# original article

# Association between gain-of-function mutations in *PIK3CA* and resistance to HER2-targeted agents in *HER2*-amplified breast cancer cell lines

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**Background:** The mechanism of resistance to human epidermal growth factor receptor 2 (HER2)-targeted agents has not been fully understood. We investigated the influence of *PIK3CA* mutations on sensitivity to HER2-targeted agents in naturally derived breast cancer cells.

**Materials and methods:** We examined the effects of Calbiochem (CL)-387,785, HER2 tyrosine kinase inhibitor, and trastuzumab on cell growth and *HER2* signaling in eight breast cancer cell lines showing *HER2* amplification and trastuzumab-conditioned BT474 (BT474-TR).

**Results:** Four cell lines with *PIK3CA* mutations (E545K and H1047R) were more resistant to trastuzumab than the remaining four without mutations (mean percentage of control with 10 µg/ml trastuzumab: 58% versus 92%; $P = 0.010$). While *PIK3CA*-mutant cells were more resistant to CL-387,785 than *PIK3CA*-wild-type cells (mean percentage of control with 1 µM CL-387,785: 21% versus 77%; $P = 0.001$), CL-387,785 retained activity against BT474-TR. Growth inhibition by trastuzumab and CL-387,785 was more closely correlated with changes in phosphorylation of S6K (correlation coefficient, 0.811) than those of HER2, Akt, or ERK1/2. Growth of most *HER2*-amplified cells was inhibited by LY294002, regardless of *PIK3CA* genotype.

**Conclusions:** *PIK3CA* mutations are associated with resistance to HER2-targeted agents. PI3K inhibitors are potentially effective in overcoming trastuzumab resistance caused by *PIK3CA* mutations. S6K phosphorylation is a possibly useful pharmacodynamic marker in HER2-targeted therapy.

**Key words:** breast cancer, HER2, *PIK3CA*, trastuzumab

## introduction

Breast cancer is the leading cause of cancer death among women worldwide, with ∼1 million new cases reported each year [1, 2]. Approximately 20% of breast cancer tumors show overexpression of the HER2 protein, which is mainly caused by gene amplification. HER2 overexpression has been repeatedly identified as a poor prognostic factor [3, 4]. Trastuzumab is a humanized mAb targeting the extracellular domain of the HER2 protein. From the late 1990s, clinical studies have intensively evaluated the therapeutic roles of trastuzumab. For the treatment of HER2-overexpressing metastatic breast cancers, studies report that a combination of trastuzumab and conventional chemotherapy shows significantly higher efficacy than chemotherapy alone [5]. The use of trastuzumab has extended to the treatment of operable HER2-overexpressing breast cancer as an adjuvant or neoadjuvant [6–8]. Despite promising usefulness in clinics, a modest percentage of patients

are reported to benefit from trastuzumab therapy, with response rates to trastuzumab as a single agent of ∼20% [9]. In addition, even when trastuzumab therapy leads to temporary tumor shrinkage, clinical relapse is observed for the vast majority of metastatic patients. To develop adequate therapies capable of overcoming primary and secondary resistance to trastuzumab, a better understanding of the resistance mechanism is crucial.

To date, several mechanisms of primary resistance to trastuzumab have been proposed. A series of studies indicated that trastuzumab resistance is due to the truncated form of HER2, which lacks an extracellular domain to which trastuzumab is indicated to attach [10, 11]. Nagata et al. [12] demonstrated that loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a negative regulator of PI3K, correlates with poor response to trastuzumab. More recently, the roles of *PIK3CA* in trastuzumab resistance have been under particular investigation. Somatic mutations of *PIK3CA* were first identified in 2004 in various malignant tumors including breast cancer [13]. Subsequent studies have reported that the E545K and H1047R hotspot mutations, found

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on exons 9 and 20, respectively, are the most frequent types of mutation, found in 8%–40% of breast cancer tumors [13–16]. Both hotspot mutations are gain-of-function mutations which transform normal mammary epithelial cells [17, 18]. Berns et al. [19] investigated the roles of gain-of-function mutations of the *PIK3CA* gene in trastuzumab resistance by transfecting wild-type and mutant (H1047R) forms of *PIK3CA* in SKBR-3 HER2-overexpressing breast cancer cells. Results showed that compared with green fluorescent protein (GFP) control, both wild-type and mutant *PIK3CA* transfections resulted in trastuzumab resistance. Further, analysis of *PIK3CA* genotypes in tumor samples obtained from breast cancer patients having undergone trastuzumab-based therapy showed an association between the presence of *PIK3CA* hotspot mutations and shorter time to progression after therapy [19].

