Breast Cancer: Targets and Therapy

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REVIEW

Multidrug-resistant breast cancer: current perspectives

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Abstract: Breast cancer is the most common cancer in women worldwide, and resistance to the current therapeutics, often concurrently, is an increasing clinical challenge. By understanding the molecular mechanisms behind multidrug-resistant breast cancer, new treatments may be developed. Here we review the recent advances in this understanding, emphasizing the common mechanisms underlying resistance to both targeted therapies, notably tamoxifen and trastuzumab, and traditional chemotherapies. We focus primarily on three molecular mechanisms, the phosphatidylinositide 3-kinase/Akt pathway, the role of microRNAs in gene silencing, and epigenetic alterations affecting gene expression, and discuss how these mechanisms can interact in multidrug resistance. The development of therapeutics targeting these mechanisms is also addressed.

Keywords: PI3K/Akt, epigenetics, miRNA, ER, HER2, triple negative

Introduction

Breast cancer affects 1.38 million women worldwide per year, making it the most common cancer in women,[1] and although the implementation of screening programs and the development of new therapeutics in the last 20 years have significantly reduced mortality rates in the Western world, resistance to these therapeutics is a growing problem.[2] Resistance can be de novo but may also be acquired. Indeed, 30% of women with early-stage breast cancer have recurrent disease, and resistance to therapeutic agents can occur in at least a quarter of all cases.[3,4] The incidence of resistance to therapeutics increases with disease progression,[4] and this refractivity contributes to breast cancer having the highest mortality rate, 12.9/100,000 population in the USA in 2008, after lung cancer.[1,5]

Breast cancer is a heterogeneous disease and is divided clinically into three basic subtypes determined by the expression of hormone receptors (estrogen and progesterone), human epidermal growth factor receptor 2 (HER2), and triple-negative breast cancer, which expresses none of these receptors.[6] Further subdivisions are now recognized, including luminal A, luminal B, basal-like, and HER2-enriched,[7] and, recently, work has classified breast cancers into ten subtypes.[8] However, to aid clarity, we will use the three basic clinical subtypes in this review. Each subtype has a different treatment strategy. For hormone receptor-positive breast cancer, the frontline treatment is endocrine therapy, whereas for HER2-positive cancers, it is trastuzumab (Herceptin; Roche, Basel, Switzerland). These specific therapies are often used in conjunction with traditional cytotoxic chemotherapeutics, such as taxanes and anthracyclines, which are the frontline therapies for triple-negative breast cancer.[4] The mechanisms underlying resistance to these different therapeutics are multiple, complex, and not mutually

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exclusive, and have recently been excellently reviewed for each of the three subtypes individually.[3,9–12] Here we will provide an overview of selected mechanisms underlying the drug resistance in each of these different groups, highlighting common mechanisms, specifically focusing on the role of the phosphatidylinositide 3-kinase (PI3K)/Akt pathway, miRNAs, and epigenetic alterations in drug resistance in breast cancer and how these interactions can contribute to a multidrug-resistant phenotype. The identification of potential targets for new therapies or as adjuvants to restore sensitivity to current treatments will be emphasized.

Hormone receptor-positive breast cancer

The predominant hormone receptor expressed in breast cancer is the estrogen receptor (ER), which is activated by the binding estrogens, predominantly estradiol.[13] Hormone receptor-positive breast cancer also expresses progesterone receptors; however, these receptors have been somewhat neglected as therapeutic targets, as the first generation of antagonists had severe side effect profiles.[14] Progesterone receptors have traditionally been considered as downstream targets of ERs, and their mechanistic roles in resistance to endocrine therapy underexplored. Consequently, we will limit our discussions here to ER-positive breast cancer (for further information on progesterone receptors in breast cancer we would like to draw the reader's attention to the recent review by Brisken[14]). Activation of the ER leads to a transcriptional response, both in genes with and without ER response elements, and also nontranscriptional cellular responses, all of which favor cell proliferation and survival (see Figure 1).[15] There are two ER forms, α and β. High expression levels of the latter have been correlated with good clinical prognosis,[16] but as the role of this receptor in breast cancer has not been as widely studied as the ERα, we will focus on the former (we refer the reader to the recent review by Haldosén et al[17] for further information). ERα-positive breast cancer accounts for approximately 70% of all breast cancer cases.[13] The expression levels of ERα determine patient response to endocrine (antiestrogen) treatment and can be used as predictor of

