Molecular predictors of response to trastuzumab and lapatinib in breast cancer

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Abstract | Trastuzumab is a monoclonal antibody directed against the human EGFR2 (HER2) protein that has been shown to improve survival in patients with HER2-positive breast cancer. Lapatinib is an oral small-molecule tyrosine kinase inhibitor directed against EGFR and HER2. Lapatinib therapy was shown to prolong the time to progression and increase the rate of response to capecitabine in patients who had received anthracycline-based and taxane-based chemotherapy, and whose tumors had progressed on trastuzumab. HER2 status, either gene copy number or the protein expression level, is the best predictive marker available for assessing response to trastuzumab and lapatinib. Whether the power of this predictive marker is the same in advanced and early-stage cancers is unknown. There is great interest in developing diagnostic tests that predict which patients are more likely to benefit from specific HER2-directed therapies. Novel therapeutics that will overcome resistance to trastuzumab and lapatinib are under intense clinical development. In the future, it will be important to characterize mechanisms of resistance in metastatic tumors to determine which novel targeted therapy will be most appropriate for individual patients.

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Introduction

The human EGFR2 (HER2; also known as HER2/neu and ErbB2) is a tyrosine kinase receptor that shares a high degree of homology with the EGFR.[1,2] The *HER2* gene is amplified in human breast cancer,[1] and its overexpression by transfection methods results in transformation of normal human fibroblasts.[2] *HER2* transgenic mice develop breast cancer at an average age of 28 weeks.[3] *HER2*-amplified breast cancer cells exhibit higher rates of DNA synthesis, proliferation, invasion and metastatic potential than breast cancer cells that have two copies of the *HER2* gene. Drebin and collaborators developed monoclonal antibodies against the murine Neu protein and showed that antibody treatment was able to inhibit cancer cell proliferation *in vitro*.[4] In the 1980s, investigators at Genentech developed monoclonal antibodies directed against the human p185HER2.[5–7] One of these antibodies, known as muMAb 4D5, was chosen for further development because of its potent antiproliferative effects against breast cancer cells that overexpressed HER2. In 1987, Slamon and colleagues reported a significant inverse correlation between *HER2* amplification and/or protein overexpression and disease-free survival in patients with early-stage breast cancer.[8] Patients whose tumors were 'HER2 positive' had a lower disease-free survival rate than those whose tumor expressed normal levels of HER2. This observation accelerated the development of HER2 monoclonal antibody therapy as a potential treatment for patients with HER2-overexpressing breast cancer.

Development of anti-HER2 antibodies

One of the most important breakthroughs in the development of successful antibody therapy for cancer was the ability to 'humanize' murine antibodies through recombinant DNA technology. Trastuzumab (Herceptin; Genentech, South San Francisco, CA) was engineered by inserting the complementarity determining regions of muMAb 4D5 antibody into the framework of a consensus human IgG1. Trastuzumab is comparable to muMAb 4D5 in blocking breast tumor cell proliferation; unlike muMAb 4D5, however, it induces antibody-dependent cell-mediated cytotoxicity against tumor cell lines in the presence of human peripheral blood mononuclear cells.[9] Moreover, the humanized form of the muMAb 4D5 antibody prevents or reduces generation of an immune response directed against the antibody itself.[10] The proposed mechanisms of action of trastuzumab are shown in Table 1.

Phase I and II trials of trastuzumab monoclonal antibody therapy showed that this drug was safe in patients with metastatic breast cancer whose tumors overexpressed HER2.[11] Phase II clinical trials showed objective responses in 11–24% of patients with HER2-overexpressing tumors who had disease progression after treatment with conventional endocrine therapy and chemotherapy given either in the adjuvant setting or for metastatic disease.[10,12] A series of phase II clinical

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trials showed that the combination of trastuzumab with chemotherapy was synergistic,[13–15] as had been shown previously in preclinical models.[16] A randomized phase III trial showed that a combination of trastuzumab with chemotherapy (either doxorubicin and cyclophosphamide or paclitaxel) increased time to progression, response rate and overall survival compared with chemotherapy alone in patients with HER2-positive metastatic breast cancer.[17] Compared with chemotherapy alone, the addition of trastuzumab to chemotherapy increased the response rate from 32% to 50% ($P<0.001$); the median time to progression from 4.6 months to 7.4 months ($P<0.001$); and the median survival from 20.3 months to 25.1 months ($P=0.046$). As a result of these data, trastuzumab was approved by the US FDA in 1998. The addition of trastuzumab to adjuvant chemotherapy, either concurrently or after completion of chemotherapy was later shown to decrease the risk of recurrence by 45–50% in patients with HER2-positive early-stage breast cancer.[18–20] Trastuzumab was subsequently approved in many countries for use in the adjuvant setting.