Tyrosine kinase inhibitors (TKIs) have also been investigated as potential agents against trastuzumab resistance [20]. A clinical study in metastatic breast cancer patients having previously experienced tumor progression under trastuzumab-based therapies showed that compared with capecitabine alone, treatment using a combination of capecitabine with lapatinib, a dual inhibitor of epidermal growth factor receptor (EGFR) and HER2 tyrosine kinase, lead to significantly longer time to progression [21]. Eichhorn et al. [22], however, demonstrated that transfection of mutant *PIK3CA* (H1047R) in BT474 HER2-overexpressing breast cancer cells resulted in resistance to lapatinib compared with parental cells. Further, results showed that resistance was overcome using NVP-BEZ235, a PI3K and mammalian target of rapamycin dual inhibitor [22].

These findings based on gene manipulations indicate that gain-of-function mutations in the *PIK3CA* gene lead to resistance to trastuzumab, as well as HER2-TKI. To our knowledge, however, these findings have not been confirmed in naturally derived breast cancer cells. Here, trastuzumab resistance due to *PIK3CA* mutations was evaluated in eight naturally derived breast cancer cell lines harboring *HER2* gene amplification. Further, possible therapeutic means to overcome primary and secondary resistance to trastuzumab were investigated, as well as potential pharmacodynamic markers correlated with the growth-inhibitory effect of HER2-targeted drugs.

## materials and methods

### cell culture

MCF-7, MDA-MB-361, HCC1954, MDA-MB-453, UACC893, CAMA-1, MDA-MB-435S, MDA-MB-415, ZR75-30, HCC70, MDA-MB-468, and HCC1419 cell lines were purchased from the American Type Culture Collection (Manassas, VA). BT474, SKBR-3, BT549, T47D, ZR75-1, and MDA-MB-231 cells were kindly provided by Ian Krop of the Dana-Farber Cancer Institute. Of the 18 breast cancer cell lines, eight (ZR75-30, BT474, SKBR-3, HCC1419, MDA-MB-453, MDA-MB-361, HCC1954, and UACC 893) were reported to have *HER2* gene amplification [23], with levels of PTEN protein expression equivalent to those reported in our previous study [24]. Among the *HER2*-amplified cell lines, ZR75-30, SKBR-3, and HCC1419 were reported to contain the wild-type *PIK3CA* gene and MDA-MB-453, MDA-MB-361, HCC1954, and UACC893 hotspot *PIK3CA* mutations (Table 1) [14]. BT474 was reported to contain a relatively rare type of *PIK3CA* mutation at exon 2, K111N (Table 1) [14]. MDA-MB-435S, MDA-MB-468, and MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle's Medium (Cellgro; Mediatech, Inc., Herndon, CA) with

**Table 1.** Genotype of *PIK3CA* in *HER2*-amplified breast cancer cell lines

| Cell line | Genotype of *PIK3CA* |
|---|---|
| BT474 | K111N |
| ZR75-30 | wt |
| SKBR-3 | wt |
| HCC1419 | wt |
| MDA-MB-361 | E545K |
| MDA-MB-453 | H1047R |
| HCC1954 | H1047R |
| UACC893 | H1047R |

wt, wild-type.

10% fetal bovine serum (FBS) (Gemini Bio-Products, Inc., Woodland, CA), 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM glutamine. The remaining cell lines were maintained in RPMI-1640 medium (Cellgro; Mediatech, Inc.) supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM glutamine. All cells were grown at 37°C in a humidified atmosphere with 5% $CO_2$ and were in logarithmic growth phase at initiation of the experiments.

### drugs

Trastuzumab was obtained from the Kobe University Hospital pharmacy. CL-387,785, a dual inhibitor of EGFR and HER2 [25], and LY294002, a PI3K inhibitor, were purchased from Calbiochem (San Diego, CA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. Before each experiment, drugs were diluted in fresh media. The final DMSO concentration was <0.1% for all experiments.

### antibodies and western blotting

Cells were washed with ice-cold phosphate-buffered saline and scraped immediately after adding lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 2 mM EDTA] containing protease and phosphatase inhibitors (100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM $Na_3VO_4$, 2 µg/ml aprotinin, and 5 µg/ml leupeptin). Lysates were centrifuged at 14 000 relative centrifugal force for 10 min. Supernatants were collected as protein extract and then separated by electrophoresis on 7.6% polyacrylamide–sodium dodecyl sulfate gels, followed by transfer to nitrocellulose membranes (Millipore Corporate Headquarters, Billerica, MA) and detection by immunoblotting using an enhanced chemiluminescence system (New England Nuclear Life Science Products, Inc., Boston, MA). The resulting signals were digitally quantified using the ImageJ software (www.nih.gov). Phospho-HER2/ErbB2 (Thr1221/1222), phospho-p70 S6 Kinase (Thr389), phospho-Akt (Ser473)(D9E), and PathScan(R) Multiplex Western Cocktail I were purchased from Cell Signaling Technology (Beverly, MA). The phospho-1/2 (pT185/pY187) antibody was purchased from Biosource International Inc. (Camarillo, CA), the c-erbb-2 antibody from Chemicon (Billerica, MA), and β-actin antibody from Sigma-Aldrich (St Louis, MO).

