Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2$^+$ breast cancer patients

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Clinical benefits from trastuzumab and other anti-HER2 therapies in patients with HER2 amplified breast cancer remain limited by primary or acquired resistance. To identify potential mechanisms of resistance, we established trastuzumab-resistant HER2 amplified breast cancer cells by chronic exposure to trastuzumab treatment. Genomewide copy-number variation analyses of the resistant cells compared with parental cells revealed a focal amplification of genomic DNA containing the cyclin E gene. In a cohort of 34 HER2$^+$ patients treated with trastuzumab-based therapy, we found that cyclin E amplification/overexpression was associated with a worse clinical benefit (33.3% compared with 87.5%, $P < 0.02$) and a lower progression-free survival (6 mo vs. 14 mo, $P < 0.002$) compared with nonoverexpressing cyclin E tumors. To dissect the potential role of cyclin E in trastuzumab resistance, we studied the effects of cyclin E overexpression and cyclin E suppression. Cyclin E overexpression resulted in resistance to trastuzumab both in vitro and in vivo. Inhibition of cyclin E activity in cyclin E-amplified trastuzumab resistant clones, either by knockdown of cyclin E expression or treatment with cyclin-dependent kinase 2 (CDK2) inhibitors, led to a dramatic decrease in proliferation and enhanced apoptosis. In vivo, CDK2 inhibition significantly reduced tumor growth of trastuzumab-resistant xenografts. Our findings point to a causative role for cyclin E overexpression and the consequent increase in CDK2 activity in trastuzumab resistance and suggest that treatment with CDK2 inhibitors may be a valid strategy in patients with breast tumors with HER2 and cyclin E coamplification/overexpression.

HER2 is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, which includes EGFR itself, HER2, HER3, and HER4. Homo- or heterodimerization of these receptors results in phosphorylation of residues in the intracellular domain and consequent recruitment of adapter molecules responsible for the initiation of several signaling pathways involved in cell proliferation and survival (1, 2). Approximately 20% of breast cancers exhibit HER2 gene amplification/overexpression, resulting in an aggressive tumor phenotype and reduced survival (3, 4). Therapy of HER2$^+$ breast cancer with anti-HER2 agents, including monoclonal antibodies and small molecule tyrosine kinase inhibitors, has markedly improved the outcome of this disease (5). Trastuzumab, a recombinant humanized monoclonal antibody that binds to the extracellular domain of HER2, improves survival in patients with HER2$^+$ breast cancer, in both the metastatic (6, 7) and adjuvant settings (8). The overall antitumor activity of trastuzumab is due to a combination of mechanisms, including inhibition of ligand-independent HER2 dimerization (9), HER2 down-regulation (10, 11), that lead to disruption of HER2-dependent PI3K/Akt signaling (12) and induction of G1 arrest through stabilization of the CDK inhibitor p27 (13). In addition, trastuzumab also mediates antibody-dependent cell-mediated cytotoxicity (ADCC) (14).

Despite the survival gains provided by anti-HER2 therapies, patients with advanced HER2$^+$ breast cancer frequently display primary resistance to trastuzumab-based therapy, and even if they initially respond, acquired resistance invariably ensues at some point. The magnitude of the resistance problem has prompted efforts at identifying the underlying mechanisms. A number of mechanisms of resistance have been described to date including hyperactivation of the phosphatidylinositol-3-kinase (PI3K) pathway (12, 15), coexpression of the truncated p95HER2 receptor (16), heterodimerization with other growth factor receptors (17–19), and loss of HER2 expression itself (20). Some, but not all, of these mechanisms have been shown to play a role in the clinic (12, 15, 16, 20). However, the described mechanisms are not prevalent enough to justify the high frequency of resistance to anti-HER2 agents. To identify additional mechanisms, we established trastuzumab-resistant HER2 amplified breast cancer cells by chronic exposure to increasing trastuzumab concentrations. Using these cells as an initial screening tool, we took an unbiased approach based on comparative genomewide copy-number analysis. Our studies revealed the presence of acquired amplification of the cyclin E gene in trastuzumab-resistant cells. We demonstrate the clinical relevance of this finding showing that cyclin E amplification/overexpression, occurring in a substantial portion of HER2$^+$ breast cancer patients, results in a lower clinical benefit rate (CBR) and progression-free survival (PFS) from trastuzumab-based therapy.

High cyclin E expression has been proposed as a marker of poor clinical outcome in breast cancer (21). Furthermore, it has been recently shown that cyclin E levels decrease upon HER2 down-regulation and HER2 inhibition, suggesting that HER2 regulates cyclin E function (22). In a reversal of roles, our study now shows that cyclin E exerts a control over HER2 function as demonstrated by cyclin E overexpression resulting in resistance to trastuzumab. Our results are indicative of a direct role of cyclin E in trastuzumab resistance and suggest that treatment with CDK2

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inhibitors should be considered in patients whose tumors display cyclin E amplification/overexpression.

