**Molecular Pathways**

**Estrogen Receptor Mutations and Changes in Downstream Gene Expression and Signaling**

Ines Barone\(^1\), Lauren Brusco\(^2\), and Suzanne A.W. Fuqua\(^2\,\(^3\)

**Abstract**

Estrogens play a crucial role in regulating the growth and differentiation of breast cancers, with approximately two thirds of all breast tumors expressing the estrogen receptor alpha (ER\(\alpha\)). Therefore, therapeutic strategies directed at inhibiting the action of ER\(\alpha\) by using anti-estrogens such as tamoxifen, or reducing estrogens levels by using aromatase inhibitors, such as letrozole, anastrozole, or exemestane, are the standard treatments offered to women with ER\(\alpha\)-positive cancer. However, not all patients respond to endocrine therapies (termed **de novo** resistance), and a large number of patients who do respond will eventually develop disease progression or recurrence while on therapy (acquired resistance). Recently, variant forms of the receptor have been identified owing to alternative splicing or gene mutation. This article reviews these variant receptors and their clinical relevance in resistance to endocrine therapy, by addressing their molecular cross-talk with growth factor receptors and signaling components. Understanding the complexity of receptor-mediated signaling has promise for new combined therapeutic options that focus on more efficient blockade of receptor cross-talk. **Clin Cancer Res;** 16(10); 2702–8. ©2010 AACR.

**Background**

Human estrogen receptors belong to a superfamily of nuclear hormone receptors that function as ligand-activated transcription factors. Two isoforms of estrogen receptor have been described: ER\(\alpha\) and ER\(\beta\). Each is encoded by unique genes, but share a common structural and functional organization. Classical estrogen receptor (ER\(\alpha\) or hER\(\alpha\)-66) contains an amino-terminal region that harbors the ligand-independent activation function (AF-1), a central DNA binding domain (DBD), and a carboxy-terminal hormone binding domain (HBD), which contains the ligand-dependent activation function (AF-2; Fig. 1). Binding of hormone to ER\(\alpha\) facilitates "classical" genomic activities of the receptor (Fig. 2), and its binding to estrogen response elements (ERE) in target genes function to either activate or repress gene expression.

Estrogen actions are also mediated by other "nonclassical" mechanisms (Fig. 2): (a) ligand-independent ER\(\alpha\) signaling, in which gene activation occurs through second-messengers downstream of growth factor signaling pathways [such as the epidermal growth factor receptor (EGFR), the insulin-like growth factor receptor (IGFR), and the G protein coupled receptor (GPCR) pathways], which alter intracellular kinase and phosphatase activity, resulting in altered phosphorylation of ER\(\alpha\) (1); (b) rapid, "nongenomic" effects through a membrane-associated receptor; and (c) ERE-independent signaling, in which ER\(\alpha\) regulates genes via protein-protein interactions with other transcription factors, such as c-Fos/c-Jun B (AP-1), Sp1, and nuclear factor-\(\kappa\)B (NF-\(\kappa\)B; refs. 2–4). These alternative mechanisms alter downstream target protein expression instrumental in cell division, angiogenesis, and survival, leading to sustained breast cancer growth and progression.

Several laboratories have evaluated the effects of phosphorylation by second-messengers on receptor action. Among the kinases that can phosphorylate ER\(\alpha\) are important signaling molecules such as Akt, extracellular regulated kinase (Erk) 1/2 mitogen-activated protein kinase (MAPK), p21-activated kinase 1 (PAK-1), and protein kinase A (PKA), resulting in diverse responses to ligands (Fig. 1; ref. 5). For example, phosphorylation of ER\(\alpha\) serine (S) 167 by Akt and S118 by Erk1/2 can result in acquired resistance to the anti-estrogen tamoxifen, and ligand-independent activation of ER\(\alpha\) (6–8). Phosphorylation of S305, which is mediated by both PKA and PAK-1 signaling, can impact estrogen hypersensitivity and tamoxifen responsiveness (9–11). These phosphorylation events are complex and interdependent. For instance, phosphorylation at ER\(\alpha\) S305 can regulate the subsequent phosphorylation of S118 (12), and receptor acetylation (9).
The region between the DBD and the LBD, known as the hinge, has long been considered to simply serve as a flexible linker to orient the other two functional domains. However, it is now thought that this region is a multifunctional domain that binds a number of coregulatory proteins and participates in the binding of estrogen receptor to DNA (9, 13, 14). The lysine residues K266, K268, K299, K302, and K303 within this domain are conserved residues that can be acetylated by the histone acetylase protein p300 (Fig. 1; refs. 14–17). Acetylation of K266 and K268 induced DNA-binding and ligand-dependent activation (14), whereas acetylation of K302 and K303 inhibited ERα activation (15). The phosphorylation status of ERαS305 coordinately regulates the acetylation of the K302/303 residues, sensitizing ERα to ligand stimulation (9). ERαK302 is also methylated by the SET7 methyltransferase (Fig. 1); this methylation stabilizes the receptor and is necessary for the efficient recruitment of ERα to its target genes and subsequent transactivation (18). Acetylation of K303 attenuates ERα-driven transcription, not just from antagonism via acetylation, but also by inhibition of K302 methylation and subsequent destabilization of ERα. Other modifications, such as ubiquitination at K302 (19) and sumoylation at K266 and K268 (20), have also been shown to affect ERα stability and activity. Thus residues in the hinge domain are frequent targets for post-translational modifications that affect hormone sensitivity through alteration of receptor stability or regulation of estrogen-dependent gene transcription. It is tempting to speculate that immunohistochemical quantitation of these post-translational modifications could provide important prognostic or predictive information in clinical samples.

