Chapter 3
Mechanisms of Action and Resistance of Trastuzumab in Breast Cancer

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Abstract Breast cancer affects approximately 1 in 8 women. It is estimated that over 252,710 women in the United States will be diagnosed with breast cancer in 2017. Breast cancer-related deaths have declined over the last two decades as a result of early detection and improved treatment, particularly targeted therapies, such as trastuzumab that targets human epidermal growth factor receptor 2 (HER2), which is frequently overexpressed in breast cancer. However, resistance to trastuzumab, either *de novo* or acquired resistance, presents a major clinical challenge. Here, we summarize the mechanisms of action and resistance of trastuzumab in breast cancer and discuss potential strategies to overcome resistance.

Keywords Receptor tyrosine kinase • Drug resistance • Therapeutic antibodies • Small tyrosine kinase inhibitors • Breast cancer

Abbreviations

ADAM10	A disintegrin and metalloproteinase domain-containing protein 10
ADC	Antibody-drug conjugate
ADCC	Antibody-mediated cellular toxicity
AMPK	Adenosine monophosphate (AMP)-activated protein kinase
CTF	Carboxy terminal fragment
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
ECD	Extracellular domain

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EGFR	Epidermal growth factor receptor
EphA2	Ephrin receptor A2
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
IGF-1	Insulin growth factor-1
IGF-1R	Insulin-like growth factor receptor 1
IGF-2	Insulin growth factor-2
IHC	Immunohistochemistry
JAK	Janus-activated kinase
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute 2
MUC4	Glycoprotein mucin-4
NK	Natural killer
NSCLC	Non-small cell lung cancer
PD-1	Programmed death-1
PD-L1	Programmed death ligand-1
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidyl-inositol-3,4,5-trisphosphate
PLCγ	Phospholipase Cγ
PTEN	Phosphatase and tensin homologue
STAT	Signal transducer and activator of transcription
T-DM1	Ado-trastuzumab emtansine
TKI	Tyrosine kinase inhibitor
TNFα	Tumor necrosis factor α

3.1 Introduction

The epidermal growth factor receptor (EGFR) family consists of four members, ERBB1 (EGFR/HER1), ERBB2 (HER2/neu), ERBB3 (HER3), and ERBB4 (HER4), which are cytoplasmic membrane-anchored receptor tyrosine kinases that regulate important biological processes, such as cell growth, differentiation, metabolism, and survival through activation of downstream signaling pathways [1–6]. All members of the EGFR family share sequence and structural similarities and contain an extracellular ligand-binding ectodomain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain [1, 7]. Following ligand binding, members of the ERBB family interact to form various combinations of homo- or heterodimers, which then induce autophosphorylation of the tyrosine residues within the kinase domain [1]. Recruitment of adaptor proteins at the phosphotyrosine residues subsequently initiates the downstream signaling cascades, such as phosphatidylinositol 3-kinase (PI3K), Ras, phospholipase Cγ (PLCγ), Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) [3, 8].

In contrast to EGFR, ERBB3, and ERBB4, which bind extracellular ligands to trigger downstream signaling, HER2 does not bind to any ligands directly. Rather, HER2 mediates downstream signaling in concert with a ligand-activated coreceptor, e.g., EGFR, ERBB3, or ERBB4 [1, 2, 9]. HER2 can also form homodimers and activate signaling cascades especially at higher concentrations as observed in cancers [2, 9]. The HER2/ERBB3 heterodimer is the most potent activator of two key pathways regulating cell survival and growth, the mitogen-activated protein kinase (MAPK) and the PI3K/Akt signaling cascades, and ERBB3 plays an essential role in HER2-mediated oncogenic signaling [10–12]. Activation of HER2 also decreases the protein levels of cell cycle negative regulator $p27^{Kip1}$ by promoting its mislocation through Jun activation domain-binding protein 1-mediated export into the cytoplasm, and subsequently its degradation via the ubiquitin-proteasome pathway [13].

HER2 is normally expressed at low levels on the cell surface, but in breast cancer, the number of HER2 receptors on the surface of each cell can reach up to 100 times more than a normal cell, which leads to aberrant activation of its downstream signaling cascades and uncontrollable cell growth [14, 15]. HER2 amplification/overexpression is observed in approximately 20% of breast cancer and is associated with poor clinical outcome and disease progression [16–18], and HER2 has proved to be one of the most successful targets in breast cancer. In this chapter, we briefly summarize the mechanisms of action and resistance of trastuzumab (Herceptin®; Genentech) as well as treatment strategies to overcome resistance in breast cancer.