Results

Generation and Characterization of Trastuzumab-Resistant Cell Lines. First, we generated trastuzumab-resistant clones by chronically exposing the HER2 amplified breast cancer cell line BT474 to increasing concentrations of trastuzumab for over 18 mo in vitro. BT474 cell clones resistant to the antiproliferative effects of trastuzumab (IC50 > 1 μM, ~100-fold higher than control BT474 cells) were identified. As shown in Fig. 1*A*, the proliferation of a representative BT474 trastuzumab-resistant cell line (BT474R) is undisturbed in the presence of increasing concentrations of trastuzumab. An emerging body of evidence suggests that cells cultured in monolayer may respond differently to trastuzumab than cells grown in 3D cultures (23, 24). Thus, we tested the capacity of our resistant cells to form tumors in the presence of trastuzumab. To do this testing, we orthotopically injected in vitro resistant clones in immunodeficient mice and treated the animals twice weekly with trastuzumab (10 mg/kg). Where appropriate, we show results from one representative in vitro and in vivo trastuzumab-resistant cell line, referred to from here on as BT474R and BT474R2, respectively (Fig. 1*A*).

We then performed genomewide copy number analyses of both BT474R and BT474R2 and compared them to parental control cells. With this approach, we identified two regions of the cell genome with copy-number alterations, 19q (gain) and 14q (loss). The focal deletion on 14q contains two transcripts GPR65 and GALC, whereas the amplification at 19q contains seven genes, two of which, UQCRFS1 and CCNE1, have previously been identified as being amplified in breast cancer (Fig. 1*B*) (25–27). We focused our work on *CCNE1*, the gene encoding cyclin E, because of its known role in the G1/S transition of the cell cycle (28). Our hypothesis was that the increased levels of cyclin E (and consequent increase in CDK2 activity) observed in our resistant cells could be responsible for the lack of trastuzumab-mediated G1 arrest, which would in turn result in the decreased sensitivity of these cells to trastuzumab.

Analysis by Western blot confirmed that both full-length and low molecular weight (LMW) isoforms of cyclin E were overexpressed in our trastuzumab-resistant cell lines (Fig. 1*C*). Histological analyses of BT474R2 cells further confirmed the increase of both nuclear and cytoplasmic expression of cyclin E (Fig. S1). Overexpression of cyclin E in the resistant cells was accompanied by enhanced cyclin E–CDK2 kinase activity, as shown by higher basal phosphorylation of the retinoblastoma protein (RB), a direct target of the cyclin E–CDK2 complex (Fig. 1*C*). In addition, whereas trastuzumab treatment of BT474 parental cells resulted in decreased RB phosphorylation, no change in RB phosphorylation was observed in the resistant cell lines. As expected, p27 levels increased in parental cells treated with trastuzumab compared with untreated cells. In contrast, BT474R and BT474R2 cells displayed high basal levels of p27, which did not change with the addition of trastuzumab (Fig. 1*C*).

As mentioned, a number of mechanisms that limit the efficacy of trastuzumab-based therapy in $HER2^+$ tumors have been previously identified. We studied whether any of these mechanisms was present in the resistant BT474R and BT474R2 cells. First, resistant cells retained high levels of HER2 expression and, as

