FOXO1A Is a Target for HER2-Overexpressing Breast Tumors

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Abstract

Trastuzumab treatment has improved the overall survival of HER2-overexpressing breast cancer patients. However, many of these patients will eventually become resistant to treatment. The mechanisms that contribute to resistance to trastuzumab are unknown. In this study, we tested the hypothesis that targeting of the FKHR transcription factor FOXO1A in HER2-overexpressing breast tumor cells can overcome the trastuzumab resistance in vitro. We have shown that overexpression of HER2 leads to activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway and subsequent inactivation of FOXO1A in HER2-overexpressing breast cancer cells SKBR3, BT474, and MCF7-HER2. In wild-type SKBR3 and BT474 cells, trastuzumab downregulates active Akt and increases FOXO1A expression that leads to increase in p27\(^{kip1}\) and decrease in cyclin D1 and finally inhibits cell proliferation. In contrast, the effect of trastuzumab was eliminated by the reduction of FOXO1A in HER2-overexpressing cells with constitutively active Akt1 (SKBR3/AA28 and BT474/AA9). The downregulation of FOXO1A resulted in nuclear export of p27\(^{kip1}\). Blocking the constitutively active Akt by a specific Akt/protein kinase B signaling inhibitor-2 (API-2) significantly increased FOXO1A expression and rendered the cells more responsive to trastuzumab-induced growth inhibition. Reactivation of FOXO1A by stable or transient transfection also restored the growth-inhibitory effects of trastuzumab in SKBR3/AA28, BT474/AA9, and MCF7-HER2 cells. Knocking down FOXO1A by small interfering RNA resulted in reducing trastuzumab-induced growth inhibition. In summary, trastuzumab can inhibit proliferation of HER2-overexpressing breast cancer cells by reactivating FOXO1A through inhibition of the PI3K/Akt pathway. FOXO1A may therefore serve as a target for HER2-overexpressing breast tumors. Cancer Res 70(13): OF1–11. ©2010 AACR.

Introduction

Overexpression of HER2 has been shown in 20% to 30% of patients with breast cancer. The overall survival and the time to relapse for patients whose tumors overexpress HER2 are significantly shorter (1, 2). The malignant phenotypes are also enhanced with HER2 overexpression (3, 4). Clinical and translational studies from our own laboratory and others have shown that an increased level of plasma HER2 in breast cancer patients is associated with poor outcome and reduction in disease-free survival (5). HER2-overexpressing tumors are more likely to be resistant to treatment with tamoxifen and standard chemotherapy (6–8).

Trastuzumab (Herceptin) is designed to target the extra-cellular domain of the HER2 receptor and block its function (9). In patients with metastatic breast cancer who over-express HER2, trastuzumab has been found to be clinically beneficial as first-line chemotherapy (10, 11). However, the response rates to trastuzumab monotherapy range from 12% to 34% for a median duration of 9 months only (12). Although current treatment regimens combining trastuzumab with the taxane paclitaxel (13, 14) or docetaxel (15) increase response rates, >70% of patients with overexpressing HER2, however, show no response to treatment (16). Many possible mechanisms have been proposed to account for the therapeutic effects of trastuzumab (17), including down-modulation of the HER2 receptor (9), interaction with immune system and enhancing cytotoxic activity of tumor-specific CTLs (9, 18), activation of apoptotic signals (19), and inhibition of HER2 receptor downstream signal transduction pathway (9, 20).

The phosphatidylinositol 3-kinase (PI3K) and its associated protein kinase B (Akt) pathway have been shown to be one of the important downstream signaling pathways that play a critical role toward antiapoptosis and pathogenesis of cancer (21). The activation of Akt results in the downstream regulation of target molecules: glycogen synthase kinase-3 (GSK-3; ref. 22), caspase-9 (23), proapoptotic Bcl-2 family member Bad (24), and FOXO (forkhead box O; forkhead members of the O subclass) family of transcription factors (21). The final outcome may result in cellular proliferation or antiapoptosis (25, 26). The FOXO family of transcription factors, consisting of FOXO1, FOXO3a, FOXO4, and FOXO6, is a direct phosphorylation target of the protein kinase Akt (27, 28).
The cell lines derived from patients who were resistant to trastuzumab treatment have shown upregulation of Akt (29). Activation of Akt followed by loss of p27Kip1 could be one of the mechanisms of trastuzumab resistance (30). FOXO1A has been suggested as a tumor suppressor gene in prostate cancer (31). Data suggest that FOXO1A is inactivated due to chromosomal deletion and/or transcriptional downregulation (31). It plays a positive role in cell differentiation by interacting with other signaling pathways (31–33). The present study was designed to understand the role of Akt mediation of FOXO1A in response to trastuzumab treatment and mechanisms of trastuzumab actions in inhibiting the HER2 receptors and their downstream events.