**Variant ERα Protein Isoforms**

Several groups have identified ERα splice variants in a number of different normal tissues such as human breast epithelium, endometrium, and pituitary, as well as various tumor types including breast cancer, endometrial carcinoma, and meningiomas; these mRNA variants are usually co-expressed along with the wild-type receptor (reviewed in ref. 21). These splice variants can confer...
either dominant-positive or dominant-negative effects on cancer cells, and are hypothesized to contribute to the hormone-independent phenotype of some breast tumors. Among these variants is ERα exon Δ3, that is missing part of the DBD (22), which showed the most significant increase in levels in breast cancer tissue (Fig. 1; ref. 23). The ERα Δ3 isoform functions as a dominant-negative receptor, able to suppress estrogen-induced transcriptional activity (24), reduce anchorage-dependent growth, soft-agar colony forming ability, and in vitro invasion when transfected in breast cancer cells (25). It is hypothesized that this reduction in estrogen receptor signaling may lead to unchecked estrogen stimulation, establishing permissive conditions for further carcinogenic events.

Despite the different estrogen receptor splice variants described thus far, relatively few variant ERα protein isoforms have been characterized, in part owing to the practical limitations of their detection (26–28). Previous reports have shown the presence of three predominant bands of 35 to 39, 46, and 66 kDa in immunoblots probed with an anti-ERα antibody raised against the LBD (26–28). There is accumulating evidence that these isoforms could play significant roles in estrogen receptor signaling events.

For instance, the hERα−46 isoform has been biochemically isolated from MCF-7 breast cancer cells (29), vascular endothelial cells (30), and osteoblasts (26). This isoform lacks the first 173 amino acids in the amino-terminal AF-1 domain because of alternative splicing of exon 1, and it copurifies with plasma membrane markers (Fig. 1; ref. 31). This altered location allows cells to mediate rapid estrogen signaling events, such as stimulation of nitrogen oxide synthesis (27). hERα−46 forms heterodimers with full-length ERα acting as a strong competitive inhibitor in ERα-positive cells, and it can promote activation of genomic activities in ERα-negative tissues (26, 29). Further studies are needed to identify the exact

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**Fig. 2.** A representation of the "classical" and "nonclassical" estrogen receptor signaling pathways. Estrogen receptor mediates transcription of its target genes using two types of mechanisms, these are known as "classical" and "nonclassical" signaling. First, "classical" signaling initiates with the binding of estrogen to estrogen receptor, causing it to bind directly to regions of DNA called EREs, located within transcriptional start sites of estrogen-regulated genes, which subsequently activate transcription of downstream genes. There are several mechanisms of "nonclassical" signaling. The first of these mechanisms is mediated by the signaling of growth factors (such as IGF and EGFR) and G-protein coupled receptors, through downstream signaling molecules to estrogen receptor. These pathways mediate estrogen receptors’ state of post-transcriptional modification (by affecting its phosphorylation, acetylation, methylation) and thus its activity, independent of estrogen binding. It is likely that crosstalk of these pathways not only results in estrogen-independent activation of estrogen receptor but also endocrine resistance. Signaling has also been shown to occur through truncated membrane bound forms of estrogen receptor; this signaling is usually inhibitory of full length estrogen receptor activity. Finally, another mechanism of "nonclassical" signaling requires the binding of estrogen receptor to other transcription factors (including SP-1 and AP-1), causing a recruitment of estrogen receptor to transcriptional start sites other than EREs and transcription of downstream genes.
function of this isoform in estrogen target cell proliferation, and to understand its potential prognostic role in clinical samples.