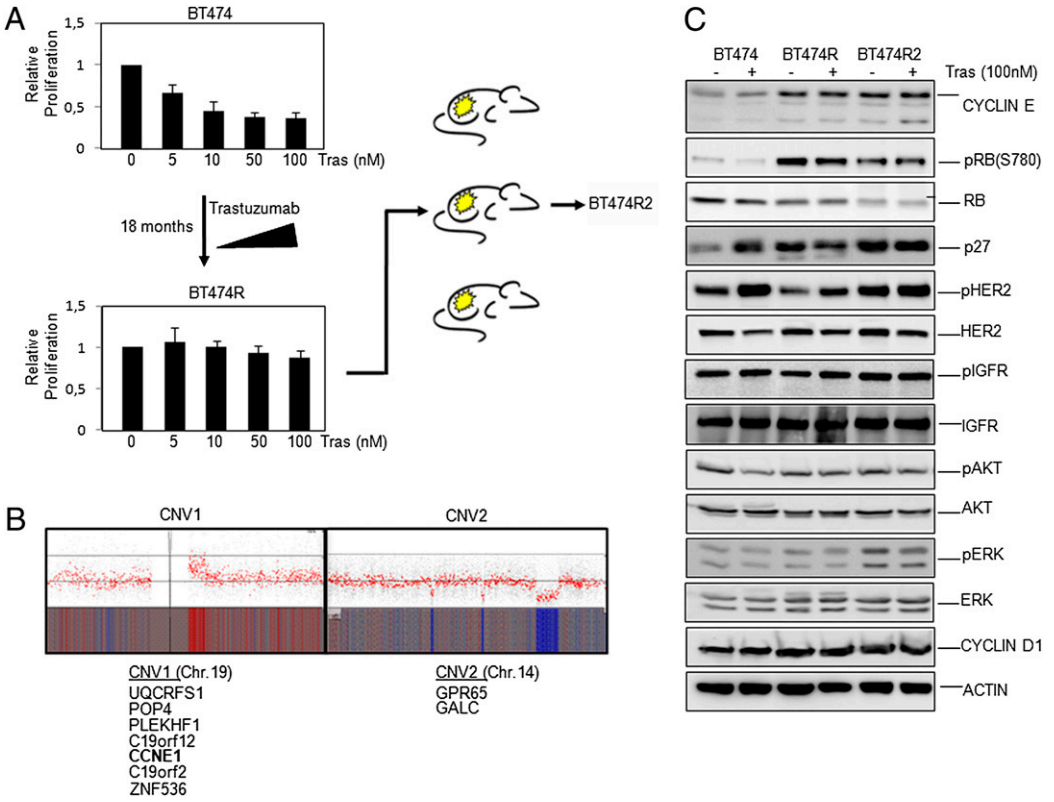

Fig. 1. Generation and characterization of trastuzumab-resistant cell lines. (*A*) Trastuzumab-sensitive breast cancer cell line BT474 was made resistant by persistent exposure to increasing concentrations of trastuzumab (*Results*). BT474 and BT474R cells were treated with trastuzumab at the indicated concentrations for 6 d and proliferation was measured. BT474R cells were subsequently injected s.c. in nude mice and animals were treated twice weekly with 10 mg/kg trastuzumab. Resistant tumors were excised and new trastuzumab-resistant cell lines (BT474R2) established. (*B*) Genomewide copy-number analyses of BT474R cells compared with parental controls. Gain of region 19q12 (*Left*) and loss of region 14q31 (*Right*). The amplification on 19q12 locus encodes the gene for cyclin E1 (CCNE1). Genetic analysis of BT474R2 cells gave identical results. (*C*) Western blot analyses of BT474 and BT474R cells. Cells were exposed to 100 nM trastuzumab for 48 h. Whole-cell extracts were analyzed with the indicated antibodies.

in the parental cells, trastuzumab increased HER2 phosphorylation while concomitantly reducing the expression of HER2 (Fig. 1*C*). Basal phosphorylation of AKT, a read-out of PI3K signaling, was not enhanced in the resistant cells, suggesting that our trastuzumab-resistant cell lines do not exhibit PI3K pathway hyperactivation. Furthermore, no changes in IGF1R phosphorylation were observed in the resistant cells, discarding an increase of HER2/IGF1R dimerization in these cells (Fig. 1*C*). At the same time, these analyses revealed that all of the in vitro and in vivo trastuzumab-resistant clones exhibited similar patterns of expression of these proteins, indicating little clonal variation among the resistant cells. In sensitive cells, trastuzumab administration results in a cell cycle blockade at the level of G1, which has been shown to occur due to a trastuzumab-induced stabilization of the CDK2 inhibitor p27(13, 29). Our parental trastuzumab-sensitive cells displayed the expected G1 blockade upon administration of trastuzumab (Fig. S2). On the other hand, under identical conditions, resistant cells failed to undergo a G1 arrest, an indication that resistant cells maintain cell cycle progression in the presence of trastuzumab despite displaying high levels of p27 (Fig. S2).

Cyclin E Amplification/Overexpression and Trastuzumab Resistance in Breast Cancer Patients. To assess the potential clinical relevance of our findings, we analyzed a cohort of patients with HER2 amplified tumors that displayed cyclin E amplification and/or overexpression and that had been treated with trastuzumab-containing therapy at our institutions. Tumors were scored as cyclin E positive if the FISH ratio cyclin E/chromosome 19 was higher than 1.5 (Fig. 2*A*) or when the H score for nuclear cyclin E staining was higher than 30 (Fig. 2*B*). We chose these cutoff values because they corresponded to the levels of cyclin E amplification and nuclear staining encountered in our resistant cells (Fig. S1). Our cutoff values are similar to those previously adopted by other groups (30, 31). The levels of cyclin E amplification and overexpression in breast tumors are lower than those described for HER2 (breast cancer) or MET (lung cancer) genes. The most likely explanation is that excessive levels of cyclin E would lead to cell death or senescence (32, 33).