### cell growth assay

Growth inhibition was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI), a colorimetric method for determining the number of viable cells based on the bioreduction of MTS to a soluble formazan product, which is detectable by spectrophotometry at a wavelength of 490 nm. Cells were diluted in 160 µl/well of maintenance cell culture media and plated in 96-well flat-bottom plates (Corning, Inc., Corning, NY). After a 96-h growth period, the number of cells required to obtain an absorbance of 1.3–2.2, the linear range of the assay, was

determined for each cell line beforehand. The number of cells per well used in the subsequent experiments were as follows: MCF-7, 2000; MDA-MB-361, 8000; HCC1954, 2500; MDA-MB-453, 7000; UACC893, 7500; CAMA-1, 6000; MDA-MB-435S, 2000; ZR75-30, 7500; HCC70, 4000; HCC1419, 8000; BT474, 3000; SKBR-3, 2500; BT549, 2000; T47D, 2500; ZR75-1, 7500; MDA-MB-415, 5000; and MDA-MB-231, 2500. At 24 h after plating, cell culture media were replaced with 10% FBS-containing media with and without trastuzumab or CL-387,785, followed by incubation for an additional 120 h. Trastuzumab and CL-387,785 concentrations ranged from 33 ng/ml to 100 µg/ml and from 3.3 nM to 10 µM, respectively. A total of 6–12 plate wells were set for each experimental point, and all experiments were carried out at least in triplicate. Data are expressed as percentage of growth relative to that of untreated control cells.

### generation of *in vitro* BT474-TR

To generate a cell line resistant to trastuzumab, BT474 cells were continuously exposed to 100 µg/ml trastuzumab. To confirm the emergence of resistant clones, MTS assays were carried out every five passages after allowing cells to grow in drug-free conditions for at least 4 days. After 11 months of drug exposure, cells showed sufficient resistance (Figure 1) and were designated as BT474-TR. For controls, BT474 parental cells were concomitantly maintained without trastuzumab, and drug sensitivity was compared with trastuzumab-conditioned cells. No significant change in the sensitivity to trastuzumab was observed in parental cells during the drug-exposure period (data not shown).

## results

### inhibitory effect of trastuzumab on growth in breast cancer cell lines

We first screened 17 breast cancer cell lines for *in vitro* growth inhibition using trastuzumab. We confirmed that all relatively sensitive cell lines were *HER2*-amplified (Figure 2A). Among eight *HER2*-amplified cell lines, those with hotspot mutations in *PIK3CA* appeared resistant compared with the remaining cell
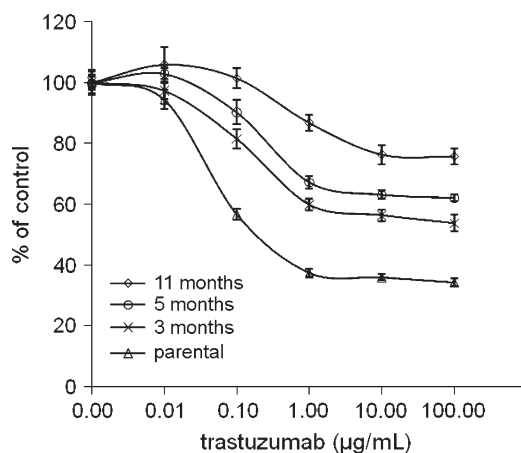


Figure 1. Development of BT474-TR. BT474 cells were continuously exposed to 100 µg/ml trastuzumab. BT-474 and trastuzumab-conditioned BT474 cells were grown in 10% serum-containing media for 5 days in the presence of various concentrations of trastuzumab. The percentage of viable cells is shown relative to that of the untreated control and plotted on the *y*-axis, whereas trastuzumab concentrations are plotted on the *x*-axis. Each data point represents the mean value and standard deviation of 6–12 replicate wells. Trastuzumab resistance increased in cells in a time-dependent manner. After 11 months, cells were designated as BT474-TR.

lines (Figure 2B and C). We categorized BT474 as a *PIK3CA*-wild-type cell line in this study, based on reports showing that the K111N mutation lack ability of transformation and its influence on downstream signaling is negligible [18, 26]. A significant difference in sensitivity at 10 µg/ml trastuzumab was observed between *PIK3CA*-wild-type and -mutant cells (Figure 2C; $P = 0.010$). Protein expression levels of p110-α, the product of *PIK3CA*, were not correlated with sensitivity to trastuzumab (Figure 2C).

### association between *PIK3CA* mutations and HER2-TKI resistance

Lapatinib, a HER2-TKI which may potentially overcome trastuzumab resistance, has been used in clinical settings [21]. We therefore tested a commercially available HER2-TKI, CL-387,785 [25], on *HER2*-amplified breast cancer cells. As shown in Figure 2D, cell lines with hotspot *PIK3CA* mutations showed resistance to CL-387,785. A statistically significant difference in sensitivity to 1 µM CL-387,785 was observed between *PIK3CA*-wild-type and -mutant cells (Figure 2C; $P = 0.001$) [24].