3.2 Trastuzumab

3.2.1 Proposed Mechanism of Action

Several HER2-specific mAbs were developed and demonstrated to effectively inhibit tumor growth of HER2-overexpressing cell lines [19]. Among them, the murine mAb 4D5, which was later humanized to become trastuzumab, selectively targets the extracellular domain IV of HER2 with high affinity and prevents ligand-induced dimerization and subsequent activation of downstream pathways [20]. Following the clinical studies which demonstrated that the addition of trastuzumab to chemotherapy, compared with chemotherapy alone, increased the response rates, time to progression, and survival in patients with HER2-positive ($HER2^+$) metastatic breast cancer, trastuzumab received approval by the FDA in 1998 for the treatment of metastatic breast cancer with HER2 overexpression [21, 22].

Trastuzumab exerts its mechanism of action through several different approaches. First, it disrupts signal transduction pathways, most notably, MAPK and PI3K/Akt signaling, leading to apoptosis and arrest of proliferation [1, 23–25]. Trastuzumab produces cytostatic effects associated with downregulation of

AKT activity and results in increased G1 growth arrest via enhanced stability of the cell cycle inhibitor $p27^{Kip1}$ [23, 26, 27]. In addition, trastuzumab can block PI3K signaling by reducing tyrosine phosphorylation of the tumor suppressor phosphatase and tensin homologue (PTEN) and increasing its phosphatase activity and membrane localization [28]. Second, trastuzumab can block proteolytic cleavage of HER2 by the metalloprotease ADAM10 [29], which liberates its extracellular domain (ECD) and produces a truncated, membrane-bound, and kinase active carboxy terminal fragment (CTF), p95HER2 [30]. Interestingly, HER2 ECD can be detected in the serum of breast cancer patients, and results from clinical studies indicated that a decline in serum HER2 ECD following trastuzumab treatment could predict clinical benefit [31]. Third, trastuzumab exerts an antitumor effect through activation of the antibody-mediated cellular toxicity (ADCC) [32, 33]. Studies have demonstrated in cell lines and xenografts that this immunological effect of trastuzumab is mainly attributed to the binding of the Fc (fragment, crystallizable) region of the antibody to Fc gamma receptor present on natural killer cells [32, 34]. Immunohistochemistry (IHC) analysis of breast tissue samples from patients with HER2-overexpressing advanced breast cancer during a neoadjuvant treatment of trastuzumab and docetaxel in a clinical trial further validated the immune cell-modulated activity of trastuzumab via an increased number of natural killer (NK) cells and cytotoxic proteins, e.g., granzyme B, in tumor infiltrates after trastuzumab treatment [33].

3.2.2 Mechanism of Resistance

Although trastuzumab in combination with chemotherapy has significantly improved the outcome of breast cancer patients, *de novo* and acquired resistance to trastuzumab pose a major challenge in the clinic [35, 36]. A large proportion of patients with $HER2^+$ breast cancer do not respond to initial trastuzumab treatment (*de novo* resistance) and those who initially responded eventually experience disease progression (acquired resistance) [37–39]. The mechanisms of trastuzumab have been extensively studied and may involve the following: (1) upregulation of downstream signaling due to genetic alterations; (2) hindrance of trastuzumab binding to HER2; and (3) overexpression of ERBB receptors or other tyrosine kinase receptors. Each will be briefly described below.

3.2.3 Upregulation of Downstream Signaling

The constitutive activation of the downstream PI3K/Akt pathway due to mutations in the gene encoding PI3K and/or inactivation or loss of PTEN have been shown to contribute to trastuzumab resistance [28, 40]. PI3K catalyzes the lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to the phosphatidyl-inositol-3,4,