In our cohort of 55 cases of HER2 amplified breast tumors, we found an incidence of cyclin E amplification/overexpression of 35%. To corroborate these findings, we explored the frequency of cyclin E overexpression/amplification in several publicly available microarray data sets (for a total of 728 tumors). Using the MDACC133 data set (34), overexpression of the CCNE1 gene was identified in 18–30% (6–10/33) of the HER2$^+$ cases (Fig. S3*A*). Interestingly, an association between ER negativity and high expression of cyclin E gene was also evident in this analysis (Fig. S3*B*). To assess cyclin E amplification, we analyzed a combined aCGH data set of 595 breast cancers (35) and estimated that amplification of the CCNE1 chromosomal region occurs in ~20% of the cases with HER2 amplification (Fig. S3*C*). Our findings are in line with previously published data in breast cancer (36–38).

We then identified and analyzed a cohort of 18 HER2$^+$ patients who scored positive for cyclin E and that had received first line therapy with a trastuzumab-containing regimen in the advanced disease setting for whom annotated clinical data were available. Clinical benefit rate from trastuzumab, defined as patients having a complete response, partial response, or stable disease ≥6 mo, was observed in only five patients (33.3%, Fig. 2*C*) and a progression-free survival of 6 mo (Fig. 2*D*). We analyzed in parallel a similar cohort of 16 HER2$^+$ patients that scored negative for cyclin E that had also been treated with first-line trastuzumab therapy. These patients had a clinical benefit rate of 87.5% and a progression-free survival time of 14 mo (Fig. 2 *C* and *D*). In summary, cyclin E amplification/overexpression was associated with a worse clinical benefit (33.3% compared with 87.5%, $P < 0.02$) and a lower progression-free survival (6 mo vs. 14 mo, $P < 0.002$). The observed clinical benefit rate in cyclin E amplification/overexpression was also lower from the expected ~60% clinical benefit in a similar population of patients with HER2

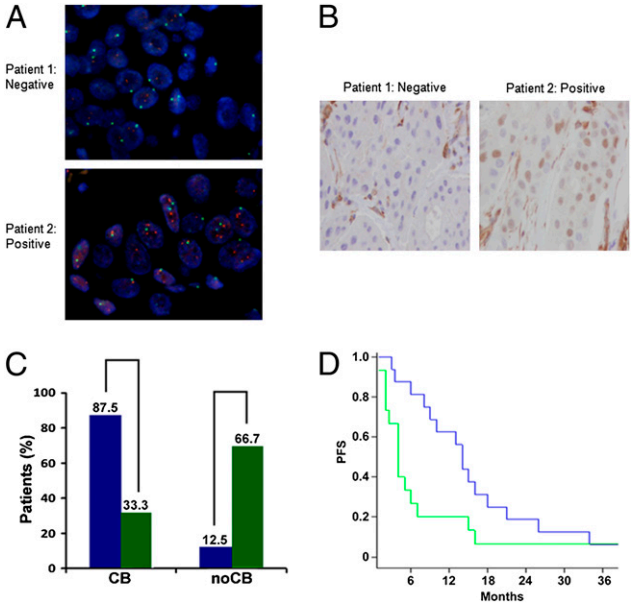

Fig. 2. Cyclin E amplification/overexpression in HER2$^+$ breast tumors and clinical trastuzumab resistance. (*A*) FISH analyses showing a representative case of a patient with no amplification of *CCNE1* (patient 1) and with amplification of *CCNE1* (patient 2). (*B*) Representative immunohistochemistry showing a tumor with negative nuclear staining for cyclin E (patient 1) and a tumor with positive nuclear staining for cyclin E (H score > 30, patient 2). (*C*) Clinical benefit rate of cyclin E positive (green, $n = 18$) vs. cyclin E negative (blue, $n = 16$) patients treated with trastuzumab-based therapy. Breslow test: $P = 0.02$. (*D*) PFS of cyclin E positive (green, $n = 18$) vs. cyclin E negative (blue, $n = 16$) patients. Median PFS was 4 mo for cyclin E positive patients and 14 mo for cyclin E negative patients. Breslow test: $P = 0.002$.

amplified tumors and unselected for cyclin E expression (5–7, 20). We also studied whether cyclin E amplification/overexpression coexisted with other potentially clinically relevant mechanisms of resistance. In 26 tumor samples (10 cyclin E positive and 16 cyclin E negative), additional slides were available and we were able to evaluate the presence of p95HER2 and the expression of PTEN. Low PTEN expression (H score ≤ 60) was found in 4 cyclin E positive tumors and the presence of truncated forms of HER2 (p95HER2) was found in 3 cyclin E positive tumors. In two cases, cyclin E amplification, low PTEN expression, and presence of p95HER2 coincided, whereas in five cases, cyclin E amplification was the only alteration present. Our results suggest that, although there is some coexistence of potential mechanisms of resistance, frequently cyclin E overexpression is the only one present of the ones that have been shown to be clinically relevant.