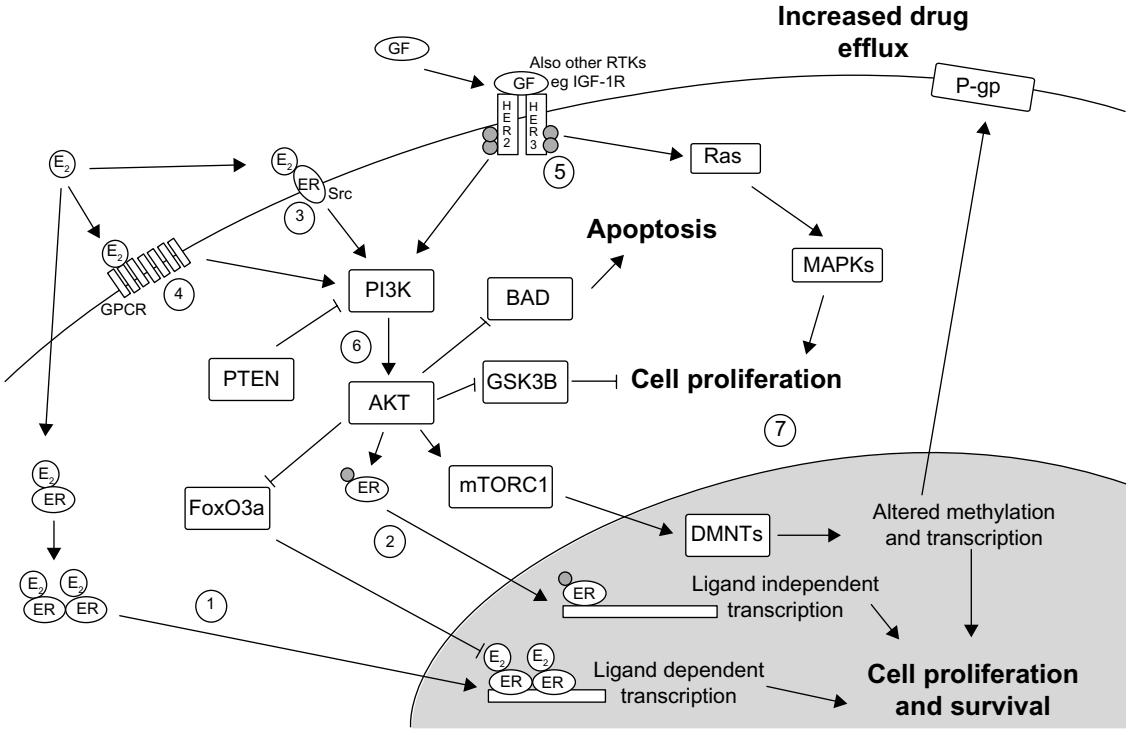

Figure 1 Estrogen, HER2 signalling, and the PI3K/Akt pathway in drug-resistant breast cancer.

Notes: ER can activate gene transcription by nuclear translocatin following ligand binding (**1**) or as a result of receptor phosphorylation in the absence of ligand (**2**). ERs may also be found associated with the plasma membrane in the presence of SRC and other adaptor proteins. Here, ligand binding triggers nongenomic effects via activation of signaling pathways, including the PI3K/Akt and the Ras/MAPK pathways (not shown) (**3**). These pathways are also activated by ligand binding to the GPR30 (**4**) and by growth factor binding to receptor tyrosine kinases, including HER2, inducing autophosphorylation and downstream signalling (**5**). The PI3K/Akt pathway (**6**) as indicated is a covergence point in the mechanisms implicated in drug resistance in the three types of breast cancer discussed here, as pathway hyperactivity frequently occurs with multiple downstream effects (**7**). Data from[9–11,25,26,30,35,36,42,57,60]

Abbreviations: BAD, Bcl-2-associated death promoter; DMNTs, DNA methyltransfereases; E_2, estrogen; ER, estrogen receptor; GF, growth factor; GPR30, G-protein coupled receptor 30; GSK3B, glycogen synthase kinase 3 beta; HER2, human epidermal growth factor receptor 2; IGF-1R, insulin-like growth factor receptor 1; MAPK, mitogen-activated protein kinase; mTORC1, mammalian target of rapamycin complex 1; P-gp, P-glycoprotein; PI3K, phosphatidylinositide 3-kinase; PTEN, phosphatase and tensin homolog; RTK, receptor tyrosine kinase; SRC, steroid receptor coactivator.

disease outcome.[18] The frontline treatment for this type of breast cancer is endocrine therapy, predominantly tamoxifen or fulvestrant, which are antagonists of ER in breast tissue (tamoxifen has agonist actions in other tissues[4]). In postmenopausal women, tamoxifen/fulverstrant is often used in conjunction with aromatase inhibitors (eg, anastrozole or letrozole) that reduce estrogen synthesis and lower the recurrence rate compared with tamoxifen alone.[13] However, many patients with metastatic ER-positive disease develop resistance to endocrine therapy,[13] so understanding the molecular mechanisms that lead to refractory disease may identify ways to circumvent resistance and restore responses to endocrine therapies. The mechanism of estrogen-induced cell responses (Figure 1) means there are many levels at which resistance to endocrine therapy can occur (excellently reviewed by Bianco and Gevry[9]). Here we will explore those that mediate tamoxifen resistance, as this is the best studied, and many of these mechanisms apply to other endocrine therapies.

Molecular mechanisms of tamoxifen resistance

Tamoxifen metabolism

Tamoxifen itself is a prodrug that requires bioactivation to the major metabolite, endoxifen, to be active against ER.[19] This process involves two members of the cytochrome P450 (CYP) family, CYP2D6 and CYP3A4. Both isoforms have several common polymorphisms.[20,21] To date, none of the common CYP3A4 polymorphisms has been associated with altered tamoxifen metabolism.[21] However, the CYP2D6 polymorphisms are well characterized and affect its catalytic activity. These polymorphisms are categorized into four groups, from the ultrarapid metabolizers with increased activity down to poor metabolizers with no CYP2D6 activity.[20] Recent work has correlated CYP2D6 metabolizer status with response to tamoxifen treatment, with poor metabolizers having greater tumor progression than extensive metabolizers.[22] Thus, determining CYP2D6 metabolizer status before tamoxifen treatment would be beneficial, allowing patients with poor metabolizer status to be treated with altered doses or other endocrine therapies, and consequently not categorized as resistant to endocrine therapy.