5-trisphosphate (PIP_3), which binds to the pleckstrin homology domain of the serine/threonine protein kinase Akt, resulting in the translocation of Akt to the membrane and its subsequent activation to promote cell survival and inhibition of apoptosis [41]. Activating mutations in the *PIK3CA* gene encoding the catalytic subunit (p110) of PI3K have been reported to induce constitutive activation of the PI3K/Akt pathway. The frequency of *PIK3CA* activating mutations in HER2+ breast cancers has been reported to be 23–33% [42]. PTEN antagonizes PI3K by dephosphorylating PIP_3 and negatively regulates AKT activities [43–46]. Hence, as PTEN normally blocks PI3K activation, the loss of *PTEN* results in constitutive activation of PI3K/Akt signaling and subsequently bypassing trastuzumab-mediated growth arrest [46, 47]. Breast cancer patients with *PTEN* deficiency demonstrated poorer response to trastuzumab compared with those with normal *PTEN* [28]. Zhang et al. reported that cytoplasmic tyrosine kinase SRC functions as a common mediator of multiple trastuzumab resistance pathways and is regulated via dephosphorylation by PTEN [48]. The increased activation of SRC was observed in both *de novo* and acquired trastuzumab resistant cells and correlated with trastuzumab resistance in patients [48]. A follow-up clinical trial indicated that patients with HER2-overexpressing metastatic breast cancer with *PTEN* loss and progressed on trastuzumab-based therapy had decreased overall survival compared with those with normal *PTEN* [49]. Moreover, studies reported that the combination of trastuzumab with everolimus, an inhibitor against AKT downstream molecule mTOR, provided an objective response rate of 15% and clinical benefit rate of 34% [49]. These findings further validated the role of *PTEN* deficiency in trastuzumab resistance. In addition, preclinical studies demonstrated that the combination of trastuzumab and the PIK3 inhibitor, GDC-0941, is highly effective against trastuzumab-resistant cells and tumors and can also overcome trastuzumab resistance caused by *PTEN* loss [24]. The PI3K inhibitors that are currently under clinical investigation for solid tumors harboring *PIK3CA* or *PTEN* mutations include buparlisib (BKM120), taselisib (GDC-0032), and GSK2636771 [50].

Akt has also been demonstrated to phosphorylate the tumor suppressor SIRT6 at Ser338, resulting in MDM2-mediated ubiquitination and subsequent degradation of SIRT6 [51]. The authors further reported a positive correlation between SIRT6 abundance and survival of breast cancer patients. Their findings suggested that stabilization of SIRT6 by preventing its degradation may be a potential therapeutic strategy to overcome trastuzumab resistance.

3.2.4 Epitope Masking

As indicated above, the ectodomain shedding of HER2 produces a truncated and constitutively active membrane-bound p95HER2 CTF of 95- to 100-kDa. In addition to the ectodomain shedding, the alternative translation initiation of *HER2* mRNA can give rise to two other p95HER2 fragments, a membrane-bound 611-CTF (100–115 kDa), which forms constitutively active homodimers, and a soluble

678-CTF (90–95 kDa), which is kinase inactive [52]. Both p95HER2 95–100 kDa and 110–115 kDa fragments lack the epitope for recognition by trastuzumab [53], and circulating HER2 ECD can compete with the full-length membrane-bound HER2 for binding to trastuzumab [54]. Pederson and coworkers found that the 611-CTF regulated genes linked to metastasis, and 611-CTF transgenic mice developed more aggressive and invasive mammary tumors compared with mice with full-length HER2 [52]. Up to 30% of HER2+ breast cancers express p95HER2 and are associated with metastasis and shorter disease-free survival [55, 56]. Retrospective studies indicated that the presence of p95HER2 fragments in tumors is associated with trastuzumab resistance [57, 58]. Interestingly, p95HER2 was shown to preferentially heterodimerize with HER3 to trigger pro-survival signaling [59]. Parra-Palau and coworkers reported that chemotherapy sensitizes p95HER2 (611CTF)-expressing patient derived xenograft from HER2+ breast cancers to trastuzumab [60].

Another mechanism contributing to trastuzumab resistance is the binding of cell surface glycoprotein mucin-4 (MUC4) to the extracellular domain of HER2, which can mask the trastuzumab-binding site on HER2 (epitope masking). Nagy et al. reported that MUC4 expression was correlated negatively with decreased trastuzumab binding, and that knocking down *MUC4* reversed trastuzumab resistance in a *de novo* trastuzumab-resistant JIMT-1 breast cancer cell line [61]. Hyperactivation of the signal transducer and activator of transcription-3 (STAT3) via a positive feedback loop was shown to upregulate MUC4 expression [62]. More recently, Mercogliano et al. reported that TNFα induces elevated expression levels of MUC4 and contributes to trastuzumab resistance in HER2+ breast cancer. The authors further identified MUC4 expression as an independent predictor of poor disease-free survival in HER2+ breast cancer patients and suggested the combination of TNFα-blocking antibodies as a therapeutic option to overcome trastuzumab resistance [63].