Cyclin E Overexpression Is Responsible for Trastuzumab Resistance. To address whether cyclin E amplification/overexpression results acquired resistance to trastuzumab, we tested the effects of modulating cyclin E expression. Ectopic expression of cyclin E in parental BT474 cells significantly decreased the sensitivity of these cells to trastuzumab (Fig. 3 *A* and *B*). Conversely, down-regulation of cyclin E by small interfering RNA (siRNA) resulted in a significant growth inhibition in BT474R cells, whereas only moderate effects were observed in parental BT474 cells (Fig. 3 *C* and *D*). Furthermore, BT474R cells were slightly more sensitive than control cells to the antiproliferative effects induced by the combination of cyclin E ablation and trastuzumab treatment. Collectively, these data indicate that cyclin E overexpression accounts for the observed resistance phenotype in BT474R trastuzumab-resistant cells.

Trastuzumab-Resistant Cells Are Sensitive to CDK2 Inhibition. The binding of cyclin E to its cognate partner CDK2 allows the cells to progress through the G1 phase of cell cycle. An excess of

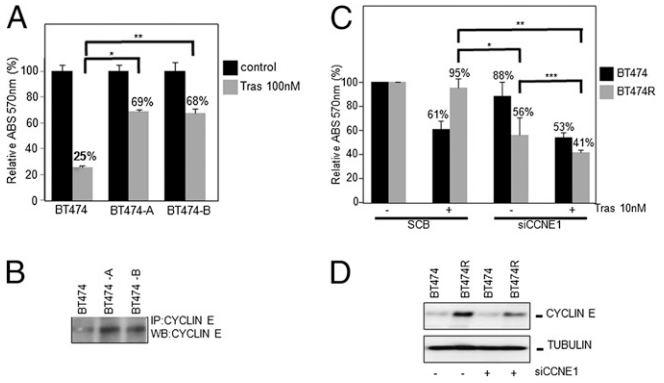

Fig. 3. BT474 and BT474R sensitivity to in vitro cyclin E manipulation. (*A*) Quantification of crystal violet staining of parental BT474 cells infected with empty vector control (BT474) compared with two independent pools stably expressing full-length cyclin E (BT474-A and BT474-B). Cells were treated for 8 d with 100 nM trastuzumab. Experiments were performed in triplicate. (Student's *t* test; $*P = 0.001$, $**P = 0.001$). (*B*) Western blot analyses showing cyclin E overexpression in BT474-A and BT474B. (*C*) Quantification of crystal violet staining of BT474 or BT474R cells upon siRNA-mediated knockdown of cyclin E (siCCNE1) or scrambled siRNA control (SCB). Cells were treated with 10 nM trastuzumab for 8 d. Experiments were performed in triplicate. (Student's *t* test; $*P = 0.013$, $**P = 0.0003$, $***P = 0.15$) (*D*) Western blot analyses of BT474 and BT474R cells transfected with siRNA-targeting cyclin E or control. Whole-cell extracts were probed with indicated antibodies.

cyclin E may therefore render cells independent from the cell cycle arrest effects of trastuzumab. As such, we reasoned that these cells were likely to be highly dependent on CDK2 activity. To stress this hypothesis, we used the selective CDK2 inhibitor CYC065 (a 2,6,9-trisubstituted purine analog; Cyclacel), an orally available ATP competitive inhibitor of CDK2 kinase activity. As expected, inhibition of CDK2 kinase activity with CYC065 resulted in a substantial decrease of cell growth/viability in the BT474 parental cell line. However, the cyclin E amplified BT474R cells showed an even higher sensitivity to the CDK2 inhibitor, the effects of which were only marginally improved by the addition of trastuzumab (Fig. 4*A*). The cell cycle profiles of cells treated with trastuzumab, CYC065, or the combination were analyzed 48 h after treatment using flow cytometry. As previously shown in Fig. 1*C*, trastuzumab had no effect on the cell cycle distribution of BT474R cells. However, treatment with CYC065 induced a marked G1 arrest, which was not further enhanced by cotreatment with trastuzumab. Importantly, in parental BT474 cells, CYC065 only induced a moderate G1 arrest, inferior to the effects obtained with trastuzumab alone (Fig. S4).

CDK2 inhibition was effective at reactivating RB function (decreasing protein phosphorylation) in both BT474 and BT474R cells (Fig. 4*B*). Phosphorylation of T187 on p27 by CDK2 results in ubiquitination and degradation of p27. Despite the expected decrease in T187 phosphorylation caused by the treatment with CYC065 (Fig. S5*A*), the total levels of p27 markedly decreased in both parental and resistant cell lines (Fig. 3*B*). This phenomenon appears to be, at least in part, proteasome dependent because coadministration of the proteosome inhibitor MG132 prevented p27 loss (Fig. S5*B*).