ERα activity

Phosphorylation is a common mechanism of post-translational modification to regulate enzyme activity, and has been linked with drug resistance in some cancers, including gemcitabine-resistant pancreatic cancer.[23] Phosphorylation regulates ERα activity and plays a role in tamoxifen resistance, specifically phosphorylation of the serine 305 residue by protein kinase A or p21-activated kinase-1. Both of these kinases show enhanced activity in tamoxifen-resistant breast cancer.[24,25] This phosphorylation changes the action of tamoxifen from antagonist to agonist; thus, the presence of tamoxifen now leads to the formation of an active transcription complex. The mechanisms underlying this change of response are not fully understood, but phosphorylation of S305 induces an altered orientation of binding between ERα and the steroid receptor coactivator-1, allowing the recruitment of RNA polymerase II and ERα-mediated gene transcription.[26] This occurs without changes in the overall levels of binding; thus, in the presence of tamoxifen, estrogen-dependent gene transcription can be induced, and in patients with overactive protein kinase A or p21-activated kinase-1, treatment with tamoxifen could enhance tumor progression. To date, though, no studies have explored this mechanism in patient tissues.

Recently, a third isoform of ER has been identified, a 36kDa protein transcribed from an alternative start site and lacking the transactivation domains of the full-length (66kDa) ER, ER-α36.[27] This isoform has a dominant negative effect on ERα activity, inhibiting both estrogen-dependent and independent effects, and levels are increased in tamoxifen-resistant MCF-7 cells[28] and have been associated with poorer disease-free survival in ER-positive and ER-negative breast cancer.[28,29] The binding of both estrogen and tamoxifen to ER-α36 stimulates activation of mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) pathway, leading to cell proliferation and thus contributing to tamoxifen resistance.[27,28]

In addition to the MAPK/ERK pathway, ERα can activate, by nontranscriptional mechanisms, the PI3K/Akt pathway (see Figure 1 for details), and alterations that increase the activity of this signaling pathway can lead to tamoxifen resistance.[30] However, ERα is not the only receptor that can activate this pathway. It is also activated by a number of growth factor receptors, including fibroblast growth factor receptors (FGFR) 1 and 3 and insulin-like growth factor receptor 1 (IGF-1R).[31] Both of these receptors have been shown to contribute to tamoxifen resistance via PI3K/Akt signaling and MAPK signaling.[32,33] Recent work suggests that microRNAs (miRNAs) also have important roles in modulating the PI3K/Akt pathway in response to tamoxifen treatment. Sachdeva et al[34] identified miR-101 as being able to confer tamoxifen-resistant and estrogen-independent growth on MCF-7 cells by suppressing membrane-associated guanylate kinase inverted 2 expression and reducing the activity of phosphatase and tensin homolog (PTEN), a negative

regulator of the PI3K/Akt pathway. Aberrations to the PI3K/Akt pathway and the roles of miRNAs in drug resistance in breast cancer are discussed in more detail in the sections PI3K/Akt pathway and miRNA-mediated resistance.

Aberrant expression of ERα target genes can lead to tamoxifen resistance. For example, overexpression of cyclin D1 drives cancer cell proliferation, circumventing the normal cell-cycle control and deregulating ERα-dependent gene responses.[3] Such alterations to the expression of ERα-dependent genes often arise from epigenetic changes, which occur in multiple formats (see section Epigenetic regulation for more details), the net effects of which are increased or decreased gene transcription. For example, transcription of the antiapoptotic gene *BCL-2*, an ERα target gene, is increased in tamoxifen-resistant cells, due to histone demethylation,[35] thus favoring cell survival and disease progression.

HER2-positive breast cancer

HER2 is a receptor tyrosine kinase and a member of the ErBb family. Dimerization of HER2 as homo- or heterodimers with other ErBb family members, in both the presence and absence of ligand, leads to receptor activation and downstream signaling (see Figure 1). In mammary tissues, HER2 activation promotes cell survival and proliferation.[36] Amplification of the HER2 (*ErbB2*) gene occurs in approximately 20% of breast cancers, and 8% of ERα-positive breast cancers are also positive for HER2.[13] ERα/HER2-positive breast cancer is associated with a poorer clinical outcome[13] due to a tendency to display intrinsic resistance to endocrine therapy, which may result from interplay between the downstream signaling pathways.[37]

The development of trastuzumab (Herceptin), a monoclonal antibody that binds HER2, has improved clinical outcomes for patients with HER2 amplification. Trastuzumab induces a cytostatic signal, G1 arrest, and induction of an immune response to destroy the cancer cell.[38] However, de novo resistance to trastuzumab occurs in approximately 65% of cases, and resistance develops in approximately 70% of patients who initially respond.[11] A number of causative mechanisms have been proposed (for an in-depth review, please see Pohlmann et al[10] and Vu and Claret[11]), including altered binding, upregulation of downstream pathways, and alterations to the immune response induced.

Molecular mechanisms of trastuzumab resistance

Epitope masking

Resistance to trastuzumab can occur due to epitope masking altering the binding to HER2. Two possible epitope masking candidates have been identified to date. Mucin 4, a large O-glycosylated membrane-associated protein, is one possible candidate, as it is upregulated in JIMT-1 cells that are trastuzumab resistant. The ratio of trastuzumab binding to HER2 binding sites was a fifth of normal on these cells, and trastuzumab–HER2 binding was increased by RNAi knockdown of Mucin 4.[39] The second candidate is the CD44/hyaluronan polymer complex, and, again, RNAi knockdown of CD44 or chemical inhibition of hyaluronan synthesis resulted in increased trastuzumab–HER2 binding in JIMT-1 cells. This effect is postulated to be due to the bulky nature of the complex sterically hindering trastuzumab binding and alterations to trastuzumab internalization,[40] thus conferring resistance, as trastuzumab cannot suppress HER2 signaling.