Lapatinib is a small-molecule inhibitor of the EGFR and HER2 receptor tyrosine kinases.[21] Fabian and colleagues evaluated the binding affinity of 20 kinase inhibitors for 100 different kinases. In this study, lapatinib was of the most specific inhibitor because it bound HER2 and EGFR kinases almost exclusively. By contrast, other inhibitors were able to bind to more kinases than previously expected (in some cases more than 10 kinases).[22] A phase II trial showed that lapatinib was effective in approximately 20% of patients with HER2-positive metastatic breast cancer who had not received first-line chemotherapy.[23] A randomized phase III clinical trial showed that a combination of lapatinib and capecitabine increased time to progression and response rates compared with capecitabine alone in patients with HER2-positive metastatic breast cancers who had been treated with an anthracycline and a taxane and had disease progression while on trastuzumab.[24] On the basis of these results, lapatinib in combination with capecitabine was approved for the treatment of patients with HER2-positive metastatic breast cancer who had previously received anthracycline-based, taxane-based, and had disease progression on trastuzumab-based therapy.

Trastuzumab and lapatinib represent advances in the treatment of HER2-positive breast cancer.[25] When using response criteria in solid tumors (RECIST),[26] the majority of HER2-positive cancers do not respond to these drugs when they are given as single agents, although clinical activity may occur in the absence of objective response as defined by RECIST. Response rates, time to progression and survival rates can be increased by giving trastuzumab or lapatinib in combination with other conventional therapies, such as endocrine therapy or chemotherapy. Most patients with metastatic breast cancer, however, develop progressive disease and die of this disease. One of the major reasons for this is the development of drug resistance. This Review discusses molecular predictors of response to trastuzumab and lapatinib and proposes ways to overcome resistance to HER2-targeted therapy in breast cancer patients. To what extent primary breast cancer exposed to adjuvant trastuzumab or lapatinib will become resistant to these agents is still unknown.

Key points

- The HER2 status of all invasive breast cancers should be assessed by immunohistochemistry (IHC) or *in situ* hybridization; the former method is most commonly used worldwide
- Trastuzumab and lapatinib are effective therapies in HER2-positive breast cancer; however, not all HER2-positive patients respond to these therapies, and progression is common in responding patients with metastatic disease
- Potential molecular predictors of resistance to HER2-directed therapies include loss of *PTEN*, PI3K/Akt hyperactivation, $p95^{HER2}$ expression, and IGF-IR overexpression
- Novel therapeutics are in clinical development to overcome resistance to trastuzumab and lapatinib, and include pertuzumab, trastuzumab-DM1, PI3K inhibitors, HSP90 inhibitors, IGF-IR inhibitors and novel tyrosine kinase inhibitors
- Randomized clinical trials have shown an improved clinical benefit in patients with HER2-positive metastatic disease who have disease progression and have been treated with drugs that inhibit HER2
- The molecular mechanism of HER2 blockade beyond disease progression is not known, and represents a new paradigm in breast cancer therapy

Table 1 | Characteristics and mechanisms of action of trastuzumab and lapatinib

Drug characteristics and mechanism of action	Trastuzumab	Lapatinib
Molecular weight (dalton)	148,000	943
Binds to target extracellularly	Yes[10]	No
Binds to target intracellularly	No	Yes[94]
Targets more than one member of the HER family	No	Yes[22]
Interference with signal transduction pathways (for example, PI3K, VEGF)	Yes[36]	Yes[95]
Induction of cell-cycle arrest	Yes[96]	Yes[97]
Induction of apoptosis	Yes[98]	Yes[43]
Impairment of extracellular domain cleavage	Yes[99]	No
Causes decreased angiogenesis	Yes[100]	No
Antibody-mediated cellular cytotoxicity	Yes[101]	No
Crosses the blood–brain barrier	No	Yes[102]

HER2 predicts response to targeted agents

HER2 DNA-based and protein-based assays have been developed to determine the HER2 status of primary breast tumors by exploiting the overexpression of the HER2 protein induced by gene amplification. To be eligible for the initial clinical trials of trastuzumab in the metastatic setting, invasive breast cancer specimens had to be HER2-positive as determined by immunohistochemistry (IHC) analysis. The IHC assay used in these clinical trials was based on two monoclonal antibodies directed against the HER2 protein (4D5 and CB11). Tumors were scored as 0, 1+, 2+ or 3+ depending on the expression level of the HER2 protein. Several IHC-based assays were subsequently approved by regulatory authorities to select patients for trastuzumab therapy. These include the HercepTest (Dako Corp., Carpinteria, CA) and CB11 (Ventana Medical Systems, Tucson, AZ) IHC tests.

A retrospective analysis of primary breast cancer specimens from patients who had participated in the

Table 2 | Definition of HER2 positivity by IHC or FISH*

Result	IHC	FISH (*HER2* copy number)	FISH (*HER2*/cep17 ratio)
Positive	3+‡	≥6 *HER2* copies per cell	*HER2*:ch 17 ratio >2.2§
Negative	0+, 1+	<4 *HER2* copies per cell	*HER2*:ch 17 ratio <1.8
Equivocal	2+	4–5 *HER2* copies per cell	*HER2*:ch 17 ratio 1.8–2.2

*Definitions according to ASCO/CAP guidelines[30]. ‡In the pivotal randomized adjuvant trastuzumab trials, IHC score 3+ was defined as strong complete membrane staining in more than 10% of tumor cells. In the ASCO/CAP guidelines, IHC score 3+ is defined as strong complete membrane staining in more than 30% of tumor cells. §In the pivotal randomized adjuvant trastuzumab trials, FISH positivity was defined HER2:cep17 ratio >2.2. Abbreviations: FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry.

