trials and inhibition of mTOR activity is thought to restore tamoxifen sensitivity in breast cancer (147).

miRNA. Recent studies have also shown the critical role of miRNAs in conferring drug resistance or responsiveness in cancer (148,149). In breast cancer, the role of miRNAs in tamoxifen resistance, through regulation of cell cycle regulatory proteins, has been suggested. In particular, ectopic expression of miR-101, miR-206 and miR-221/miR-222 was found to render ER-positive MCF-7 cells resistant to tamoxifen (150-153). These miRNAs were also found to be significantly increased in Her2-positive primary human breast cancer tissues indicating an interrelationship between miR-221/222 expression and Her2 overexpression in primary breast tumors that are generally resistant to tamoxifen therapy. In tamoxifen-resistant breast cancer, it has been shown that miRNA-221/222 play a critical role in the development of

resistance by targeting p27/kip1, a cell cycle inhibitor (153). When miRNA-221/222 downregulate p27/kip1, p27/kip1 can no longer bind to the CDK2/cyclin-E complex, allowing the cells to progress through cell cycle and facilitating growth of cancerous cells, even when estrogen receptors are blocked by tamoxifen (153).

miRNA-mediated targeting of ERα cofactors, which influence agonistic and antagonistic effects of ligand binding, has also been linked to impaired chemotherapeutic response. Indeed, one of the major co-activators for ERα, AIB1, is a target for miR-17 and miR-106, which are found to be dysregulated in breast cancer cells exhibiting drug resistance (154,155). The transcriptional co-repressor, receptor interacting protein 140 (RIP140), was found to be targeted by miR-346, which is downregulated in endocrine-resistant cell lines (156), indicating a role for miRNAs in regulating estrogen-responsive genes.

4. Signaling pathways that regulate EMT in tumor microenvironment

The tubular EMT (TEMT)-related signaling associated with endocrine resistance are WNT, nuclear factor-κβ (NFκβ), Notch, keratinocyte growth factor (KGF), the platelet-derived growth factor (PDGF)/Abl signaling pathway and enolase-1.

Wnt signaling pathway is an important developmental pathway that is frequently dysregulated in human cancers including breast cancer (157-160). Wnt signaling is important for cell migration, invasion, adhesion and survival. Wnt ligands primarily signal via membrane bound Frizzled receptors through a number of different but interconnected signaling pathways, including the β-catenin, Wnt/Ca^{2+}, and planar-cell polarity pathways (1,161). Wnt signaling is altered in nearly one half of all breast cancers. Both upregulation of Wnt pathway activators and downregulation of pathway inhibitors have been identified in breast cancer. Previous studies have reported that the activity of β-catenin is altered in acquired tamoxifen-resistant (TamR) breast cancer cells compared to endocrine-sensitive parental MCF7 cells, with an increase in β-catenin-mediated gene transcription. These observations suggest that deregulated Wnt signaling may play a role in acquired tamoxifen resistance in breast cancer where it may act to promote growth and the development of a more aggressive phenotype.

Keratinocyte growth factor/fibroblast growth factor-7 (KGF/FGF-7) is a member of the FGF family, which interacts with fibroblast growth factor receptors (FGFRs). KGF has a stromal origin and appears to act specifically on epithelial cells in human tissue and is therefore an exclusively paracrine growth factor in human tissues (162,163). KGF stimulates normal human breast and human breast cancer epithelial cell proliferation in a dose-dependent manner. KGF may increase endocrine resistance via decreasing ER, progesterone receptor (PR) and protein tyrosine phosphatases γ (PTPγ). Results suggested that the presence of ER and PR is a good prognostic factor and indicator of benefit from endocrine therapy. Low levels of ER and PR in human breast cancers have been associated with resistance to tamoxifen and increased risk of breast cancer. The signal transduction of KGF/KGFR can proceed via the Ras/MAPK and PI3K/Akt pathways (164,165). The findings on KGF stimulation of cell proliferation and motility via Ras/MAPK suggest that KGF/KGFR could potentially influence the development and progression of breast cancer (166).

The Notch pathway has been reported to be involved in drug resistance. Studies have demonstrated that Notch regulates the formation of cancer stem cells (CSCs) and contributes to EMT leading to the acquisition of the invasive phenotype, which are associated with drug resistance (167,168).