HER2 signaling

Blockade of HER2 signaling with trastuzumab can be bypassed, to some degree, by the upregulation of other ErBb family members and increased heterodimer formation. In addition, in up to 30% of HER2-enriched breast cancers, an amino-terminal truncated form of HER2 is expressed, p95-HER2. This form of HER2 possesses constitutive kinase activity, triggering downstream signaling, but lacks the trastuzumab binding site, thus generating trastuzumab resistance.[33,41] In these cases, treatment with another monoclonal antibody, pertuzumab, which blocks dimer formation, or with lapatnib, which inhibits the tyrosine kinase activity of HER2 dimers, may restore clinical responsiveness to anti-HER2 therapy.[42] However, even if HER2 signaling is effectively blocked, cancer proliferation may continue, as downstream pathways are activated by alternative routes, as described for tamoxifen resistance, again via IGF-1R, which is often overexpressed in trastuzumab-resistant cells, and its inhibition can restore sensitivity to trastuzumab in SKBR3 cells.[43] Signals from IGF-1R are transduced, in part, by the PI3K/Akt pathway, which is also activated by HER2 and ERα. Trastuzumab treatment can induce alterations to this pathway, giving rise to resistance via sustained Akt activation. One mechanism that may mediate this is the upregulation of c-Met, which physically interacts with HER2, enhancing growth in HER2-overexpressing cells. This effect is abrogated upon treatment with a c-Met inhibitor such as SU11274.[44] Trastuzumab treatment also induces upregulation of a number of miRNAs, notably miR-21, that can influence the PI3K/Akt pathway by inhibiting PTEN.[45] In trastuzumab-resistant cells, overactivity of the PI3K/Akt pathway leads to epigenetic changes, including repression of FoxO, and

subsequently the increased transcription of the antiapoptotic gene survivin.[46] It can also downregulate $p27^{Kip1}$, which has been proposed as a critical downstream effector of trastuzumab, leading to increased cyclin-dependent kinase 2 expression and cell proliferation.[42] The aberrations in this pathway contributing to multidrug-resistant breast cancer are discussed further in the section PI3K/Akt pathway.

Altered immune response

As a monoclonal antibody, trastuzumab-induced cell death is partially mediated by antibody-dependent cell-mediated cytotoxicity, a process by which natural killer cells/monocytes bind to the Fc region of trastuzumab with Fcγ receptors and initiate tumor cell death.[47] FcγRIIIa has a well-known polymorphism at position 158, where either a valine or a phenylalanine is expressed, which has been linked to clinical prognosis. Indeed, the latter is less effective at inducing antibody-dependent cell-mediated cytotoxicity upon trastuzumab binding to HER2 and shows poorer clinical outcome than the valine/valine genotype in patients.[48] This mechanism of resistance is specific to monoclonal antibody therapies and not related to the common mechanisms discussed in the section Common mechanisms leading to drug-resistant breast cancer.

Triple-negative breast cancer

Triple-negative breast cancer does not express ERα or progesterone receptors (PR) or show HER2 amplification, and accounts for 10%–20% of breast cancers.[49] To date, there are no targeted therapies for this breast cancer subtype. Consequently, frontline treatments are limited to surgical approaches and chemotherapeutics, with the taxanes and anthracyclines being the chemotherapeutics of choice.[50] Taxanes (eg, paclitaxel) act as mitotic poisons by stabilizing microtubules, leading to abnormal spindles and induction of apoptosis,[51] whereas anthracyclines (eg, doxorubicin) act as DNA intercalators and topoisomerase II inhibitors and they can also generate reactive oxygen species via semiquinone formation.[52] These chemotherapeutics are often given in conjunction with the targeted therapies for the other subtypes of breast cancer discussed previously, and thus cellular alterations induced by targeted therapies can contribute to chemotherapeutic resistance and vice versa.

Molecular mechanisms of resistance to chemotherapeutics

Resistance to both the taxanes and the anthracyclines predominantly arises from decreased drug intracellular concentrations due to increased efflux.[12] This increased efflux is mediated by a small number of adenosine triphosphate (ATP)-binding cassette proteins that utilize ATP hydrolysis to translocate a variety of substrates, and increased expression of these correlates with poor clinical prognosis in breast cancer irrespective of subtype.[53] The taxanes and anthracyclines are substrates for P-glycoprotein (P-gp), the best studied of the ABC transporters responsible for drug efflux and encoded by the *MDR1* gene (*ABCB1*), as well as other transporters, notably multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein, that contribute to resistance to chemotherapeutics.[54] We will focus on P-gp here as an example, as many of the mechanisms discussed may be applicable to MRP2 and breast cancer resistance protein. Resistance to one chemotherapeutic commonly means resistance to many, often structurally distinct compounds, as P-gp can transport a diverse range of molecular structures. Indeed, the tamoxifen metabolite endoxifen is a P-gp substrate.[55]

Alterations to drug efflux levels

The primary mechanism underlying increased efflux is upregulation of the drug transporter proteins. This phenomenon occurs in a wide variety of cancers and has consequently been excellently reviewed by Chen and Sikic.[12] Subsequently, we will cover this topic briefly here to highlight the commonalities with tamoxifen and trastuzumab resistance, specifically focusing on miRNAs, the PI3K/Akt pathway, and epigenetic alterations (discussed in more detail in the section Common mechanisms leading to drug-resistant breast cancer).