pivotal phase III trial of chemotherapy with and without trastuzumab showed that the survival advantage of chemotherapy plus trastuzumab was limited to patients whose primary tumors carried *HER2* amplification as demonstrated by fluorescence *in situ* hybridization (FISH).[27] Specialized FISH assays, Pathvysion (Abbott Laboratories, Abbott Park, IL), Inform (Ventana Medical Systems, Tucson, AZ), and HER2 FISH pharmDx (Dako Corp., Carpinteria, CA) have been approved for selecting patients for trastuzumab therapy. Chromogenic *in situ* hybridization (CISH) is commercially available (Spot-Light HER2 CISH; Invitrogen, Carlsbad, CA), although this assay is rarely used in the US to determine HER2 status.[28] One advantage of CISH is that it doesn't require a fluorescence microscope. The concordance between CISH and FISH is excellent (97–99%).[28] One of the disadvantages of CISH is that it uses a single *HER2* probe and the number of chromosomes 17 (cep17) is not assessed.

To be eligible for adjuvant trastuzumab clinical trials, the primary tumors had to be HER2-positive by either IHC or FISH. Unfortunately, the implementation of IHC-based assays resulted in a false positive rate of 18% when tumors were tested in community-based laboratories; FISH data were more accurate than IHC data when compared with repeat testing at a large volume central laboratory.[29] To address the discrepancy between small-volume and large-volume laboratories, ASCO and the American College of Pathology (ACP) joined forces to standardize HER2 testing by IHC and FISH and/or CISH, and changed the definition of HER2 positivity from what was used previously.[30] For example, an IHC test was initially considered positive if 10% of the cells exhibited complete membrane staining; according to the ASCO/ACP criteria, 30% of cells must have this feature if the case is to be considered HER2 positive. For an invasive breast cancer to be considered HER2 positive when using FISH, the HER2/cep17 ratio must be greater than 2.2. If a chromosome 17 probe is not used (for example, Inform FISH or CISH), the *HER2* copy number must be greater than six. Several studies have shown that the presence of more than six copies of the *HER2* gene does not correlate with added benefit from trastuzumab in the neoadjuvant or adjuvant setting.[31,32] For both IHC and FISH, the ASCO/ACP guidelines introduced the concept of 'equivocal' HER2 status (Table 2).

We believe that assessment of HER2 status according to the ASCO/ACP guidelines will improve HER2 testing accuracy, and improve patient selection for trastuzumab and/or lapatinib therapy.[30] Assessment of HER2 by IHC and FISH is not enough, however, to identify the optimum treatment for an individual patient. Only one-third of patients with obvious *HER2* amplification or IHC scores of 3+ overexpression benefit from HER2-directed therapy in the metastatic setting and perhaps only half of patients in the adjuvant setting. Therefore, additional markers of response or benefit from HER2-directed treatments must be identified. Furthermore, the role of aneuploidy in response to HER2-targeted therapy is not well defined. The Cancer and Leukemia Group B (CALGB) 9,840 trial randomized patients with metastatic breast cancer to receive paclitaxel either once weekly or every 3 weeks. Patients with HER2-positive disease received trastuzumab, and patients with HER2-negative disease were randomly assigned to receive trastuzumab or paclitaxel.[33] Among patients with HER2-negative disease expressing polysomy of chromosome 17, the response rate was higher in the group receiving trastuzumab plus paclitaxel.[34] This finding needs further confirmation before it can be used to make treatment decisions in routine clinical practice.

Recent data from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-31 trial indicate that patients with 'negative' HER2 expression levels may also benefit from adjuvant trastuzumab. Paik and colleagues conducted a central review of tumor blocks from patients who had been entered in the NSABP B-31 trial, and found that 10% of the tumors were HER2 negative according to IHC and FISH analysis.[35] Trastuzumab reduced the risk of recurrence in this group of patients, conferring a benefit similar to that observed in patients with HER2-positive tumors. This study was retrospective, and therefore hypothesis-generating. It should be noted that the patients found to be HER2-negative by the NSABP 'central' laboratory had been reported originally as HER2-positive by 'local' laboratories. Although the NSABP investigators went to great lengths to confirm the HER2 negativity of the samples using complementary methods, they have not yet convincingly excluded errors in the central laboratory as the source of the 'HER2-negative' status. If this finding is confirmed by an independent review, a clinical trial to determine the efficacy of adjuvant trastuzumab in patients with low-level HER2 expression would be justified.

HER2 dimers and response to therapy

Activation of the type I family of tyrosine kinase receptors (EGFR, HER2, HER3, HER4) occurs via receptor dimerization between similar or different EGFR type I family members.[36] Dimerization of these proteins, with the exception of HER2, occurs upon ligand binding. For example, EGF binds EGFR, causing a conformational change that allows EGFR to heterodimerize with other receptors, such as HER2.[37,38] Crystallographic studies revealed that HER2 is always in the activate conformation in the absence of ligand and is ready to interact with other ligand-activated HER proteins.[39]

The mechanisms of inhibition of HER2 and EGFR signaling by trastuzumab and lapatinib are different.