In addition to cell cycle arrest, treatment with CYC065 induced gross apoptosis in BT474R cells as determined by accumulation of cells in sub-G1 and annexin V staining (Fig. 3 *C* and *D*). In contrast, parental cells treated with CYC065 exhibited only a minor increase in sub-G1 and annexin V staining. In both sensitive and resistant cells, the addition of trastuzumab did not significantly increase cell death. This difference in susceptibility to apoptosis was confirmed by the levels of cleaved caspase 3, a known proapoptotic marker that significantly increased only in BT474R cells following exposure to CYC065 (Fig. 4*B*).

To explore the consistency of our findings in an in vivo model, we evaluated tumor growth inhibition of BT474R2-derived xenografts in response to trastuzumab, CYC065, or the combination (Fig. 4*E*). Therapy was started when tumors were well established (~300 mm^3). As a defining feature, BT474R2 xenografts responded poorly to trastuzumab treatment. Conversely, treatment with CYC065 alone or in combination with trastuzumab significantly decreased tumor growth. Histological analyses of CYC065-treated tumors showed reduced expression of phosphorylated RB compared with control and trastuzumab-

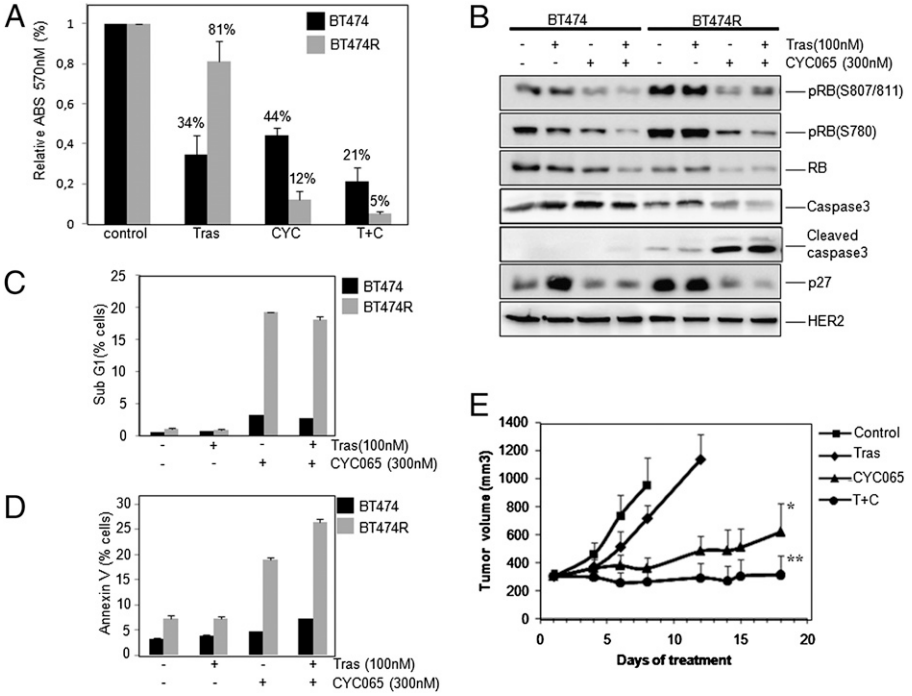

Fig. 4. BT474 and BT474R sensitivity to CDK2 inhibition. (*A*) Quantification of crystal violet staining of BT474 or BT474R cells treated for 8 d with trastuzumab, CYC065, or the combination (T+C) at the indicated concentrations. Proliferation assays were performed in triplicate. (*B*) Western blot analysis of BT474 parental and resistant cell lines exposed to trastuzumab, CYC065, or the combination for 48 h at the indicated concentrations. Whole-cell extracts were analyzed with the indicated antibodies. Similar results were obtained comparing BT474 with BT474R2. (*C*) Cell death of BT474 or BT474R cells as delineated by sub-G1 population following treatment of trastuzumab, CYC065, or the combination for 48 h at the indicated concentrations. Similar results were obtained comparing BT474 with BT474R2. (*D*) Cell death of BT474 or BT474R cells as delineated by annexin V staining following treatment of trastuzumab, CYC065, or the combination for 48 h at the indicated concentrations. Similar results were obtained comparing BT474 with BT474R2. (*E*) Tumor growth inhibition in response to trastuzumab, CYC065, and the combination of the two agents. Student's *t* test was used to compare tumor sizes between the groups and data are expressed as mean ± SE $*P = 0.0019$ vs. trastuzumab; $**P = 0.00085$ vs. trastuzumab on day 12. The experiment was repeated two times with similar results.

treated mice (Fig. S6), suggesting that this parameter could be used as a molecular biomarker of CDK2 inhibition in vivo.