3.2.5 Expression of Other Receptor Tyrosine Kinases

HER2 can form heterodimers with other receptor tyrosine kinases to activate downstream signaling cascades to compensate for the inhibition of HER2 signaling by trastuzumab [10, 11, 64–67]. Ritter et al. demonstrated that trastuzumab-resistant cells exhibited higher levels of EGFR phosphorylation and EGFR/HER2 heterodimers, and the addition of EGFR TKIs, erlotinib, gefitinib, or lapatinib (a dual EGFR/HER2 inhibitor), induced apoptosis in those resistant cells [64]. The HER2-HER3 heterodimer potently activates the PI3K/Akt and MAPK pathways, and trastuzumab is unable to block the ligand-induced HER2/HER3 heterodimer [68]. The HER-2 targeting monoclonal antibody, pertuzumab (see later for more details), was developed to prevent HER2 dimerization with EGFR and HER3 [69].

The receptor tyrosine kinase Eph receptor A2 (EphA2) is overexpressed in many cancer cell lines and human tumor tissue specimens and can form a complex with

HER2 and activate signaling promoting cell proliferation and motility [70–72]. Eliminating EphA2 expression in ERBB2-driven murine mammary tumor models impaired tumor initiation and metastatic progression [72]. In addition, Zhuang and colleagues found that high levels of EphA2 expression in HER2+ breast cancer patients predict poor prognosis and identified a mechanism by which EphA2 contributes to trastuzumab via EphA2-mediated amplification of the PI3K/Akt and MAPK cascades [65]. Their findings suggested targeting EphA2 as a therapeutic strategy to overcome trastuzumab resistance. Amato et al. demonstrated that the EphA2 kinase inhibitor, ALW-II-41-27, inhibited cell viability of non-small cell lung cancer (NSCLC) cells *in vitro* and induced tumor regression in a NSCLC xenograft tumor model [73]. Targeting EphA2 was shown to overcome primary and acquired resistance to anti-EGFR therapy, cetuximab, in metastatic colorectal cancer [74]. Whether the addition of ALW-II-41-27 could overcome trastuzumab resistance in breast cancer remains unclear.

The overexpression of the insulin-like growth factor receptor 1 (IGF-1R) and its ligands, insulin growth factor-1 (IGF-1) and IGF-2, is often observed in breast tumors [75]. Activation of IGF-1R following ligand binding triggers cell survival signals, and overexpression of IGF-1R has been shown to confer resistance to trastuzumab via hyperactivation of SRC [48, 76]. Specifically, ectopic expression of IGF-1R in trastuzumab-sensitive breast cancer cells in the presence of IGF-1 ligand rendered trastuzumab ineffective in reducing cell proliferation, and the addition of IGF-binding protein-3, which suppresses IGF-1R signaling, reversed resistance [76]. The inhibition of SRC renders trastuzumab-resistant IGF-1R breast cancer cells sensitive to trastuzumab [48]. IGF-1R is also reported to form a heterodimeric complex with HER2 and HER3 in breast cancer cells resistant to trastuzumab through enhanced PI3K/Akt signaling and SRC activation [77]. Liu et al. reported that metformin, a type 2 diabetes drug with antitumor effects, reduces HER2 and IGF-1R interactions in trastuzumab-resistant breast cancer cells [78]. Metformin has been shown to activate the adenosine monophosphate (AMP)-activated protein kinase AMPK, which plays a critical role as a regulator of cellular energy homeostasis [79]. Interestingly, metformin inhibits the insulin/IGF signaling by decreasing insulin metabolism in the liver or by reducing IGFR expression. Whether AMPK regulates HER/IGF-1R interaction remains unclear.