Trastuzumab treatment disrupts HER2/EGFR heterodimers in breast cancer cells.[40] In contrast to lapatinib, however, short-term trastuzumab treatment increases HER2 phosphorylation.[41,42] The agonistic-like activity of trastuzumab is not well understood, and it is probably a time-dependent phenomenon because trastuzumab downregulates the PI3K pathway presumably by de-phosphorylation of key signaling proteins.[42] Lapatinib is a potent kinase inhibitor of EGFR and HER2 kinases.[21,43] Protein-protein interactions in response to trastuzumab and lapatinib treatment is an active area of investigation. HERmark (Monogram Biosciences, South San Francisco, CA) is a commercially available protein proximity assay designed to assess HER2 protein expression individually as well as HER2 homodimers and heterodimers in formalin-fixed, paraffin-embedded tissue.[44] Retrospective studies using this assay are ongoing to determine the predictive roles of total HER2, HER2/HER2 homodimers, HER2/HER3 heterodimers, and HER3/phosphatidylinositol-3 kinase (PI3K) dimers in patients treated with trastuzumab-based therapy for early-stage or metastatic breast cancer. Other applications of this technology include the potential ability to measure the HER3/PI3K complex as a marker of PI3K pathway activation in primary breast cancer tissues.

Truncated HER2 protein

The extracellular domain of HER2 can be shed into the circulation, resulting in a truncated HER2 protein that is 95 kDa ($p95^{HER2}$), which retains kinase activity.[13,45,46] Overexpression of $p95^{HER2}$ has been associated with poor clinical outcome in patients with early-stage breast cancer.[47] Trastuzumab binds to the extracellular domain of HER2, and a predominance of $p95^{HER2}$ would prevent trastuzumab binding. Scaltriti and colleagues showed that $p95^{HER2}$ is associated with trastuzumab resistance *in vitro*, as well as in a small number of patient samples.[48] The authors demonstrated that treatment of $p95^{HER2}$-expressing cells with lapatinib inhibited $p95^{HER2}$ phosphorylation and reduced downstream phosphorylation of Akt, whereas trastuzumab treatment had no effect on any of these parameters. Breast cancer cells that express $p95^{HER2}$ may still be sensitive to lapatinib.[49] A validated and reproducible assay of $p95^{HER2}$ expression would be of interest, because it may help select alternative therapies in those patients unresponsive to trastuzumab and help select patients to receive single-agent trastuzumab, single-agent lapatinib, or perhaps a combination or sequence of both therapies.[48,50]

Predictive role of *HER2* mRNA

Analysis of mRNA from breast cancer tissue allows measurements of individual genes (for example, estrogen receptor, HER2) as well as measurement of entire gene-expression pathways. HER2 is considered a good target for this type of analysis because mRNA levels correlate with *HER2* copy number.[8] Microdissection may be necessary, however, to examine the contribution of stroma to the overall gene-expression profile of a tumor.[51] Using cDNA microarrays, Gong and colleagues showed that HER2 expression can be assessed reliably from gene-expression profiling data.[52] In this study, samples were obtained by fine-needle aspiration of primary tumors, which were previously shown to contain less stroma than core biopsies.[53] Oncotype DX (Genomic Health, Redwood City, CA) is a reverse-transcription PCR assay that evaluates the expression of 16 cancer-related genes and 5 control genes. One of the genes is HER2, however, its value to date is mostly as a prognostic marker.[54]Although these techniques are promising, none of the available mRNA-based assays have been tested prospectively in patients treated with trastuzumab or lapatinib. Therefore, their role in predicting response or resistance to HER2-directed therapies is unknown.

Response and HER2 phosphorylation

Phosphorylation of the C-terminal domain of the HER2 receptor is required for active signaling, and it has been proposed that measuring phosphorylated HER2 may be more clinically relevant than measuring total HER2 levels.[55] DiGiovanna and colleagues, however, found no correlation between phosphorylated HER2 status and improved benefit from anthracycline therapy in HER2-overexpressing tumors.[56] Frogne and collaborators used IHC to evaluate the predictive role of phosphorylated HER2 in 268 primary breast tumors from postmenopausal women with hormone receptor-positive breast cancer who had received adjuvant tamoxifen therapy. In this study, phosphorylated HER2 expression was an independent predictor of poor rates of disease-free survival and overall survival.[57]

Johnston and colleagues conducted a retrospective correlative study to assess primary tissue from patients with inflammatory breast cancer who had participated in a phase II trial of lapatinib. Patients were included if their primary tumor was either HER2 positive or HER2 negative but EGFR positive. Objective clinical responses were limited to patients with HER2-positive tumors. In this study, co-expression of phosphorylated HER2 and phosphorylated HER3 was highly predictive of response to lapatinib.[58]

There are several caveats regarding assessment of HER2 phosphorylation as a predictive marker of response. The IHC assay has not been standardized, and it is unclear how reproducible it would be in clinical practice. In terms of the predictive role of phosphorylated HER2, it should be noted that short-term treatment with trastuzumab can induce HER2 phosphorylation in breast cancer cells.[42] Furthermore, it is well-known that multiple tyrosine residues of HER2 can be phosphorylated and whether a specific phospho-tyrosine residue could serve as a better predictor is still unknown.