hERα−36 is a naturally occurring isoform that is expressed in both ERα-positive and -negative breast cancer cells, and is generated from a promoter located in the first intron of the hERα−66 gene (32, 33). It lacks both AF-1 and AF-2, but retains the DBD and portions of the HBD (Fig. 1). It possesses an extra, unique 27-amino-acid domain that replaces the last 138 amino acids encoded by exons 7 and 8 of the hERα−66 gene (28). The hERα−36 isoform also contains three potential myristoylation sites located near the amino-terminal region, which are postulated to direct it to the plasma membrane. This isoform lacks intrinsic transcriptional activity, but it efficiently suppresses the transactivation activities mediated by full-length ERα, suggesting that it is a potent inhibitor of genomic estrogen signaling. Interestingly, the hERα−36 isoform primarily localizes to the plasma membrane, where it transduces “nongenomic” signaling cascades initiated by both estrogens and anti-estrogens, such as activation of the MAPK/ERK signaling pathway, thus stimulating cell proliferation (34). In clinical samples, overexpression of hERα-36 was associated with poorer disease-free survival in patients, identifying a subset of patients who are less likely to benefit from tamoxifen treatment (33). In summary, ERα protein isoforms capable of modulating ERα-mediated signaling have been identified, and their integration into the accurate classification of ERα status may be warranted.

### Table 1. Clinical trials using drugs that target pathways known to interact with the ERα pathway in patients with ERα-positive breast disease

<table>
<thead>
<tr>
<th>Drug/Combination</th>
<th>Pathway Target(s)</th>
<th>Patient Disease Information</th>
<th>Phase</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-325901*</td>
<td>MEK (MAPK pathway)</td>
<td>Advanced breast cancer, colon cancer, and melanoma</td>
<td>I-II</td>
<td>Terminated</td>
</tr>
<tr>
<td>Bevacizumab + sorafenib tosylate†</td>
<td>RAF (MAPK pathway)</td>
<td>Refractory, metastatic, or unresectable solid tumors</td>
<td>I</td>
<td>Ongoing</td>
</tr>
<tr>
<td>Paclitaxel and RAD001 followed by FEC (chemotherapy)‡ §</td>
<td>mTOR</td>
<td>Triple negative breast tumors</td>
<td>II</td>
<td>Recruiting</td>
</tr>
<tr>
<td>Ritonavir (preoperative)†</td>
<td>Akt</td>
<td>Newly diagnosed breast cancer patients</td>
<td>I-II</td>
<td>Not yet open</td>
</tr>
<tr>
<td>GSK2141795§</td>
<td>Akt</td>
<td>Solid tumors/lymphomas not responsive to other therapies</td>
<td>I</td>
<td>Recruiting</td>
</tr>
<tr>
<td>GDC-0941 + bevacizumab + paclitaxel**</td>
<td>PI3K</td>
<td>Locally recurrent or metastatic breast cancer</td>
<td>Ib</td>
<td>Recruiting</td>
</tr>
<tr>
<td>BGT226§</td>
<td>PI3K</td>
<td>Advanced solid malignancies including breast cancer</td>
<td>I-II</td>
<td>Recruiting</td>
</tr>
<tr>
<td>BEZ235§</td>
<td>PI3K</td>
<td>Advanced solid malignancies including breast cancer</td>
<td>I</td>
<td>Recruiting</td>
</tr>
<tr>
<td>Temsirolimus† †</td>
<td>mTOR</td>
<td>Locally recurrent or metastatic breast cancer</td>
<td>II</td>
<td>Ongoing</td>
</tr>
<tr>
<td>XL147+ XL647‡‡</td>
<td>PI3K</td>
<td>Solid tumors including breast cancer</td>
<td>I</td>
<td>Suspended</td>
</tr>
</tbody>
</table>

**NOTE:** Information from http://ClinicalTrials.gov. Study sponsors footnoted below.

Abbreviation: mTOR, mammalian target of rapamycin.

*Pfizer.
†NCI.
‡MD Anderson, Houston, TX.
§Novartis.
††GlaxoSmithKline.
**Genentech.
†††University of Chicago.
‡‡Exelixis.