The Notch pathway is implicated in both cell fate in normal human mammary gland and regulation of CSCs in both ductal carcinoma *in situ* and invasive carcinoma of the breast (169-171). Binding of Notch ligand (Jagged or Δ) to the Notch receptor cleaves its intracellular domain (NICD), which translocates to the nucleus where it binds with co-activators to induce transcription of its target genes and regulates migration and invasion of breast cancer cells. Estradiol inhibits Notch activity and affects Notch receptor cellular distribution. Tamoxifen and raloxifene block this effect, reactivating the Notch pathway. Pharmacologic inhibition of Notch activation with γ-secretase inhibitors (GSI) was more effective in combination with tamoxifen than tamoxifen alone (172). These data indicate that GSIs block the proliferative effect of tamoxifen through the Notch pathway, and at the same time allow tamoxifen to exert its antagonistic effect. In a previous study, Rizzo *et al* found that downregulation of Notch-1 by siRNA or GSI potentiated the effects of tamoxifen in breast cancer cells. Moreover, GSI in combination with tamoxifen caused regression of breast cancer cell growth in mice. These data indicate that the combinations of tamoxifen and Notch inhibitors may be effective in ERα (+) breast cancer, and such a combination treatment could eliminate the emergence of tamoxifen-resistance (172,173).

PDGF/Ableson (Abl) canonical signaling pathway has also been associated with resistance (174). PDGF receptor (PDGFR) is classified as a TRK whose activation is dependent on the binding of PDGF resulting in stimulation of several intracellular pathways. PDGF can promote tumor growth via autocrine stimulation of malignant cells, overexpression or overactivation of PDGFRs, or by stimulating tumor angiogenesis. Abl is an Src-like non-receptor protein kinase that acts downstream of the PDGFR (175). Its corresponding gene *c-ABL* is a proto-oncogene with multiple functions; it regulates a variety of cellular activities, including cell migration, response to oxidative stress and DNA damage, cell proliferation and survival. Data revealed the PDGF/Abl canonical pathway as significantly upregulated as early as one-week post-estrogen deprivation and revealed that this could be the top adaptive pathway at the point of full resistance. In studies of molecular changes occurring in tumors in a cohort of patients treated with an aromatase inhibitor in the neoadjuvant setting, it was found that PDGFR β expression was significantly associated with poor antiproliferative response to therapy (176).

α-enolase (ENO1) is a glycolytic enzyme that converts 2-phosphoglycerate into phosphoenolpyruvate in glycolysis and a multifunctional protein that plays a crucial role in a variety of biological and pathophysiological processes (177). ENO1 may act as a stress protein that promotes hypoxic tolerance in tumor cells by increasing anaerobic metabolism (178). ENO1 may also function as a plasminogen receptor on the

surface of a variety of hematopoetic, epithelial, endothelial and cancerous cells (179,180). Previously, several lines of evidence suggested that ENO1 may contribute to tumor malignancy (180). Upregulation of the *ENO1* gene has been observed in several highly tumorigenic or metastatic cell lines (181,182) and its enzymatic activity in breast cancer suggests a role of ENO1 in tumor progression. Increased expression of enolase α in human breast cancer confers tamoxifen resistance in humans. The treatment with 4-OH tamoxifen-induced ENO-1 overexpression, which results in endocrine resistance in human breast cancer (MCF-7) cells. ENO-1 was induced by 4-OH tamoxifen treatment through transcriptional upregulation of ERα and NFκB. The enhanced expression of ENO-1 exerts a negative regulatory effect on c-Myc transcription that counteracts the 4-OH tamoxifen-induced apoptosis of breast cancer cells. In contrast, inhibition of ENO-1 transcriptional regulation, either by blocking the NFκB signal pathway or by siRNA knockdown, significantly sensitized human breast cancer cells to 4-OH tamoxifen-induced cytotoxicity (183). These results suggest that ENO-1 may facilitate resistance to 4-OH tamoxifen-induced cell death and that the combined use of 4-OH tamoxifen and an NFκB pathway inhibitor (PDTC) may provide a novel approach to overcome tamoxifen resistance in breast cancer (184).

5. Conclusion

Endocrine treatment of ER-positive breast cancer with tamoxifen, and later with aromatase inhibitors and the estrogen receptor antagonist fulvestrant, are the first target-based therapeutic strategies in oncology. However, a substantial proportion of patients, despite being ER and/or PR-positive, are either primarily resistant or will develop resistance during the course of their disease. Delineation of the molecular mechanisms underlying the development of resistance will allow the development of therapeutic strategies to improve the efficiency of treatment and to prevent or overcome the drug resistance.

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