Predictive markers in PI3K/mTOR pathway

Upregulation of the PI3K pathway is an important survival mechanism used by cancer cells to escape the effects of chemotherapy, endocrine therapy and HER2-targeted therapy.[42,59–61] The most common mechanisms of PI3K pathway hyperactivation in cancer cells are mutations in genes encoding the PI3K catalytic domain[62] and loss

of the phosphatase and tensin homolog (*PTEN*) tumor suppressor gene.[42] PTEN dephosphorylates PI3K, resulting in inhibition of Akt signaling.[63] PTEN was initially discovered as a tumor suppressor gene,[64] and downregulation of PTEN expression has been reported in approximately 50% of human breast cancers. One of the proposed mechanisms of action of trastuzumab and lapatinib is inhibition of PI3K activity and activity of the downstream kinase Akt in *HER2*-amplified breast cancer cells.[61,65] Thus, PI3K and Akt are potentially attractive therapeutic targets.[66]

Our group has shown that *PTEN* loss is associated with trastuzumab resistance.[42] Berns and colleagues conducted high-throughput screening using a short-hairpin RNA library and confirmed that PTEN loss was associated with resistance to trastuzumab-based therapy.[60] Using the same library, Eichhorn and colleagues also identified PTEN as a marker of resistance to lapatinib *in vitro* and *in vivo*. In this study, mutations in *PI3K* were also associated with lapatinib resistance.[62] However, Xia and collaborators showed no correlation between *PTEN* expression and response to lapatinib *in vitro*.[67] Furthermore, PTEN expression in primary breast cancer tissue as assessed by IHC showed no correlation with clinical response to lapatinib.[58] The differences between these studies may be explained by the use of different cell lines, tissues and testing methods by the different laboratories. Alternatively, *PTEN* loss might be a marker of resistance to trastuzumab and not to lapatinib therapy. Retrospective analysis of tissues collected during the conduct of prospective clinical trials of trastuzumab and lapatinib should be extremely useful to address this question.

Predictors of response to therapy

As only 20–30% of patients with metastatic breast cancer are considered HER2-positive by standard IHC and FISH methods, it is possible that HER2 status is necessary but not sufficient to induce response to trastuzumab and/or lapatinib. Various signal transduction pathways have been associated with resistance to these biologic targeted therapies, including MAPK and PI3K. Our group showed that *PTEN* loss is associated with trastuzumab resistance.[42] In a small cohort of patients, we found that *PTEN* loss was even more predictive of trastuzumab resistance than HER2 itself. One of the limitations of this study and other similar studies is establishing the optimal method to assess PTEN in primary and/or metastatic tumors.

In the NSABP-B31 and NCCTG N9831 studies, patients with early-stage HER2-positive breast cancer were treated with four cycles of doxorubicin and cyclophosphamide followed by paclitaxel, with or without trastuzumab.[18] Kim and colleagues showed that *c-MYC* amplification was highly predictive of benefit from adjuvant trastuzumab in patients who participated in the NSABP-B31 trial.[68] However, this finding was not confirmed by a similar evaluation by NCCTG N9831 investigators.[69] Therefore, the role of *c-MYC* amplification in response to trastuzumab remains investigational.

Gene-expression profiling of primary tumors in the neoadjuvant setting has been used to identify novel marker of response to therapy. Using this approach, we reported that loss of CD40 signaling may result in a low pathologic complete response after administration of anthracycline-based and taxane-based trastuzumab neoadjuvant chemotherapy.[31] CD40 signaling plays an important role in cell growth of macrophages, but how CD40 signaling regulates or contributes to HER2 signaling is unknown. It is possible that upregulation of macrophages and other immune cells play a role in the response to trastuzumab *in vivo*. Harris and colleagues reported a correlation between high IGF-IR expression levels and resistance to trastuzumab and vinorelbine in patients with locally advanced breast cancer undergoing neoadjuvant systemic therapy.[70]

Overcoming resistance in metastatic disease

Most patients with HER2-positive breast cancer develop progressive disease after trastuzumab or lapatinib therapy, so there is great interest in the development of novel HER2-directed therapies.[25] This section discusses new monoclonal antibodies (either as passive immunotherapy or bound to a toxin), small-molecule tyrosine kinase inhibitors, and heat-shock protein (HSP)-90 inhibitors (Figure 1) as potential new therapies to overcome resistance. Interestingly, most clinical trials are evaluating novel agents in combination with trastuzumab, despite disease progression in patients treated with trastuzumab (Table 3).

Trastuzumab-DM1

HER2 is an excellent target for antibody–drug conjugate therapy because it is expressed at high levels in cancer cells that carry *HER2* amplification. Trastuzumab-MCC-DM1 (T-DM1) is an antibody–drug conjugate consisting of trastuzumab, a nonreducible linker and DM1, a microtubule inhibitor.[71] T-DM1 is internalized by the cancer cell and the DM1 moiety is released, resulting in microtubule damage and cell death. The mechanism of action of DM1 is similar to that of the vinca alkaloids, which are known to be synergistic with trastuzumab.[72,73]

The concept of using immunotoxins to treat cancer is well established.[74] Despite many attempts, however, only one antibody–drug conjugate has been approved for cancer treatment, that is, gemtuzumab ozogamicin for recurrent acute myeloid leukemia. One of the main technological advances in the field of antibody–drug conjugate therapy was development of appropriate linkers for conjugating the antibody to the chemotherapeutic agent. In phase I clinical trials of T-DM1, thrombocytopenia was reported as a dose-limiting toxic effect. Preliminary data from phase I and II trials showed significant clinical efficacy in heavily pretreated patients. In a multicenter phase II trial, an objective response was seen in over 30% of patients with HER2-positive metastatic breast cancer whose disease had progressed following multiple lines of trastuzumab therapy, including patients who had also received lapatinib.[75] Phase III trials are ongoing in patients with metastatic breast cancer to determine the efficacy of T-DM1 not only with respect to trastuzumab and lapatinib resistance, but also in the front-line setting.