Recent work has identified miRNAs as having an important role in multidrug resistance, with over 50 different miRNAs linked to this phenotype (see Kutanzi et al[56] for a review). A number of these miRNAs, including miR-19, miR-21, and miR-205, target PTEN, suggesting that the PI3K/Akt pathway is important in resistance to chemotherapeutics as well as for the more targeted therapies discussed previously. Indeed, use of the Akt inhibitor perifosine in multidrug-resistant MCF-7 cells improved response to doxorubicin treatment.[57] It remains to be seen whether this is via a direct effect of the PI3K/Akt pathway on *MDR1* expression or an indirect effect possibly via a reduction in cell survival signaling. In addition to altered miRNA levels, chemotherapeutic-resistant cancers, both in vitro and in patients, show diminished epigenetic repression of *MDR1* with promoter hypomethylation and histone H3 lysine 9 acetylation.[58–60]

Common mechanisms leading to drug-resistant breast cancer

As can be seen from these discussions, the three clinical subtypes of breast cancer have distinct therapeutic approaches, but the molecular mechanisms that give rise to refractory disease have common facets, notably alterations to the PIK3/Akt pathway, miRNA levels, and epigenetic modulation of gene transcription. These common facets will now be discussed in more detail, together with their potential as targets for adjuvant therapies to circumvent drug resistance and restore clinical responsiveness.

PI3K/Akt pathway

The PI3K/Akt pathway is an important signaling mechanism regulating many cellular responses, including cell proliferation and survival (Figure 1) in normal as well as neoplastic breast tissue. It forms a convergence point between all three clinical subtypes of breast cancer, and aberrations in this pathway occur in 70% of breast cancers irrespective of subtype.[61] As highlighted previously, aberrations in this pathway are important in resistance to both tamoxifen and trastuzumab, especially as this pathway forms a crosslink between HER2 signaling and ERα-regulated gene transcription,[37] and have also been linked to *MDR1* upregulation and resistance to chemotherapeutics. Thus, understanding this pathway is paving the way for new adjuvant treatments in resistant breast cancer. A number of changes can occur, but all result in sustained pathway activity. Common aberrations include activating mutations or amplification of any of the PI3K subunits p110α, p110β, or p85α or loss of PTEN activity and its inhibition of PI3K, via inactivating mutations, overexpression of miRNAs, or promoter hypermethylation.[31] Both of these scenarios result in increased Akt phosphorylation and sustained Akt activation, the net effects of which are inhibition of apoptosis, transcription of ERα-dependent genes, and cell proliferation.[61] A major downstream effector of Akt activation that mediates a number of these responses is mammalian target of rapamycin complex 1 (mTORC1). mTORC1 also acts as a signaling integration node receiving inputs from the MAPK pathways that may be disrupted in drug-resistant breast cancer. Sustained PI3K/Akt/mTORC1 activity may also be due to alterations in miRNA expression and can induce a number of epigenetic changes that perpetuate drug resistance, which are discussed here.

miRNA-mediated resistance

In the last decade it has become clear that alterations to miRNA expression levels can contribute to cancer prognosis and outcome.[62] miRNAs are small, noncoding RNAs or approximately 22 nucleotides, which bind to mRNA, preventing translation and accelerating mRNA deadenylation and subsequent degradation, thus having a gene silencing effect.[63] Several miRNAs have been associated with drug resistance in breast cancer (see Table 1), and these target a variety of genes, including *PTEN*, *ESR1* (ERα), *FoxO3*, and DNA (cytosine-5)-methyltransferases (*DNMTs*).[56] The mechanisms that lead to miRNA upregulation in drug-resistant breast cancer are currently unclear, but they have powerful effects. One miRNA that is overexpressed

Table 1 miRNA associated with drug resistance in breast cancer

miRNA	Expression change associated with resistance	Target genes	References
Lin 28	Upregulated	*CDKN1A* (p21), *RB1, Let-7*	91
miR-10a	Upregulated	Not stated	92
miR-21	Upregulated	*PTEN, PDCD4*	92,93
miR-22	Upregulated	Not stated	92
miR-29a	Upregulated	*PTEN*	92,94
miR-30c	Downregulated	*TWF1* (Twinfilin 1)	95
miR-31	Downregulated	*PKC epsilon (PRKCE)*	96
miR-34a	Downregulated	*NOTCH1*	97
miR-93	Downregulated	Not stated	92
miR-125b	Upregulated	*E2F3*	92,98
miR-128	Downregulated	*BMI1, ABCC5*	99
miR-137	Downregulated	*YB-1* (P-gp indirectly)	100
miR-181	Upregulated	Not stated	92
miR-181a	Downregulated	*ABCG2* (BCRP)	101
miR-200a and miR-200b	Downregulated	*ZEB1/2*	92,102
miR-200c	Downregulated	*ZEB1, CDH1* (E-cadherin), *PTEN, NTRK2* (TrkB), *BMI1*	62,63,92, 102,103
miR-203	Upregulated	*SOC3*	104
miR-205	Downregulated	Not stated	92
miR-210	Upregulated	Not stated	105
miR-222	Upregulated	*PTEN*	92,94
miR-298	Downregulated	*MDR1* (P-gp)	106
miR-375	Downregulated	*MTDH* (metadherin)	107
miR-487a	Downregulated	*ABCG2* (BCRP)	108
miR-505	Downregulated	*AKT3*	109
miR-633	Upregulated (hypomethylated)	*HSPG2*	110

Notes: A number of miRNAs have shown altered expression levels in drug-resistant forms of breast cancer in both cells and patients. The table contains those reported since 2011 (for prior studies we refer the reader to Kutanzi et al[56]) together with their delineated target genes.