Materials and Methods

Chemicals and antibodies
The PI3K inhibitor LY294002 was obtained from Cell Signaling Technology; heregulin β-1 was bought from NeoMarkers, and trastuzumab was received as a gift from Genentech. The following antibodies were used and their source is indicated: anti–phospho-Akt (pAkt; Ser473), anti-Akt, anti–phospho-GSK-3β (pGSK-3β), and p27Kip1 from Cell Signaling Technology; anti-phosphotyrosine, anti–cyclin D1, anti-ErbB2-Neu, and anti–c-erbB3 from Oncogene Science; FOXO1A antibody from Abcam; and anti–β-actin from Sigma.

Cell lines and cell culture
Human breast cancer cell lines SKBR3 and BT474 were obtained from the American Type Culture Collection. Unless otherwise stated, monolayer cultures of SKBR3 and BT474 cells were maintained in DMEM/F12 media with 10% fetal bovine serum. MCF7-HER2 and MCF7-neo cells (MCF7 cells transfected with HER2 and empty vector) were kind gift from Dr. Kent Osborne (Baylor College of Medicine, Houston, TX). SKBR3/A28 and BT474/A9 cells were generated by transfection of Myr-Akt1 in SKBR3 and BT474 cells; SKBR3/DN9 and BT474/DN5 cells were created by transfection of dominant-negative Akt1 in SKBR3 and BT474; and SKBR3/V33 and BT474/V were generated by transfection of pUSEamp(+) vector in SKBR3 and BT474 (Supplementary Figs. S1-2). The transfected cells were maintained in growth media with 400 μg/mL G418.

Quantitative real-time reverse transcription-PCR
Quantitative real-time PCR was performed with iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories) using SYBR Green Master Mix (Qiagen). The mRNA levels of Akt1, HER2/neu (HER2), and FOXO1A were quantified by measuring the threshold cycle (Ct) and adjusted with the level of 18S for each sample.

Overexpressing FOXO1A
Overexpressing FOXO1A was done by either transient transfection or stable transfection of the 2-kb pcDNA3 Flag FKHR (pcDNA3 Flag FOXO1A; Addgene plasmid 13507; ref. 33) into the cells. Lipofectamine PLUS reagent (Invitrogen) was used for transfection following the manufacturer’s instruction.

Short hairpin RNA/small interfering RNA transfection
The HER2 short hairpin RNA (shRNA) and negative control shRNA were expression Arrest shRNA constructs, cloned into pSHAG-MAGIC2c (pSM2c) retroviral vector, and purchased from Open Biosystems (Supplementary Fig. S1). The plasmid DNA was prepared by using a kit for plasmid DNA extraction (Ultrapure, Qiagen) according to the manufacturer’s protocol. The transfection was performed by using Arrest-in transfection reagent (Open Biosystems) following the manufacturer’s instructions.

The FOXO1A was knocked down by transfecting chemically synthesized small interfering RNA (siRNA) of FOXO1A (ID s5259; Ambion) into the cells. The siPORT NeoFX reagent (Ambion) was used for transfection following the manufacturer’s instructions.

The HER2 and FOXO1A mRNA expression after shRNA/siRNA knockdown were determined by quantitative reverse transcription-PCR (RT-PCR) at 48 to 72 hours of transfection.

Immunoblotting and immunoprecipitation
Cells were either treated with trastuzumab at 10 μg/mL for 72 hours or preincubated with different PI3K or Akt inhibitors for 16 hours and then treated with trastuzumab. The growth medium containing trastuzumab was refreshed every 24 hours. For immunoblot analysis, total protein (30–50 μg) from cell lysates was used, and for immunoprecipitations, 250 μg of protein from whole-cell lysates were used.

Immunohistochemistry and immunofluorescence
Immunofluorescence was performed by incubating the slides with FITC or Texas red fluorescence-conjugated secondary antibodies for 30 minutes and then mounted with Vectashield mounting medium. The cells with positive staining were counted in five different areas under a fluorescence microscope and adjusted with total number of cells using DigiPro software (Labomed, Inc.).

Statistical analysis
The values were expressed as mean ± SD and/or mean fold change. The statistical significances of mean values among different cell lines were determined by one-way ANOVA first and then by Student’s t test. P value of ≤0.05 was considered significant for a Student’s t test.