If these trials confirm the promising results reported to date, it would be reasonable to compare T-DM1 to trastuzumab-based chemotherapy in the metastatic and adjuvant settings. Studies are also ongoing to determine the safety and efficacy of T-DM1 in combination with pertuzumab monoclonal antibody therapy.

Pertuzumab

Pertuzumab (Genentech, South San Francisco, CA) is a humanized monoclonal antibody directed against the extracellular heterodimerization domain of HER2 (domain II).[76] Pertuzumab effectively blocks HER2/HER3 heterodimers and subsequent signaling.[77] By contrast, trastuzumab binds to domain IV of the HER2 extracellular domain and does not inhibit dimerization of HER2 with ligand-activated HER3.[77] Whether trastuzumab blocks dimerization between HER2 and EGFR is controversial.[40,77]

Our group showed that targeting *HER2*-amplified breast cancer cells with both trastuzumab and pertuzumab resulted in synergistic induction of apoptosis *in vitro*.[78] This observation was confirmed by subsequent, independent *in vivo* studies.[79,80] A phase II clinical trial of pertuzumab showed objective responses and stable disease in patients with metastatic breast cancer who had disease progression after trastuzumab-based therapy.[81] Randomized clinical trials are ongoing in patients with HER2-positive metastatic breast cancer to determine whether the trastuzumab and pertuzumab combination is superior to trastuzumab alone.

PI3K/mTOR pathway as a therapeutic target

The HER2/PI3K/PTEN pathway is commonly altered in breast cancer cells, and it is not unusual for cancer cells to have mutations at different levels within this pathway.[60,82] Mutation in the catalytic p110α subunit of PI3K seems to be a late effect in breast cancer development, and a potential target for therapy.[82,83] Folkes and collaborators synthesized a series of small molecules that block PI3K and selected the inhibitor GDC-0941 for clinical development.[84] Preclinical studies have shown that this novel inhibitor is synergistic with trastuzumab *in vitro* and *in vivo*.[83] The combination of trastuzumab with GDC-0941 resulted in apoptosis, identified by accumulation of cleaved caspase-3 fragments and a cleaved 89-kDa poly ADP-ribose polymerase fragment.[83] Clinical trials are exploring the safety and efficacy of GDC-0941 as a single agent and in combination with T-DM1.

The mammalian target of rapamycin (mTOR) functions downstream of PI3K/Akt in the EGFR pathway. The mTOR inhibitor RAD001 (everolimus) is moderately active against PTEN-deficient, HER2-positive breast cancer xenografts.[66] One of the limitations of single-agent RAD001 is development of a feedback loop resulting in Akt activation after mTOR blockade.[85] On the basis of these data, we and others are conducting clinical trials of RAD001 in combination with trastuzumab in breast cancer patients previously treated with trastuzumab. In addition, novel inhibitors that can block both PI3K and mTOR are in clinical development.[62]

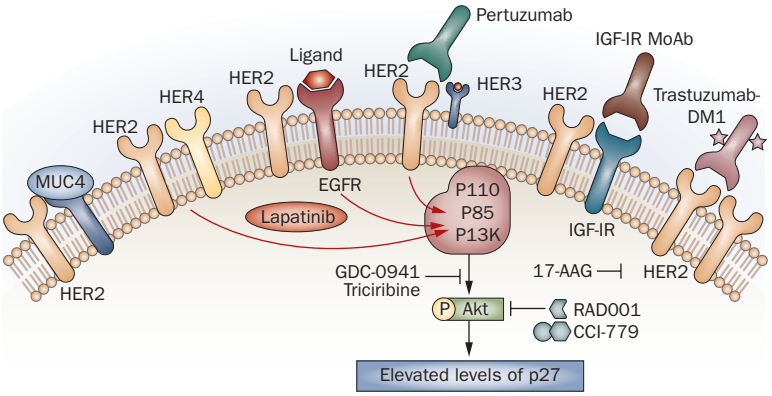

Figure 1 | Molecular targets and therapeutic approaches in trastuzumab and lapatinib resistance. The EGFR family consists of four different members: EGFR, HER2, HER3, and HER4. Upon ligand binding, EGFR, HER3 and HER4 can homodimerize or heterodimerize to form several receptor combinations. There is no known ligand for HER2, which can be activated by constitutive overexpression and homodimerization or heterodimerization with other receptors. Receptor phosphorylation results from tyrosine kinase activity within the cytoplasmic domains of these receptors, which activates downstream signaling pathways, in particular the PI3K/Akt pathway. Activation of these pathway components leads to elevated levels of the p27Kip1 protein. MUC4 can block inhibitory actions of trastuzumab by binding to HER2, thereby preventing interaction between the drug and its molecular target. Furthermore, in trastuzumab-resistant cells HER2 and IGF1R physically interact and are involved in a cross-talk that results in p27Kip1 downregulation. Potential novel agents for trastuzumab and lapatinib resistant tumors include the following: trastuzumab-DM1, an antibody–drug conjugate that binds HER2 and delivers the DM1 toxin intracellularly; pertuzumab, a HER2-targeted antibody that disrupts receptor heterodimerization between EGFR/HER2 and HER2/HER3; IGF1R-targeted antibodies; the mammalian target of rapamycin inhibitors RAD001 and CCI-779; GDC-0941 and triciribine, small molecule inhibitors of PI3K; 17-AAG and other HSP90 inhibitors. Abbreviations: IGF1R, insulin-like growth factor 1 receptor; MUC4, membrane-associated glycoprotein mucin-4; PI3K, phosphatidylinositol 3-kinase.