Abbreviations: BCRP, breast cancer resistance protein; miRNA, micro-RNAs; PDCD4, programmed cell death 4; P-gp, P-glycoprotein; PTEN, phosphatase and tensin homolog.

in both trastuzumab-resistant cells and cells resistant to chemotherapeutics is miR-21, which targets *PTEN* and results in sustained PI3K/Akt pathway activity as discussed previously.[44,64] It also downregulates the apoptotic gene programmed cell death 4 (*PDCD4*), allowing cancer cells to evade apoptosis. This protein is also inactivated by phosphorylation by S6K1, a downstream effector of the PI3K/Akt pathway.[65] Another prominent miRNA that appears to be important in drug resistance in both ERα-positive and triple-negative breast cancer is miR-221, which targets the cell-cycle inhibitory protein p27Kip1, among others.[56,66] Thus, it can be seen that miRNAs have important roles in mediating drug resistance in breast cancer. However, the mechanisms leading to miRNA overexpression are not yet fully understood.

Epigenetic regulation

There are three main interlinked mechanisms by which epigenetic modulation leads to transcriptional regulation, chromatin remodeling, modification of nucleosome composition, and modification of epigenetic marks, all of which have been implicated in resistance to breast cancer therapies. ATP-dependent chromatin remodeling allows transcriptional complexes to access the highly coiled genomic DNA to initiate gene transcription. This can be achieved by selected transcription factors, known as pioneer factors. One such family, the Forkheads (Fox), is highly involved in breast cancer.[67] Indeed, FoxA1 controls approximately 50% of ERα target genes,[68] and its expression, along with that of FoxP1, has been correlated to a favorable response to tamoxifen treatment.[69] In contrast, FoxM1 has a role to play in trastuzumab and paclitaxel resistance, as knockdown increases drug sensitivity in multidrug-resistant cell lines.[70] FoxO1 expression is also associated with chemotherapeutic and tamoxifen resistance, as it regulates the transcription of both the *MDR1* (P-gp) and *ABCC2* (MRP2) drug efflux pumps.[71] The nuclear translocation of another FoxO isoform, FoxO3a, is inhibited by phosphorylation by Akt, which acts to drive cell proliferation and tamoxifen resistance, as FoxO3a has cytostatic actions via p27 upregulation and cell-cycle inhibition[72] and by decreasing the expression of ERα-regulated genes.[73] The biology of this complex family of transcription factors is not fully understood, but it has become clear that the balance of expression of the different isoforms is important, and further studies are needed to fully delineate their roles in drug-resistant breast cancer.

Nucleosomes are duplicates of the canonical histones H2A, H2B, H3, and H4 contained in a DNA loop, and modification by substitution with noncanonical histones regulates chromatin compaction and thus the transcription factor access to the genomic DNA.[74] One such noncanonical histone implicated in ERα-positive breast cancer is H2A.Z, which stabilizes the nucleosome at the promoter of ERα-dependent genes. Overexpression of H2A.Z increased cell growth in MCF-7 breast cancer cells in the presence of tamoxifen and the absence of estrogen, suggesting that this histone can drive cell growth in an ERα-independent manner, and high expression levels of H2A.Z in patients have been associated with poor clinical prognosis.[75] Post-translational modification of histone residues, predominantly by methylation and acetylation, also contributes to alterations in gene transcription. Methylation of lysine 27 of histone H3 governs the ligand dependency of ERα-mediated transcription of the *BCL-2* gene, an important driver of the antiapoptotic response of breast cancer cells. Both tamoxifen-resistant and triple-negative breast cancer have been shown to have demethylation of H3K27 and consequently constitutive activation of *BCL-2* and cancer cell survival.[35,76] Changes to histone acetylation also occur in breast cancer, and hyperacetylation of histones H3 and H4 of the HER2 promoter may contribute to trastuzumab resistance by driving increased HER2 expression.[77] Histone hyperacetylation is also involved in the overexpression of *MDR1* (P-gp) in chemotherapeutic-resistant breast cancer, and the levels of acetylation of the lysine 9 residue of histone H3 are elevated by two orders of magnitude in drug-resistant MCF-7 cells.[60]

In addition to histone residues, DNA bases can also be methylated, specifically at CpG sites, which are often found to be associated with gene promoter elements. The formation of methylated CpGs is catalyzed by the DNMT enzymes, and methylation levels are inversely correlated with gene transcription.[78] Both hypermethylation and hypomethylation occur in drug-resistant breast cancer, as seen in Table 2. *PTEN* is frequently hypermethylated in drug-resistant breast cancer, and this silencing is maintained by a positive feedback as reduced PTEN levels lead to increased Akt activity and increased activity of DNMT1, the DNMT that methylates the *PTEN* promoter expression,[79] thus bypassing HER2 or ERα induction of PI3K/Akt pathway activation. Hypermethylation of the *ESR1* (ERα) promoter by DMNT3B contributes to tamoxifen resistance by reducing expression of tamoxifen's target, ERα.[80] In contrast, hypomethylation and increased expression