The hepatocyte growth factor (HGF) receptor (also known as c-Met), which regulates important biological processes, including morphogenesis, cell proliferation, survival, differentiation, and anti-apoptosis, is also implicated in the progression and metastasis of many human cancers [80]. Overexpression of c-Met is observed in 20–30% of breast cancers and it has been reported to be an independent prognostic of poor prognosis for breast cancer patients [81–83]. Shattuck et al. reported the co-expression of c-Met and HER2 in HER2-overexpressing breast cancer cells and HER+ breast cancer tumor tissues [67]. Moreover, the inhibition of c-Met sensitized HER2-overexpressing breast cancer cells to trastuzumab, suggesting that c-Met contributes to trastuzumab resistance [67]. High risk of trastuzumab treatment failure in breast cancer patients has been reported to associate with high *MET* and *HGF* gene copy numbers [84].

3.3 Treatment Strategies to Overcome Resistance

Below we describe some strategies to overcome trastuzumab resistance.

3.3.1 Pertuzumab

The humanized monoclonal antibody pertuzumab (Perjeta®; Genentech) binds to domain II (trastuzumab binds to domain IV) of HER2 and blocks ligand-dependent HER2 heterodimerization with EGFR, HER3, or HER4, but most potently targets the heregulin-mediated HER2/HER3 signaling heterodimer [69, 85]. Inhibiting the formation of the HER2/HER3 heterodimer prevents the activation of downstream signaling, e.g., PI3K and MAPK, that regulate cell survival and growth [1, 11]. Similar to trastuzumab, pertuzumab also triggers ADCC [86]. Clinical studies of patients with advanced HER2+ breast cancer after trastuzumab treatment demonstrated that pertuzumab in combination with trastuzumab was more efficacious than pertuzumab alone [87]. In 2012, the FDA approved pertuzumab in combination with trastuzumab and docetaxel for the treatment of HER2+ metastatic breast cancer patients who have not received prior anti-HER2 therapy or chemotherapy for metastatic disease based on a phase III multicenter randomized clinical trial [88]. More recently, follow-up data extended the results of previous analyses demonstrating the efficacy of the pertuzumab plus trastuzumab and docetaxel combination [89]). Pertuzumab was later approved in 2013 for use in combination with trastuzumab and docetaxel as neoadjuvant treatment of patients with HER2+, locally advanced, inflammatory, or early stage breast cancer [90]. Interestingly, tumor gene expression analyses indicated high expression of the programmed death ligand 1 (PD-L1), an immune checkpoint protein that facilitates cancer immunesurveillance escape, is associated with resistance after neoadjuvant treatment with regimens containing HER2-targeted treatments [91]. A phase I trial is currently underway to evaluate the safety and pharmacokinetics of the PD-L1 monoclonal antibody, atezolizumab, in combination with trastuzumab and pertuzumab in HER2+ breast cancer (NCT02605915).

3.3.2 Lapatinib

Lapatinib (Tykerb®; Novartis) is a reversible ATP-competitive small molecule tyrosine kinase inhibitor (TKI) which binds to the intracellular ATP binding domain of EGFR and HER2 and inhibits the activation of downstream signaling [92–94]. The combination of lapatinib and capecitabine in a randomized phase III trial was found to be superior than capecitabine alone in patients with metastatic breast cancer who progressed after treatment with regimens that included an anthracycline, a taxane,

and trastuzumab [95, 96]. On the basis of the phase III data, the FDA approved lapatinib in combination with capecitabine for the treatment of patients with advanced or metastatic HER2+ breast cancer and who have received prior therapy including an anthracycline, a taxane, and trastuzumab. Synergistic growth inhibition was observed in trastuzumab-treated HER2+ breast cancer cell lines for the lapatinib and trastuzumab combination [93]. Lapatinib combined with trastuzumab was later evaluated in a phase III clinical trial with results showing a significant overall survival advantage of the combination compared with lapatinib alone in HER2+ metastaic breast cancer patients whose disease progressed during trastuzumab treatment [97]. These findings further supported the dual blockage of HER2 as an approach to overcome resistance.

3.3.3 Neratinib

Neratinib (Puma Biotechnology) is an irreversible ATP-competitive small molecule TKI that blocks the intracellular ATP-binding site of EGFR, HER2, and HER4 [92–94]. The results from preclinical studies demonstrated that neratinib inhibits proliferation of HER2-overexpressing human breast cancer cell lines *in vitro* as well as an EGFR-overexpressing epidermal carcinoma cell line [92]. Phase II studies showed that neratinib was well tolerated among advanced HER2+ breast cancer patients with or without prior treatment with trastuzumab [98]. In a phase III (ExteNET) study, neratinib treatment significantly improved the 2-year disease-free survival of HER2-positive breast cancer patients after chemotherapy and trastuzumab-based adjuvant therapy [99]. Currently, neratinib is being evaluated in a number of clinical trials as a neoadjuvant therapy for patients with HER2+ breast cancer and as a treatment for patients with metastatic HER2+ breast cancer (clinicaltrials.gov). A new drug application for neratinib for extended adjuvant treatment of HER2+ early stage breast cancer has been accepted by the FDA and awaiting approval.