Results
FOXO1A expression is regulated by PI3K/Akt pathway
We first examined the constitutive expression of FOXO1A in HER2-overexpressing cells SKBR3 [estrogen receptor (ER) negative], BT474 (ER positive), and MCF7-HER2 (ER positive); HER2 moderately expressing cells MCF7 (ER positive); and nontumorigenic breast cells MCF12A. The constitutive expression of FOXO1A was lower in all breast cancer cells than that in nontumorigenic breast cells MCF12A.
HER2-overexpressing cells SKBR3, BT474, and MCF7-HER2 had lower FOXO1A compared with HER2 moderately expressing cells MCF7 (Fig. 1A). Conversely, the pAkt was higher in the HER2-overexpressing cells than that in MCF7 (Fig. 1A, bottom). We then used SKBR3 cells to examine the regulation of FOXO1A gene expression by different growth factors. FOXO1A expression decreased significantly in response to heregulin (HRG) treatment. Similarly, epidermal growth factor (EGF) also reduced FOXO1A expression compared with untreated cells (Fig. 1B). EGF can lead to an efficient signaling complex that can enhance PI3K signal transduction pathway (34). In contrast, FOXO1A expression did not change significantly in response to insulin-like growth factor-I (IGF-I). Similarly, Fig. 1C shows that HRG induces phosphorylation of HER3 receptor and formation of HER3/HER2 protein complex and further phosphorylates HER2 receptor in SKBR3 cells. This results in activation of pAkt, whereas total Akt levels did not change (Fig. 1C). The PI3K inhibitor wortmannin increased FOXO1A expression significantly (Fig. 1A).

Surprisingly, LY294002 (40 μmol/L), another PI3K inhibitor, did not restore FOXO1A expression significantly. Figure 1C confirms that wortmannin treatment completely blocks pAkt expression and inhibits HRG-induced SKBR3 cell proliferation (Fig. 1D).

Effect of downregulating HER2 gene on pAkt and FOXO1A expression

Faltus and colleagues (35) showed that silencing the HER2 gene by siRNA/shRNA inhibits proliferation of HER2-overexpressing breast cancer cells. Using a similar approach, we transfected shHER2 into SKBR3 cells and tested the hypothesis that downregulation of HER2 receptors results in decrease in pAkt and increase in FOXO1A expression. As expected, after 72 hours of shHER2 transfection, HER2 mRNA level decreased in SKBR3 cells and this was associated with decrease in HER2 extracellular membrane receptor expression (Fig. 2A). SKBR3 cells transfected with shHER2 showed a decrease in pAkt but not total Akt (Fig. 2B). Nontransfected

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**Figure 1.** FOXO1A expression is regulated by PI3K/Akt pathway in HER2-overexpressing cells. A, FOXO1A (top) and Akt (bottom) protein expression was examined by Western blot analysis, and β-actin antibody was used for loading control. B, SKBR3 cells were treated with HRG, IGF-I, and EGF in serum-free medium with LY294002 (LY) or wortmannin (Wor). The bar graph indicates the fold change of FOXO1A mRNA level in treated cells compared with nontreated cells (control) adjusted for 18S. Each bar represents the mean of three determinations. *, P < 0.05, compared with control. RT-Q-PCR, quantitative RT-PCR. C, cells were induced with HRG for 20 min or treated with wortmannin (200 nmol/L, 16 h). D, cells were treated with or without HRG and wortmannin for 3 d. MTT assay was performed at day 3. The bar graph indicates the percentage changes of cell growth in treated cells compared with untreated cells. Columns, mean of six determinations; bars, SD. The statistical significance was determined by ANOVA analysis. *, P < 0.05, compared with control.
SKBR3 cells had FOXO1A expression mainly in the cytoplasmic and membrane regions (Fig. 2C, left, a–c). However, on shHER2 transfection, there was a significant increase in nuclear uptake of FOXO1A (Fig. 2D, left, d–f). FOXO1A nuclear protein expression increased by ∼25% after silencing HER2 gene with shHER2 (Fig. 2C, right).

To further confirm these results, pAkt and FOXO1A expression was examined in MCF7-overexpressing HER2 cells, MCF7-HER2, and compared with MCF7 transfected with empty vector, MCF7-neo cells. Figure 2D shows that MCF7-HER2 cells had significant increase in HER2 receptor and upregulated pAkt expression than that in MCF7-neo. The phosphorylated FOXO1A was increased and total FOXO1A was decreased in MCF7-HER2 compared with MCF7-neo (Fig. 2D).