Table 2 Genes with altered methylation status in drug-resistant breast cancer

Gene	Protein	Hypermethylated/hypomethylated	References
ABCB1 (MDR1) Upstream promoter	P-glycoprotein	Hypomethylated	57,58,111–114
ABCB1 (MDR1) Alternative Downstream promoter	P-glycoprotein	Hypermethylated	111
ACVR1	Activin A receptor	Hypomethylated	115
APC	Adenomatous polyposis coli	Hypomethylated	113
TUBA1A	α-Tubulin	Hypermethylated	115
AVEN	Cell death regulator Aven (PDCD12)	Hypermethylated	115
BAD	BCL2-associated agonist of cell death	Hypermethylated	115
BRCA1	BRAC1	Hypermethylated	113
CDH1	E-cadherin	Hypermethylated	113
CDK10	Cyclin-dependent kinase 10	Hypermethylated	116
CXCR4	Stromal cell-derived factor 1 receptor	Hypomethylated	115
ESR1	ERα	Hypermethylated	113,115
ESR2	ERβ	Hypermethylated	117,118
FTH1	Ferritin heavy chain	Hypomethylated	115
FOXK1	Forkhead box protein k1	Hypomethylated	115
GSTP1	Glutathione S-transferase pi 1	Hypomethylated	57,113
HIC1	Hypermethylated in cancer 1	Hypomethylated	113
IL2	Interleukin 2	Hypermethylated	115
LEP	Leptin	Hypomethylated	115
MGMT	6-O-methylguanine-DNA methyltransferase	Hypomethylated	57,119
NAT1	N-acetyltransferase 1	Hypermethylated	120
CDKN1A	p21	Hypermethylated	121
TP73	p73	Hypermethylated	115
PLAU	Plasminogen activator urokinase	Hypomethylated	57,113
PGR	Progesterone receptor	Hypermethylated	122
TFF1	pS2 (trefoil factor 1)	Hypermethylated	123
ATP2B4	Sarcolemmal calcium pump	Hypermethylated	123
DUSP7	Dual-specific phosphatase 7	Hypermethylated	123
GDF15	Growth differentiation factor 15	Hypermethylated	123
PSAT1	Phosphoserine aminotransferase 1	Hypomethylated	124
PTEN	PTEN	Hypermethylated	78
RAB6C	WTH3	Hypermethylated	113,125,126
RASAL1	Ras protein activator-like 2 (GAP1)	Hypermethylated	113
ARHGEF2	Rho/rac GEF2	Hypermethylated	115
RASSF1	Ras association domain containing protein 1	Hypomethylated	113
RFC1	Replication factor C1	Hypermethylated	127
SDK2	Sidekick 2	Hypomethylated	115
SULF2	Sulfatase 2	Hypermethylated	113
TGM2	Tissue transglutaminase	Hypomethylated	113,115
UTRN	Utrophin	Hypomethylated	115

Note: Hypomethylation results in gene overexpression as DNA methylation reduces gene transcription; consequently, hypermethylation effectively leads to gene silencing.
Abbreviations: ER, estrogen receptor; PTEN, phosphatase and tensin homolog.

of *MDR1* (P-gp) play an important role in resistance to chemotherapeutics.[58] Thus, it is clear that DNA methylation status has important roles in mediating drug resistance in breast cancer and, to this end, Rhee et al[81] have recently published an integrated analysis correlating DNA methylation status with gene expression data for the different subtypes of breast cancer. This study, together with the comprehensive molecular analysis of the subtypes complied by the Cancer Genome Atlas Research Network,[82] will allow other genes with altered methylation states to be identified and their roles in drug resistance explored, especially for drug-resistant HER2-positive breast cancer, as this area has not been explored in this subtype.

Future perspectives for the treatment of drug-resistant breast cancer

The studies discussed here delineating the molecular mechanisms underlying drug resistance in breast cancer, in terms of both single and multidrug resistance, have identified a

number of pathways that offer potential routes to circumvent resistance to the current therapies.

The PI3K/Akt/mTORC1 signaling axis offers targets for therapeutic interventions, and a number of clinical trials are ongoing using PI3K, AKT, mTOR, or dual inhibitors in combination with endocrine or chemotherapies (Table 3). However, caution is required, as the clinical response may depend on the specific aberration and the subtype of breast cancer, as inhibition of Akt may induce apoptosis by release of Bcl-2-associated death promoter (BAD) inhibition, but Akt inhibition can also permit FoxO3a nuclear translocation, potentially leading to the transcription of ERα-dependent genes encouraging cell proliferation. Also, upregulation of growth factor receptors (eg, FGFRs and IGF-1R) may favor activation of other signaling cascades, such as the MAPK pathways, which could be exacerbated by inhibition of