We then established a trastuzumab-resistant BT474 cell line (BT474-TR), a model of secondary resistant cells, by continuous exposure to trastuzumab (see 'Materials and methods' section). In contrast to *PIK3CA*-mutant cells, which showed primary resistance to trastuzumab, BT474-TR cells remained sensitive to CL-387,785 (Figure 3), which indicates that secondary resistant cells maintain dependency on HER2 signaling for growth.

### association between phosphorylation change in S6K and growth inhibition by HER2-targeted agents

To identify potential pharmacodynamic markers of sensitivity to HER2-targeted therapy, we examined changes in phosphorylation of HER2 and representative downstream signaling molecules in 10% FBS-containing media with or without 10 µg/ml trastuzumab or 1 µM CL-387,785 (Figure 4A). The trastuzumab concentration was selected based on maintained growth inhibition (Figure 2B) and wide use in previous studies [11, 19]. The 1-µM CL-387,785 concentration was selected based on the approximate maximum plasma concentration of most TKIs available in clinics to date, including lapatinib [27], and use in previous studies [28, 29].

Trastuzumab treatment resulted in moderate phosphorylation inhibition of Akt and/or S6K in cell lines with wild-type *PIK3CA*. In contrast, no significant changes in Akt and S6K phosphorylation were observed in cell lines with hotspot mutant *PIK3CA*, as well as in BT474-TR cells. Although in ZR75-30, trastuzumab treatment appeared to inhibit phospho-ERK1/2, no significant changes were observed in other sensitive cells, namely BT474 and SKBR-3 (Figure 4A). In addition, phospho-ERK1/2 levels increased in MDA-MB-361 and UACC893, which indicates the presence of compensational cell signaling. Further, with the exception of HCC1419, treatment with CL-387,785 resulted in significant inhibition of Akt and S6K phosphorylation in BT474-TR and *PIK3CA*-wild-type cells, whereas residual phosphorylation signals were observed in all *PIK3CA* hotspot mutant cells.
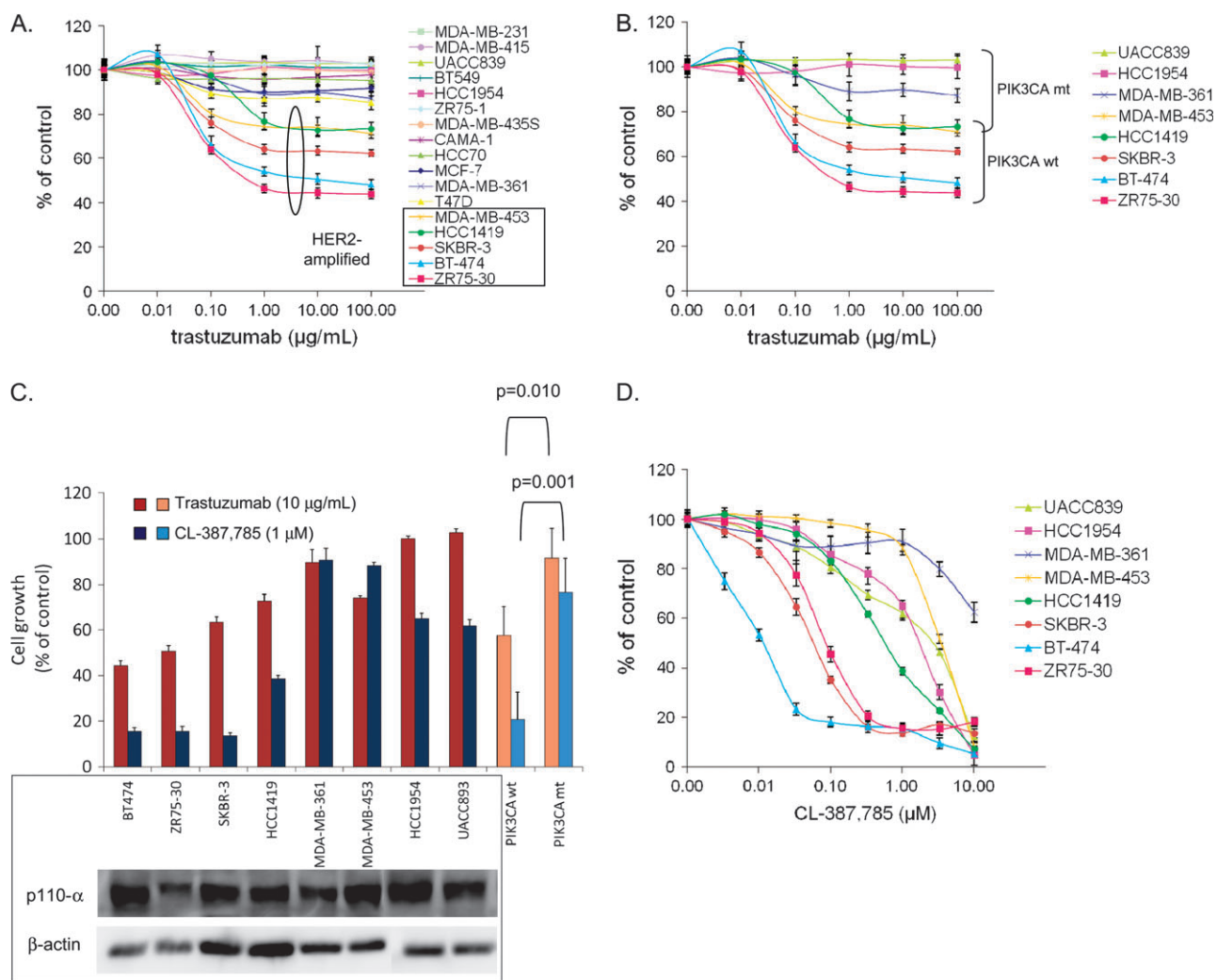
**Figure 2.** Effect of trastuzumab and CL-387,785 on growth inhibition in breast cancer cells *in vitro* [(**A**) trastuzumab on 17 breast cancer cell lines; (**B**) and (**D**) trastuzumab and CL-387,785 on eight *HER2*-amplified cell lines, respectively]. Breast cancer cells were grown in 10% serum-containing media for 5 days in the presence of various concentrations of trastuzumab (**A** and **B**) or CL-387,785 (**D**). The percentage of viable cells is shown relative to that of the untreated control and plotted on the *y*-axis, whereas trastuzumab and CL-387,785 concentrations are plotted on the *x*-axis. Each data point represents the mean value and standard deviation of 6–12 replicate wells. (**C**, top) Mean percentage of control and standard deviation of 6–12 replicate wells treated with 10 μg/ml trastuzumab and 1 μM CL-387,785, as well as those of *PIK3CA*-wild-type and -mutant cell lines (bottom), were plotted. (**C**, bottom) Protein expression of p110-α in *HER2*-amplified breast cancer cells. Blots were stripped and re-probed for β-actin as loading control.