3.3.4 Ado-Trastuzumab Emtansine (T-DM1)

T-DM1 is an antibody-drug conjugate (ADC) containing trastuzumab covalently linked to the cytotoxic microtubule inhibitor, emtansine (DM1), via a thioester linker MCC (4-[N-maleimidomethyl] cyclohexane-1-carboxylate). T-DM1 contains about 3.5 molecules of DM1 per antibody and is internalized following binding of trastuzumab to HER2 on the cell surface [100, 101]. After binding of T-DM1 to HER2 on the cell surface, the HER2-T-DM1 complex is internalized via receptor-mediated endocytosis followed by lysosomal degradation, resulting in the release of intracellular DM-1-containing catabolites that bind to and inhibit microtubule polymerization, and subsequently induce cell cycle arrest and cell death [101]. In retaining the activity of trastuzumab, T-DM1 also disrupts the PI3K/Akt signaling

cascade, inhibits the HER2 ectodomain shedding, and induces ADCC [100, 101]. Preclinical studies of T-DM1 indicated greater activity compared with trastuzumab with retained selectivity toward HER2 [100]. Favorable results from clinical studies led to the approval of T-DM1 in second-line therapy by the FDA in 2013 for patients whose advanced HER2+ breast cancer progressed after trastuzumab treatment [102–104]. A phase III study (MARIANNE) evaluating T-DM1 for first-line treatment of HER2-positive, advanced breast cancer indicated that T-DM1 and T-DM1 plus pertuzumab did not achieve superiority compared with trastuzumab plus a taxane [105]. The acquired resistance to T-DM1 has been reported, and factors contributing to T-DM1 resistance include poor internalization and defective intracellular trafficking of the T-DM1-HER2 complexes, inefficient lysosomal degradation of T-DM1, expression of drug efflux proteins, and altered tubulins in addition to those mechanisms known to induce trastuzumab resistance [106]. To circumvent resistance, clinical studies to evaluate T-DM1 in combination with other targeted therapies, for example, immunotherapy (pembrolizumab or atezolizumab), HER/HER3 antibody (pertuzumab), TKIs (lapatinib or neratinib), and cyclin D kinase 4/6 inhibitor (palbociclib or ribociclib) as well as in triple combination (chemotherapy and TKI), for metastatic breast cancer are currently underway. Studies on T-DM1 combined with PI3K inhibitors (taselisib) are also ongoing (clinicaltrials.gov).

3.4 Conclusion

Trastuzumab has demonstrated remarkable clinical success and increased patient outcome. However, acquired and *de novo* resistance via multiple mechanisms remain a clinical challenge. Furthering our understanding of the resistance mechanisms has led to the development of therapeutic strategies to overcome this resistance and improve patient outcome. *HER2* somatic mutations have been reported in breast cancer, but these mutations occur almost always in the absence of *HER2* gene amplification [107, 108]. HER2 mutations were functionally characterized in breast cancer without *HER2* amplification [109]. While many of the identified mutations were found to be sensitive to HER2-targeted therapies in cell lines, those harboring the L755_T759 deletion mutation were resistant to lapatinib [109]. How this mutation affects patients treated with the combination therapy containing lapatinib should be further evaluated. Antibodies against immune checkpoints, e.g., PD-L1, PD-1, and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), to unleash T cell-mediated anti-tumor activity have demonstrated success as a cancer treatment in recent years [110]. Preclinical studies indicated that PD-1 antibodies significantly improved the therapeutic activity of trastuzumab [111]. A phase Ib/II clinical trial is currently underway to evaluate the efficacy of PD-1 antibody (MK-3475) and trastuzumab in patients with trastuzumab-resistant, HER2+ metastatic breast cancers (NCT02129556). As more combination therapies are being evaluated, optimizing patient selection and predictive biomarkers are required to maximize clinical efficacy.

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