Taken together these results demonstrate that our trastuzumab-resistant cells display exquisite sensitivity to CDK2 inhibitors, an indication of acquired dependency to the cyclin E–CDK2 signaling pathway.

Discussion

Primary and secondary resistance to anti-HER2 agents is a major limitation in the current treatment of patients with $HER2^+$ breast tumors. The identification of critical mechanisms of resistance to anti-HER2 therapies could lead to the design of successful strategies to circumvent them. Following this line of thought, we established trastuzumab-resistant cells by chronic exposure to trastuzumab in vitro (BT474R) and further subselection by prolonged trastuzumab exposure in vivo (BT474R2). This approach to develop resistant cells is remarkably similar to what occurs in the clinic, where acquired resistance arises frequently in the setting of prolonged administration of trastuzumab. Using an unbiased approach based on comparative genomewide copy-number analyses, we found that resistant cells had an amplified region in 19q where *CCNE1*, the cyclin E encoding gene, resides and confirmed that these cells had cyclin E amplification/overexppresion. Cyclin E and its associated CDK2 are essential for cellular progression through the G1 phase of the cell cycle and initiation of DNA replication. On the other hand, the antiproliferative effects of trastuzumab are mediated by accumulation of cells in the G1 phase, which can be accompanied by decreased cyclin E-associated kinase activity (39). An excess of cyclin E may therefore render cells independent from trastuzumab-mediated cell cycle arrest.

We provide evidence that overexpression of cyclin E and corresponding CDK2 activity is sufficient to counteract the antiproliferative effects of trastuzumab. Previous studies have also described an oncogenic role for cyclin E (40, 41). In fact, cyclin E and LMW cyclin E are frequently overexpressed in breast cancer, resulting in poor overall survival (21). Recently it has also been shown that cyclin E and LMW cyclin E overexpression confers a worse prognosis in patients with $HER2^+$ breast cancer that had not received therapy with trastuzumab (22).

Here, we demonstrate that cyclin E amplification/overexpression results in a decreased sensitivity to trastuzumab (Fig. 5). Thus, in addition to being a prognostic factor in $HER2^+$ breast cancer, cyclin E may also be a predictive factor of poor response to anti-HER2 therapy. We fully acknowledge the retrospective nature of our clinical findings and that our findings need to be prospectively validated in a larger cohort of patients.

This type of drug-mediated cell adaptation differs substantially from the previously described mechanisms of resistance to anti-HER agents. For example, in a similar study conducted in lung cancer, the amplification of MET protooncogene has been described to mediate resistance to anti-EGFR tyrosine kinase inhibitors. In this case, however, concurrent inhibition of both EGFR and MET upstream receptors was required to achieve down-regulation of the PI3K/AKT pathway and subsequent growth inhibition. In our case, loss of cyclin E–CDK2 activity alone, either by chemical inhibition of CDK2 or knockdown of cyclin E expression, dramatically suppressed the proliferation potential of trastuzumab-resistant cells compared with their parental counterparts. It is therefore tempting to speculate that chronic exposure to trastuzumab with consequent cyclin E amplification/overexpression renders resistant cells addicted to cyclin E.

Although a functional cyclin E–CDK2 activity appears to be critical for both parental and trastuzumab-resistant cells, its inhibition leads to different phenotypic outcomes. Whereas exposure to a CDK2 inhibitor moderately increased cell death in BT474 parental cells, identical treatment conditions resulted in massive apoptosis in cyclin E overexpressing cells. Thus, the enhanced cyclin E–CDK2 kinase activity present in our trastuzumab-resistant cells, witnessed by the higher basal phosphorylation of RB, may be essential for the suppression of proapoptotic signals. This finding opens up a window of opportunity because cyclin E amplified $HER2^+$ breast cancers refractory to trastuzumab may be exquisitely sensitive to CDK2 inhibition.

An open question is whether CDK2 inhibition should be given alone or in combination with trastuzumab in cyclin E amplified patients after relapse to first line trastuzumab-based therapy. Our in vitro data would suggest that the combination may not be necessary but we acknowledge that the dual approach may still benefit from the immune-mediated trastuzumab cytotoxicity in the clinical setting.

Intriguingly, we observed constitutively elevated p27 levels in our cyclin E amplified trastuzumab-resistant cell lines. These findings diverge with previous data originated from using pools of SKBR-3 cells with acquired resistance to trastuzumab in vitro (42). In this model, Nahta et al. showed increased CDK2 activity of the resistant cells but accompanied by decreased levels of p27. This discrepancy may be explained, at least in part, by the substantial differences existing between their and our models. Nahta et al. (42) used a different cell line and exposed it to trastuzumab for a shorter period (3 mo) before selecting and characterizing the resistant pools. Moreover, trastuzumab treatment was cytotoxic in their parental SKBR-3 cells, whereas, in our parental BT474 cells, trastuzumab was mainly cytostatic.