Phosphorylation signals were then quantified and correlated with growth inhibition caused by trastuzumab and CL-387,785. As shown in Figure 4B, the closest association was observed between phospho-S6K changes and growth inhibition caused by trastuzumab and CL-387,785 [correlation coefficient (*r*), 0.811]. Further, close associations between phospho-S6K and cell growth were consistent when analyzed for trastuzumab and CL-387,785 separately (*r* for phospho-S6K versus growth: 0.8487 and 0.6970 for trastuzumab and CL-387,785, respectively).

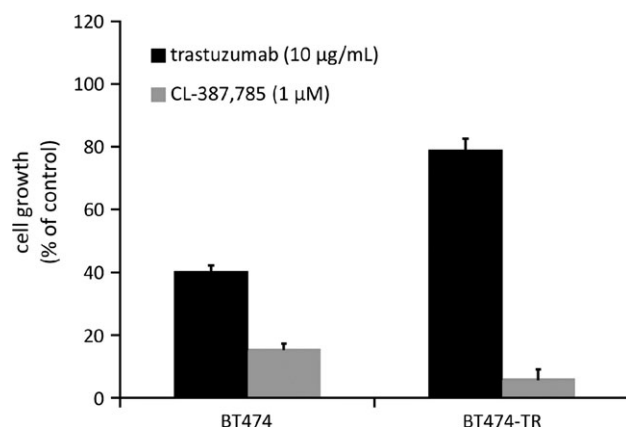## dependency of *HER2*-amplified breast cancer cells on PI3K pathway

Given that inhibition of the PI3K pathway is critical in distinguishing cells sensitive from resistant to HER2-targeted agents (Figure 4B), we evaluated cell lines for the effects of

LY294002, a PI3K inhibitor. As shown in Figure 5A, with the exception of ZR75-30, LY294002 induced a >30% growth inhibition compared with control in all cell lines. No significant difference in LY294002 sensitivity was observed between *PIK3CA*-mutant and -wild-type cell lines (Figure 5; $P = 0.655$). These results indicate that most *HER2*-amplified cells at least partly depend on the PI3K pathway regardless of the presence or absence of *PIK3CA* hotspot mutations.

To further gain insight into this concept, we evaluated phosphorylation levels of Akt and ERK1/2 in protein extracts obtained from cells under serum-starved conditions for 24 h. As shown in Figure 5B, despite the absence of serum factors, all *HER2*-amplified breast cancer cells showed a high level of phospho-Akt, regardless of *PIK3CA* genotype. High levels of phospho-Akt were also observed in MDA-MB-468, which lacks PTEN [30], and T47D, which harbors a *PIK3CA* mutation

(H1047R) [14]. These two cell lines do not show *HER2* amplification [23]. In contrast, no significant levels of phospho-Akt were observed in MDA-MB-231 and MDA-MB-435S, which show no *HER2* amplification, *PIK3CA* mutation, or PTEN loss [14, 23]. Further, with the exception of MDA-

MB-231, all cell lines showed very low levels of phospho-ERK1/2 under serum-starved conditions. MDA-MB-231, in particular, was reported to contain double activating mutations in KRAS (G13D) and BRAF (G464V), whereas MDA-MB-435S showed an activating mutation in BRAF alone (V600E) [31]. These findings further support the concept that *HER2*-amplified cells tend to have HER2-PI3K signaling axis and they are thus dependent on the PI3K pathway rather than on extracellular signal-regulated kinase pathway.