**ERα Mutations in Tumors**

The number of naturally occurring mutations identified in breast cancers to date is relatively low (35), which is surprising because mutation of the clinical target is a common resistance mechanism in tumors. The Y537N (Tyr537Asn) mutation was discovered in a metastatic breast tumor (36). This mutation eliminates a carboxy-terminal
tyrosine residue that is considered to be an important c-Src phosphorylation site with potential roles in regulating ligand binding, homodimerization, and transactivation of ERs. It was shown that the Y537N ERα mutant exhibits constitutive transactivation activity, and that this activity was only slightly affected by estradiol, tamoxifen, or the steroidal anti-estrogen ICI 164,384 (36). A mutation at this site may allow ERs to escape phosphorylation-mediated controls, providing cells with a potential selective advantage, but unfortunately, only a few metastatic breast tumors have yet been examined for mutations at this site.

A somatic mutation at nucleotide 908 of ERα (A908G) has been identified in about a third of premalignant breast hyperplasias and one half of invasive breast tumors from untreated patients (37, 38). The A908G mutation introduces a lysine-to-arginine transition at residue 303 (termed K303R) within the hinge domain. Molecular analyses of the K303R ERα mutation have shown that the mutated arginine at the 303 position allows ERα to be more highly phosphorylated by PKA (9) and Akt kinase signaling (39), and alters the dynamic recruitment of co-activators and corepressors, such as BRCA-1 or calmodulin (40, 41).

Overexpression of the K303R ERα mutation in ERα-positive MCF-7 breast cancer cells confers estrogen hypersensitivity (37), and decreased sensitivity to tamoxifen treatment when engaged in cross-talk with growth factor receptor signaling pathway (42). Enhanced growth factor receptor cross-talk with ERα is a known mechanism of hormone resistance in breast cancer (43). Expression of the K303R ERα mutation also conferred resistance to the nonsteroidal aromatase inhibitor anastrozole in ERα-positive cells, via a dynamic interaction between the K303R ERα mutation, S305 phosphorylation, and the IGF-1R signaling pathway (39, 44). Signaling components both upstream and downstream of the IGF-1R were altered in mutant-overexpressing cells. The frequency of the mutation is still contentious (45–49), however, the sequencing method used in some of these studies might not have been sensitive enough for straight-forward detection of this specific mutation (38). The presence of the K303R ERα mutation was associated with poor outcomes in univariate analyses of tumors from untreated breast cancer patients, and its presence was correlated with older age, larger tumor size, and lymph node-positive disease, all clinical factors associated with worse outcomes (38). Collectively these data suggest that this mutation could play an important role as a predictive marker in breast cancer, and strategies to accurately measure it in clinical samples are currently underway.

**Clinical-Translational Advances**

*Combination therapies: How do ERα variants come into play?* Because a number of different signaling pathways can be simultaneously active in ERα-positive breast tumors, and a variety of estrogen receptor activities, such as receptor turnover, cellular localization, and hormone responsiveness can all be influenced by the presence of ERα variants, strategies to prevent signaling to these variants may present a unique opportunity for complete blockade of estrogen receptor. Future directions include peptide mimetics capable of blocking altered post-translational modifications on the receptor (39, 42). We speculate that the ERα hinge region may be a particularly attractive target to block the multiple post-translational modifications, including phosphorylation, acetylation, methylation, sumoylation, and ubiquitination, occurring in this important regulatory region.

Major breakthroughs in understanding the molecular dynamics of cell-signaling networks operative in tumors are rapidly being translated into the clinic, with a number of potent drugs selectively targeting the MAPK and PI3K/Akt pathways entering into clinical trials (Table 1). There is also evidence of potential cross-talk between these signaling pathways, necessitating horizontal blockade, or combined use of multiple signaling inhibitors. The complex bidirectional cross-talk that exists between growth factor receptors, these second-messenger signaling pathways, and ERα, along with the variant ERα forms suggests that simultaneous blockade will be required to bypass resistance mechanisms or restore hormone sensitivity in some breast cancer patients. The potential utility of these new therapeutics to signaling components, along with combined ERα-directed therapy is predicted to improve clinical care and reduce mortality from breast cancer.

**Conclusions**

A significant challenge for effective blockade of ERα signaling is the inherent cellular heterogeneity present in breast tumors. Not only can variant forms of ERα be expressed along with wild-type receptor in tumors, but ERα-positive patients can present with alterations in the growth factor receptors themselves, as well as critical signaling molecules such as PI3K and Akt (50). Accurate determination of this molecular heterogeneity is requisite for accurate and effective treatment decisions. The development of new biomarkers to detect this heterogeneity is key.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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References


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