Table 3 Inhibitors of the PI3K/Akt pathway currently undergoing clinical trials

Drug	Target	Breast cancer selection criteria	Combination therapies	Phase	Trial identifiers
AZD5363	Akt	ER+	Paclitaxel/none	I	NCT01625286, NCT01226316
GSK2110183	Akt	Drug resistant	None	I	NCT01476137
GSK2141795	Akt	Not stated	None	I	NCT00920257
MK2206	Akt	ER+	Lapatnib ditosylate/paclitaxel/ anastrozole/letrozole/exemestane/ fulvestrant/none	II	NCT01245205, NCT01277757, NCT01776008, NCT01344031
		HER2+	Lapatnib ditosylate/trastuzumab	I	NCT01705340, NCT01281163
		Not stated	Paclitaxel	Ib	NCT01263145
Triciribine Phosphate Monohydrate	Akt	Not stated	Paclitaxel/doxorubicin/ cyclophosphamide	I/II	NCT01697293
BAY 80-6946	PI3K	Not stated	Paclitaxel	I	NCT01411410
BKM120	PI3K	ER+	Fulvestrant/letrozole	I, III	NCT01339442, NCT01248494, NCT01633060, NCT01610284
		HER2	Lapatnib/trastuzumab/capecitabine	I/II	NCT01589861, NCT01132664
		Trastuzumab-resistant HER2+	Trastuzumab + paclitaxel	I, II	NCT01285466, NCT01816594
		HER2–	Paclitaxel	II	NCT01572727
		Triple-negative	Postchemotherapeutics	I, II	NCT01629615
BYL719	PI3K	ER+/HER2–	Letrozole/fulvestrant	I	NCT01791478, NCT01219699
GDC-0941	PI3K	Not stated	Trastuzumab, paclitaxel, bevacizumab	I	NCT00960960
		ER+	Fulvestrant	II	NCT01437566
XL147	PI3K	ER+ HER2+	Letrozole	I	NCT01082068
CC-223	mTOR	ER+	Unresponsive tumors	I/II	NCT01177397
Everolimus	mTOR	ER+/HER2+/–	Endocrine therapies (tamoxifen)/ bevacizumab, lapatnib	II, III	NCT01298713, NCT01805271
		ER+, AI–resistant	Fulvestrant/ chemotherapeutics/exemestane	II, III	NCT01797120, NCT01088893, NCT00863655 and NCT01626222
		HER2+	Paclitaxel, trastuzumab	III	NCT00876395
		HER2–	Vinorebine	II	NCT01520103
		Triple-negative	Gemcitabine, cisplatin	I	NCT01939418
Rapamycin	mTOR	HER2+	Trastuzumab	II	NCT00411788
Temsirolimus	mTOR	ER+	Letrozole	II	NCT00062751
		HER2+/triple negative	Neratinib	I/II	NCT01111825
Ridaforolimus	mTOR	ER+	Dalotuzumab, exemestane	II	NCT01234857
BEZ235	PI3K/mTOR dual inhibitor	ER	Letrozole/everolimus	I	NCT01248494, NCT01482156
		HER2+	Paclitaxel, trastuzumab	I	NCT01285466
		HER2–	Paclitaxel	I/II	NCT01495247
GDC-0980	PI3K/mTOR dual inhibitor	ER+	Fulvestrant	II	NCT01437566
XL765	PI3K/mTOR dual inhibitor	ER+ HER2–	Letrozole	II	NCT01082068

Notes: The alterations to the PI3K/Akt pathway are common factors in the different subtypes of breast cancer and also have important roles in mediating drug resistance (see text for details). Subsequently, this pathway is a promising therapeutic target to overcome drug resistance with a variety of compounds in clinical trials. Data obtained from the ClinalTrials.gov database.

Abbreviations: AI, aromatase inhibitor; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; mTOR, mammalian target of rapamycin complex 1.

PI3K/Akt/mTORC1. In addition, the effects of this pathway on transcription via epigenetic changes need to be considered to prevent the selection of tumor subpopulations that are resistant to therapy, especially the FoxO family, as targeting these transcription factors directly is not a viable option currently, due to their complexity. Thus, the combination of therapies needs to be carefully considered and appropriate for the cancer subtype.

Overcoming drug resistance that results from P-gp overexpression may require a different approach to that of the PI3K/Akt pathway, as several P-gp inhibitors have been trialed without satisfactory clinical outcomes.[83] To this end, a number of compounds that are microtubule stabilizers, like the taxanes, but are not P-gp substrates have been trialed with promising clinical results (see Nobili et al[83] for details). Other drugs that actually exploit P-gp overexpression are being considered, notably NSC73306, the cytotoxic capacity of which correlates with P-gp expression levels. The more P-gp expressed, the more toxic NSC73306 is. NSC73306 does not appear to be a P-gp substrate. Understanding the way this drug utilizes P-gp overexpression may help identify mechanisms to circumvent multidrug resistance involving P-gp.

As yet, no therapeutics targeting specific miRNAs have made it into the clinic. However, one antagonist to miR-122 is undergoing Phase II clinical trials for use in hepatitis C,[84] suggesting that miRNAs could be valid therapeutic targets in breast cancer. To this end, therapeutics targeting miR-21 and miR-221, which are implicated in drug resistance in breast cancer, are being commercially developed for use in hepatocellular carcinoma and other cancers,[85,86] and so it may not be too long before clinical trials of miRNA inhibitors for the treatment of multidrug-resistant breast cancer become a reality.

Reversing the deleterious alterations to epigenetic regulation that are associated with drug resistance in breast cancer is a challenging proposition. However, histone deacetylation inhibitors are showing promise in all subtypes of breast cancer,[87–89] but caution is required, as these approaches may alter the expression of ERα, HER2, and MDR1, as discussed previously, which could actually enhance drug resistance. It will be interesting to see how the large volume of preclinical data available on this topic translates to clinical outcomes, as trials of a number of different histone deacetylation inhibitors are ongoing.[87]

Other approaches to circumvent multidrug resistance in breast cancer, especially the triple-negative subtype, are being pursued and include inhibitors of angiogenesis, epidermal growth factor receptor, poly(adenosine diphosphate-ribose) polymerase, and FGFRs, which are outside the scope of this review (we refer the reader to Bayraktar and Glück[90]).

Conclusion

Multidrug-resistant breast cancer is a complex clinical condition arising from a diverse range of molecular perturbations, yet several common mechanisms have been identified. The PI3K/Akt pathway discussed here is just one. By identifying these common mechanisms and developing therapeutics targeting them, an armory for overcoming drug resistance in different clinical situations can be created. It will be interesting to see how the PI3K/Akt/mTOR inhibitors perform in the ongoing clinical trials to ascertain whether such global approaches are useful for circumventing resistance to frontline therapies. Further studies are required before therapeutics targeting other common mechanisms highlighted here, miRNA upregulation and epigenetic alteration, may be targeted, as these are not yet as well understood.

Disclosure

The authors report no conflicts of interest in this work.

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