## discussion

In this study, we show that gain-of-function mutations in *PIK3CA* genes are associated with trastuzumab resistance in naturally derived breast cancer cell lines showing *HER2* amplification. This finding is consistent with a recent study by Berns et al. [19] reporting trastuzumab resistance in SKBR-3 cells transfected with mutant *PIK3CA* (H1047R) compared with GFP control. Transfection of wild-type *PIK3CA*, however, appeared to equally cause trastuzumab resistance [19]. This observation does not identify either quantitative or qualitative changes in *PIK3CA* mutation as the major factor in developing trastuzumab resistance. In the present study, no clear association was observed between *PIK3CA* protein (p110-α) expression levels and *in vitro* sensitivity to trastuzumab
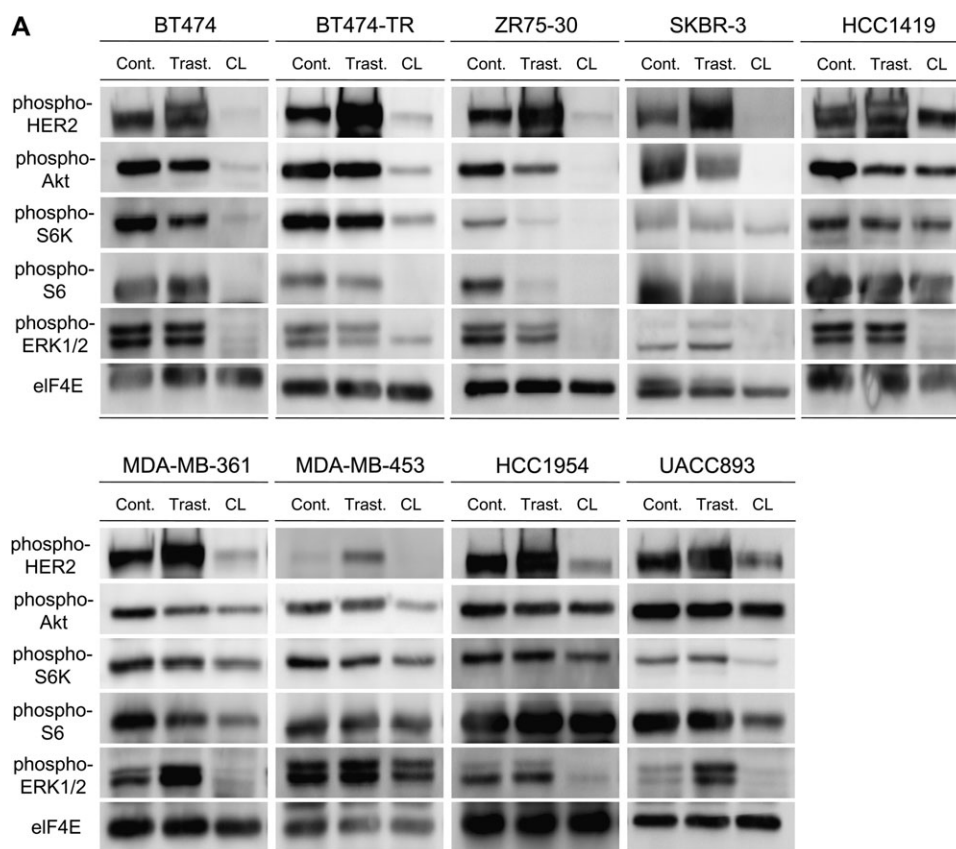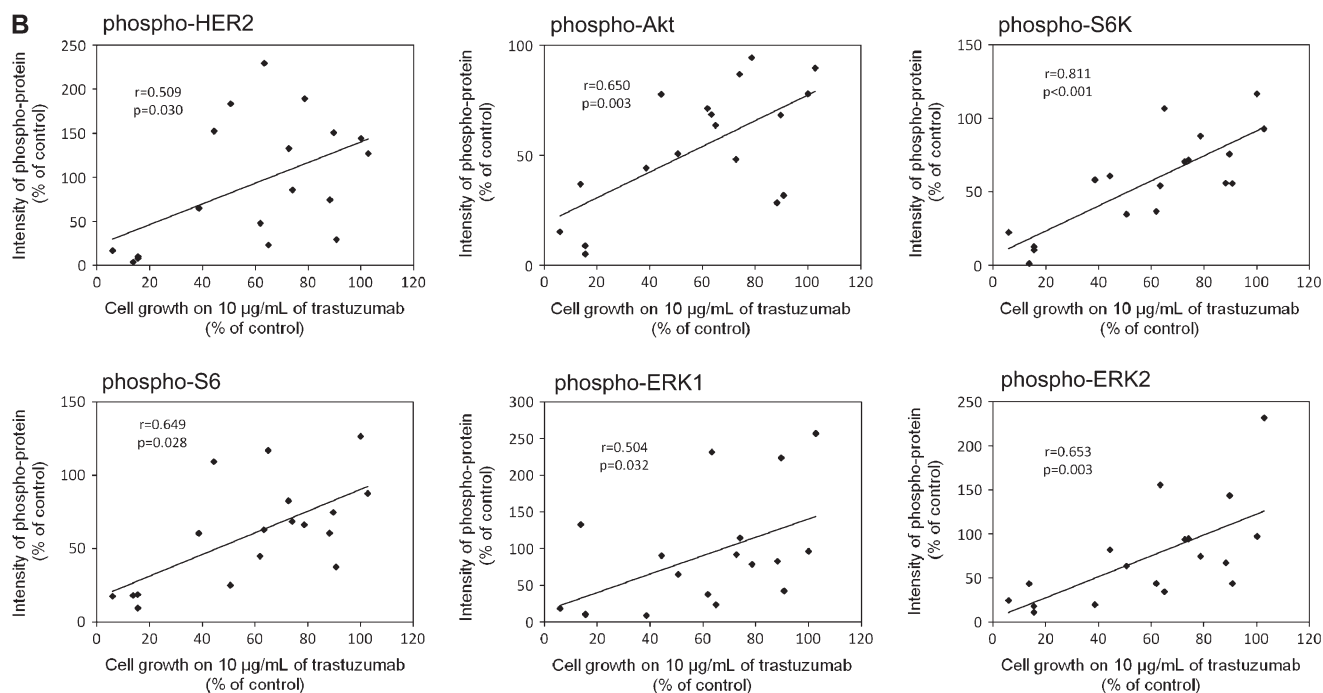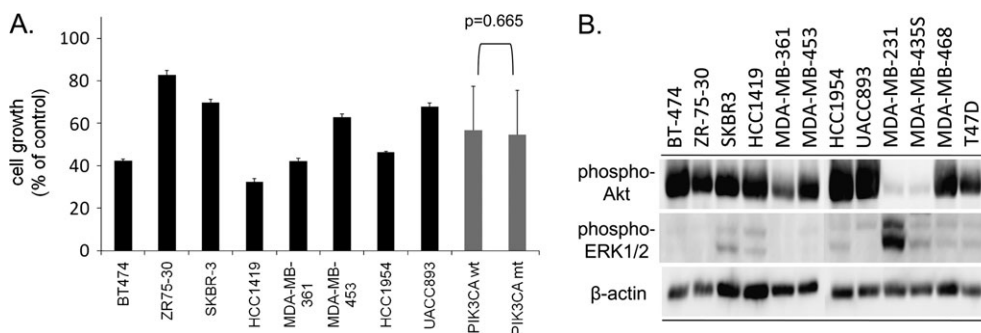