Inhibitors of IGF-1R

The insulin-like growth factor receptor (IGF-1R) pathway plays important roles in cell proliferation and survival. Dysregulation of this pathway has been associated with poor prognosis, attributed in part to its association with drug resistance. IGF-1R is a homodimeric receptor with tyrosine kinase activity that can be activated by IGF-1 and IGF-2. Ligand-dependent activation results in phosphorylation of adaptor proteins (such as IRS-1, IRS-2) and activation of downstream components including PI3K/Akt. The final result is cell growth and proliferation, mediated by inhibition of apoptosis and activation of cyclins.

Overexpression of IGF-1R in HER2-overexpressing breast cancer cell lines by transfection of *IGF-IR* results in trastuzumab resistance.[86] Harris and colleagues conducted a phase II clinical trial of neoadjuvant vinorelbine and trastuzumab in patients with HER2-positive breast cancer, and found that overexpression of IGF-1R in the primary tumor was associated with drug resistance.[70] Our laboratory developed an *in vitro* model using the breast cancer cell line SKBR3 to generate acquired trastuumab resistance by long-term trastuzumab treatment. Using this experimental system we discovered heterodimerization of HER2 and

Table 3 | Efficacy of HER2-directed therapy in trastuzumab-resistant metastatic breast cancer

Study	Drug regimen	Trial phase	Number of patients	Tumor stage	ORR (%)	CBR (%)	Median TTP (months)	Median OS (months)
Kaufman *et al.* (2009)[103]	Lapatinib	II	126	IIIB	39*‡	NA	3.5	18
Burstein *et al.* (2008)[104]	Lapatinib	II	140	IV	1.4‡	5.7‡	2.2‡	7.3
Geyer *et al.* (2006)[24]	Lapatinib + capecitabine	III	163	IV	22	27	8.4	NA
Cameron *et al.* (2008)[105]	Lapatinib + capecitabine	III	198	IV	23.7	29.3	6.2	15.6
von Minckwitz *et al.* (2008)[91]	Trastuzumab + capecitabine	III§	78	IV	48	75	8.1	25.5

*Clinical response, inflammatory breast cancer. ‡HER2+ patients only. §Designed as phase III trial but closed earlier than planned because of poor accrual. Abbreviations: CBR, clinical benefit rate (complete response, partial response, or stable disease for ≥24 weeks); NA, not available; ORR, overall response rate; OS, overall survival; TTP, time to progression.

IGF-IR in trastuzumab-resistant cells but not in the parental, trastuzumab-sensitive SKBR3 cells. Based on these preclinical studies we hypothesized that HER2-overexpressing tumors use the IGF-IR pathway as a mechanism of escape in the presence of trastuzumab therapy. We are currently conducting a phase I/II clinical trial of IGF-IR monoclonal antibody (AMG-471) in combination with trastuzumab in patients who have developed progressive disease on trastuzumab-based therapy. In future studies it will be important to collect biopsies from metastatic tumors to determine whether the IGF-1R pathway is activated and how interference of this pathway affects tumor response and survival rates. Small molecules that target IGF-1R have proven more challenging than antibody therapy for the development of targeted agents because of the similarity of IGF-1R and the insulin receptor.

Inhibitors of heat-shock protein 90

HSP-90 is a chaperone protein that is critical for the folding of other proteins. HER2 is one of the most important HSP-90 'clients'. Inhibition of HSP-90 by geldanamycin can induce apoptosis in breast cancer cells, particularly if the cells are dependent on the HER2 pathway.[87] Clinical trials are ongoing to determine the efficacy and safety of HSP-90 inhibitors in a variety of situations, including trastuzumab resistance. Modi and collaborators completed a phase I trial of tanespimycin (17-AAG) in combination with trastuzumab in patients with HER2-positive metastatic breast cancer who had progressed on trastuzumab therapy. In this study, the combination of tanespimycin and trastuzumab was well tolerated and clinical activity was noted.[88]

Novel HER2 tyrosine kinase inhibitors

Neratinib (HKI-272) is an irreversible inhibitor of EGFR and HER2 kinases. A phase II trial of neratinib as front-line therapy for HER2-positive metastatic breast cancer has produced a response rate of 26%. Preliminary data from a phase I–II trial showed that a combination of neratinib and trastuzumab is safe and effective in patients with HER2-positive metastatic breast cancer who had not received prior trastuzumab. No significant cardiac toxic effects have been observed to date.[89]

ARRY-380 is an inhibitor of HER2, with minimal effect on EGFR. Phase I trials are ongoing to determine the safety profile of this novel small molecule inhibitor. One of the potential advantages of this agent is the lack of toxic effects caused by EGFR blockade, such as diarrhea or skin rash.