In an earlier work, trastuzumab-induced p27 up-regulation was found to be long lasting and irreversible (43). Maybe, in our case, the protracted exposure to trastuzumab led to increased steady-state levels of p27 that, over time, acted as a selection pressure for cyclin E overexpressing cells. On the other hand, the high levels of p27 encountered in our resistant cells could occur as a consequence of a yet undefined adaptative response to abnormally high CDK2 activity. In favor of the latter is a previous report that described increased p27 levels in transgenic mice overexpressing cyclin E (44).

Pharmacological inhibition of CDK2 was found to trigger p27 down-regulation in our model. This finding is surprising, given that p27 degradation is known to be mediated by CDK2 kinase activity (45). However, these findings are consistent with previous work describing p27 down-regulation in response to the CDK inhibitor roscovitine (46). Although the mechanistic details of this paradox need further biochemical characterization, we found that inhibition of CDK2 affects proteasome-dependent p27 stability.

In conclusion, our work proposes that cyclin E amplification/overexpression leads to trastuzumab resistance. These tumors

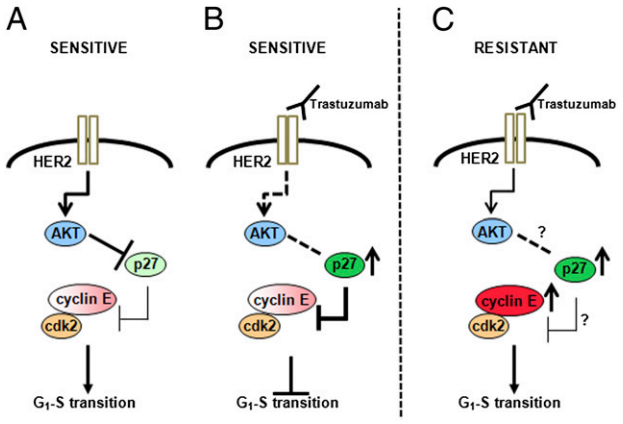

Fig. 5. (*A*) In unperturbed conditions HER2-dependent activation of AKT inhibits the cyclin E/CDK2 inhibitor p27. (*B*) Inhibition of HER2 suppresses AKT activity leading to increased levels of p27 and inhibition of the cyclin E/Cdk2 activity. (*C*) Trastuzumab-resistant cells harboring an amplification of cyclin E renders these cells insensitive to the negative regulation by p27.

may be exquisitely sensitive to CDK2 inhibitors, an observation that could have therapeutic implications.

Materials and Methods

Cell Culture, Transfections, and Proliferation Analyses. BT474 cells were obtained from ATCC. Cells were maintained in Dulbecco's modified Eagle medium/Ham F12 1:1 (DMEM/F12) supplemented with 10% FBS and 2 mM L-glutamine (Life Technologies) at 37 °C in 5% CO_2. Trastuzumab-resistant BT474R and BT474R2 cells were maintained in 1 μM of trastuzumab. Cells were infected as previously described (47). Trastuzumab (Herceptin; kindly provided by F. Hoffmann-La Roche, Basel, Switzerland) was dissolved in sterile apyrogen water and stored at 4 °C. CYC065 (Cyclacel) was dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C.

Cell growth and cell cycle analyses were performed as previously described (16, 47).

Protein Extraction and Western Blot. Proteins were extracted with 20 mM Tris-HCl pH 7.4, 137 NaCl, 2 mM EDTA, 10% glycerol, 1% Nonidet P-40 supplemented with 25 mM NaF, 50 μg/mL leupeptin, 50 μg/mL aprotinin, 0.5 mM orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. Protein extracts were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Protein detection was performed as previously described (11).

Reagents, SNP Array, Tumor Xenografts, Immunohistochemical (IHC) Staining and Evaluation, Fluorescence in Situ Hybridization and Interpretation, and Patients. See *SI Materials and Methods*.

Statistical Analysis. Associations between CBR and cyclin E status in $HER2^+$ breast cancer patients were studied by contingency tables and analyzed by Fisher's exact test. Results were considered to be statistically significant when P value was <0.05. The Breslow test in Kaplan–Meier survival analysis was performed because our data did not fulfill the proportional hazards assumption. All statistical analyses were performed using the SPSS 15.0 statistical software. For in vitro assays and nude mice experiments, comparisons between groups were made using a two-tailed Student's t test. Differences for which P was <0.05 were considered statistically significant.

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