**Figure 3.** Effect of trastuzumab and CL-387,785 on growth inhibition in BT-474 and BT474-TR cells. Mean percentage of control and standard deviation of 6–12 replicate wells treated with 10 µg/ml trastuzumab and 1 µM CL-387,785 were plotted. BT474-TR remains sensitive to CL-387,785.



**Figure 4.** (**A**) Expression of phosphorylated-HER2, -Akt, -S6K, -S6 and -ERK1/2 in HER2-amplified breast cell lines with and without treatment with trastuzumab (10 µg/ml) and CL-387,785 (1 µM). Breast cell lines grown in 10% serum-containing media were lysed and immunoblotted for each protein. Blots were stripped and re-probed for eIF4E as loading control.

**Figure 4.** (Continued) (**B**) Correlation between changes in phosphorylation of HER2 signaling molecules and cell growth. Immunoblot quantification was carried out by densitometry using ImageJ software. Correlations were analyzed by calculating Pearson's correlation coefficient.



**Figure 5.** (**A**) Effect of LY294002 on growth inhibition in HER2-amplified breast cancer cell lines. Mean percentage of control and standard deviation of 6–12 replicate wells treated with 10 µM LY294002 were plotted. (**B**) Protein expression of phospho-Akt and phospho-ERK1/2 in HER2-amplified breast cancer cells under serum-starved condition. Blots were stripped and re-probed for β-actin as loading control.

(Figure 2C). A study by Haverty et al. [32], which analyzed copy number alterations in 51 breast tumors using a high-resolution single nucleotide polymorphism array, showed no gain in copy number on chromosome 3p, the location of the *PIK3CA* gene. These results indicate that qualitative changes in the *PIK3CA* gene itself may cause trastuzumab resistance in naturally derived breast cancer cells.