Trastuzumab beyond disease progression

When the phase III randomized trial of trastuzumab was underway in the early 1990s, patients were allowed to continue trastuzumab beyond disease progression. As responses were observed, it was believed that treatment beyond progression was justified.[90] Recently, results from two randomized clinical trials shed further light on this controversial issue. Von Minckwitz and colleagues randomly assigned patients with HER2-positive metastatic breast cancer who had developed progressive disease after first-line trastuzumab-based therapy to receive capecitabine or capecitabine plus continuation of trastuzumab. This was supposed to be a phase III trial, but the study was closed earlier than planned because of slow accrual. The primary endpoint was time to progression. Efficacy analysis of 154 patients treated on this clinical trial showed a longer time to progression in the group of patients who received trastuzumab beyond progression.[91]

O'Shaughnessy and colleagues reported preliminary results from a randomized clinical trial of trastuzumab and lapatinib in 269 patients who had received several trastuzumab-based regimens. In this study, patients were randomized to receive single-agent lapatinib or lapatinib plus continuation of trastuzumab. Patients were heavily pretreated with a mean of three prior trastuzumab-based regimens and other chemotherapies (approximately 30% of patients had received as many as six prior treatments for metastatic breast cancer). The combination of lapatinib and trastuzumab yielded a significantly greater rate of progression-free survival than lapatinib alone (12 weeks versus 8.1 weeks). This outcome translated to a 27% reduction in the risk for disease progression (hazard ratio 0.73; $P = 0.008$). The overall rate of clinical benefit (response rate and rate of durable stable disease) for the combination was double that for monotherapy (24.7% versus 12.4%; $P = 0.01$).[92] Adverse events were similar

in both groups, including cardiovascular toxic effects. This study demonstrates that lapatinib and trastuzumab are synergistic in breast cancer, a finding supported by preclinical data. Our laboratory showed that lapatinib induces apoptosis in combination with trastuzumab in HER2-overexpressing breast cancer cell lines.[65] Scaltriti and colleagues demonstrated that lapatinib stabilizes HER2 protein at the cell membrane, which results in enhanced antibody-dependent cell-mediated cytotoxicity induced by trastuzumab.[65] A large trial of adjuvant lapatinib and/or trastuzumab treatment optimization (ALTTO) is ongoing to determine whether these agents should be given in combination in the adjuvant setting.

There are no tests available that can predict which patients would benefit from trastuzumab beyond disease progression, in part because decisions are based on the HER2 status of the primary tumor and not on that of the metastatic tumor. Interestingly, data from the neoadjuvant setting suggest that HER2 expression might change in the primary tumor after trastuzumab-based chemotherapy. Our group assessed HER2 expression in primary breast cancers before initiation of neoadjuvant trastuzumab-based chemotherapy and at the time of definitive surgery. Twenty-five patients were found to have residual disease, and HER2 was negative by FISH in eight (32%) of these patients.[93] This is most likely because of the erradication of HER2-overexpressing cells by trastuzumab-based therapy, leaving HER2-negative cells as residual disease. As more therapies become available, a biopsy of metastatic tumors may be necessary to determine the optimal therapy for patients with progressive metastatic breast cancer.

Conclusions

Assessment of *HER2* amplification by FISH/CISH, and HER2 protein overexpression by IHC, are the current standard methods to establish HER2 status in the clinic. The negative predictive value of these tests is excellent. Therefore, patients whose tumors are HER2-negative by IHC and/or FISH should not be considered for trastuzumab or lapatinib outside of a clinical trial. However, the positive predictive value of IHC and FISH is limited, and there is great interest in developing novel diagnostic approaches to assess HER2 expression and function. Emerging new technologies that assess HER family homodimers and heterodimers are promising because dimerization is critical for receptor function *in vivo*. Activation of the PI3K pathway is one of the most important mechanisms of escape or resistance to trastuzumab and lapatinib. PI3K/Akt can be activated by mutations in the catalytic domain of PI3K, loss of PTEN by mutation or postranslational modifications or IGF-1R overexpression. Mutations in genes encoding the EGFR, HER2 and HER4 kinase domains are under investigation. Potential approaches to overcome primary resistance to trastuzumab and lapatinib include combination of both therapies upfront. For patients who develop progressive metastatic disease after treatment with trastuzumab and lapatinib, it will be important to obtain biopsies of accessible metastatic tumors to identify the critical pathway driving metastasis and design intelligent clinical trials to determine the appropriate targeted therapy for each individual patient.

Review criteria

Information for this Review was compiled by searching the PubMed and MEDLINE databases for articles published before 30 June 2009. Only articles published in English were considered. The search terms used were "trastuzumab" and "lapatinib" in association with the search terms "breast neoplasm", "drug resistance" and "tumor markers, biological". Whenever possible primary sources have been used. Full articles were downloaded and references were checked for additional material when appropriate. We reviewed abstracts presented at the 2007, 2008 and 2009 ASCO annual meetings or the 2007 or 2008 San Antonio Breast Cancer Symposium.

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