The CL-387,785 HER2-TKI was first evaluated to identify groups of compounds which may overcome trastuzumab resistance. Of note, results show an association between *PIK3CA* hotspot mutations and CL-387,785 resistance. Further, the difference in sensitivity between *PIK3CA*-wild-type and -mutant cell lines was more significant for CL-387,785 than for trastuzumab (Figure 2C). These results are consistent with a recent study by Eichhorn et al. [22], which showed that transfection of mutant *PIK3CA* (H1047R) in BT474 cells, which are sensitive to lapatinib, results in drug resistance. In contrast,

the results of the present study show that BT474-TR cells remain highly sensitive to CL-387,785, which is consistent with a previous study by Konecny et al. [20] which reported that lapatinib remains active against cell lines selected by long-term exposure to trastuzumab. Although the study did not show the effect of lapatinib on cell signaling in secondary resistant cells, our present findings indicate that BT474-TR remains dependent on HER2/PI3K signaling and sensitive to HER2-TKI (Figure 4A).

We then evaluated LY294002 as a model PI3K inhibitor. Results show that *HER2*-amplified cells are generally sensitive to LY294002 regardless of *PIK3CA* genotype (Figure 5A), which indicates that *HER2* amplification is associated with dependency on PI3K pathway. Supporting this notion, all *HER2*-amplified breast cancer cells have high level of phosphorylation of Akt even in serum-starved condition. The Akt phosphorylation levels observed in *HER2*-amplified cells

were equivalent to those in MDA-MB-468 and T47D cells, which were reported to contain PTEN loss and a *PIK3CA* hotspot mutation without *HER2* amplification, respectively [23]. These findings therefore indicated that *HER2* amplification itself may have equivalent biological effect on PI3K signaling with PTEN loss or *PIK3CA* hotspot mutation. In addition, our results are consistent with a recent study by Oda et al. [33], in which they showed that HER2 and/or HER3 overexpression, PTEN, or *PIK3CA* mutations occur almost exclusively in breast and other cancer cell lines.

Findings in past and present studies may potentially lead to beneficial clinical applications. For *HER2*-amplified breast cancer showing no *PIK3CA* mutations, trastuzumab is likely to be effective, with possible rescue using HER2-TKIs in cases of relapse. For *HER2*-amplified breast cancer with *PIK3CA* mutations, inhibitors against molecules of the PI3K pathway are possibly more effective than anti-HER2 agents, which are unlikely to be beneficial.

In addition to pharmacogenetic approaches, including *PIK3CA* genotyping, pharmacodynamic markers are potentially powerful tools in individualized use of molecularly targeted therapy. In a number of previous pharmacodynamic studies on HER2- or EGFR-targeted therapy, phospho-Akt was used as a surrogate marker for PI3K pathway activity [34, 35]. In the present study, however, growth inhibition is more closely associated with changes in phospho-S6K than that in phospho-Akt. These findings indicate that the prediction of tumor response to trastuzumab may strongly benefit from measurements of S6K phosphorylation levels. The cause of the discrepancy between the association of cell growth with phospho-Akt and that with phospho-S6K, however, remains unclear. It may be due to the difference in sensitivity of phospho-specific antibodies used in the present study or the higher sensitivity of phospho-Akt to positive feedback signals following initial inhibition of the PI3K pathway compared with phospho-S6K.

The present study shows several limitations. First, although a relatively large panel of *HER2*-amplified breast cancer cell lines ($N = 8$) were used, the properties of all HER2-overexpressing breast tumors are not necessarily represented. Despite HER2 amplification being retained, particular tumor subtypes may have been selected in the establishment of cell lines. Secondly, in addition to inhibition of HER2 signaling, a few studies have indicated the contribution of antigen-dependent cellular cytotoxicity (ADCC) in the antitumor effect of trastuzumab. Because ADCC only works in *in vivo* conditions, our current data do not necessarily deny the potential effect of trastuzumab on tumors showing *PIK3CA* mutations [36]. Thirdly, although wild-type *PIK3CA* appeared necessary for trastuzumab sensitivity *in vitro*, other factors may be involved, as shown by results showing moderate resistance of HCC1419 to trastuzumab (Figure 2C). The mechanisms of *PIK3CA*-unrelated resistance remain unknown but are under current investigation in our laboratory.

In conclusion, our findings show an association between the presence of *PIK3CA* hotspot mutations and resistance to not only trastuzumab but also HER2-TKI in naturally derived *HER2*-amplified breast cancer cell lines. Further, PI3K inhibitors are potentially effective in overcoming trastuzumab resistance caused by *PIK3CA* mutations. Assessment of S6K

phosphorylation levels may be a useful pharmacodynamic marker correlated to the antitumor effect of HER2-targeted therapy. A better understanding of these findings, however, may require further investigation in clinical trials and concomitant translational studies.

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