Data from Fig. 2 suggest that FOXO1A expression is inversely related to active Akt (pAkt), suggesting that tumors with activated Akt will have low levels of FOXO1A and this may contribute to resistance to therapy. The elevated Akt1 kinase activity has been shown to be important for development and proliferation of human cancers, including breast cancer (25). Several laboratories have used either transient...
Akt transfection (36) or stably transfected Akt cell models. To understand the mechanisms associated with decrease in FOXO1A in relation to increased Akt1 in HER2-overexpressing cells, we created stable cell transfectants with either myrAkt1 (active Akt1) or K179M (dominant-negative mutant) Akt1 (DNAkt1). The empty vector, pUSEamp(+), was also transfected. The stable transfectants have been confirmed by RT-PCR and sequencing analysis (Supplementary Fig. S2). Protein level of Akt1 expression was also analyzed by Western blot analysis with antibodies against pAkt (Ser473) and total Akt for all positive clones. SKBR3 transfected with myrAkt1 clones (SKBR3/AA28, SKBR3/AA29, and SKBR3/AA9) showed a significant increase in pAkt (Ser473) compared with vector-transfected clone (SKBR3/V33) or dominant-negative K179M Akt transfectants (SKBR3/DN8 and SKBR3/DN9) but did not change in total Akt expression (Supplementary Fig. S2). Similar to investigations on wild-type SKBR3 cells (Fig. 2), we examined FOXO1A and p27kip1 protein expression in SKBR3 cells with activated Akt (SKBR3/AA28) and treated with shHER2. Figure 3A shows that FOXO1A protein expression decreased significantly in SKBR3/AA28 and increased in SKBR3/DN9 slightly compared with SKBR3 and SKBR3/V33.

Figure 3. Myr-Akt1 inhibits FOXO1A and p27kip1 protein expression, and overexpressing FOXO1A restores nuclear expression of p27kip1. A, FOXO1A protein expression in SKBR3/AA28, SKBR3/DN9, SKBR3/V3, and parental SKBR3 cells was determined by Western blot analysis (top) and immunohistochemistry (bottom). B, p27kip1 expression in total cell lysate and in cytoplasm (c) or nucleus (n). C, MCF7-HER2 cells were transfected with either FOXO1A or empty vector for 72 h. FOXO1A mRNA was determined by quantitative RT-PCR. Poly(ADP-ribose) polymerase (PARP) and α-tubulin were examined to confirm the separation of nuclear and cytoplasmic protein. D, SKBR3/AA28 cells were transfected with either shHER2 or negative sequence for 72 h. Bottom left, fold changes of HER2 mRNA level in the shHER2-transfected cells were compared with nontransfected cells. HER2 membrane receptors in the same cells were determined by immunofluorescence analysis with FITC-labeled anti-HER2 antibody (green), and the cell nucleus was labeled by propidium iodide (red). The merged FITC (green)–labeled and propidium iodide (red)–labeled cells were presented in a to c. Top, the arrows indicate the FITC-labeled HER2 membrane receptor (green). Bottom right, pAkt and total Akt were examined in the same transfected cells.
cells. Immunohistochemistry analysis further confirmed loss of nuclear FOXOA1 in SKBR3/AA28 (Fig. 3A, bottom).

Decrease in FOXOA1 in Akt activated cells results in decreased expression of the cell cycle inhibitor protein p27

p27kip1, a member of the Kip family of cyclin-dependent kinase inhibitors, plays an important role in cell cycle arrest (37). Its promoter activity has been associated with FOXOA1 and FOXO3 (38, 39). Cytoplasmic translocation of p27kip1 has been increasingly recognized to be associated with an aggressive phenotype of tumors, whereas nuclear expression confers a more favorable outcome (40). Data from our current study show that total p27kip1 protein expression was significantly decreased in SKBR3/AA28 cells (Fig. 3B). As shown in Fig. 3B, p27kip1 was present in both nuclear and cytoplasm of SKBR3/V33 and SKBR3/DN9 cells. In contrast, p27kip1 was exclusively localized in the cytoplasm of SKBR3/AA28 cells (Fig. 3B).

To further confirm if FOXOA1 influences the expression of p27kip1, the full-length FOXOA1 gene was transfected into MCF7-HER2 cells. The FOXOA1 mRNA level was significantly increased in the FOXOA1-transfected MCF7-HER2 cells (MCF7-HER2/FOXOA1), and FOXOA1 in MCF7-HER2/FOXOA1 cells was exclusively located in the nucleus (Fig. 3C). Figure 3C shows that the p27kip1 protein was mainly expressed in the nucleus in MCF7 cells; however, it was translocated in both nuclear and cytoplasmic space in MCF7-HER2 cells. Overexpressing FOXOA1 in the MCF7-HER2 cells (MCF7-HER2/FOXOA1) restored the nuclear expression of p27kip1 protein (Fig. 3C).

Effect of shHER2 transfection on HER2 mRNA, HER2 protein, and pAkt expression on SKBR3/AA28 cells

Our data from Fig. 3D confirm downregulation of HER2 mRNA and HER2 receptor protein by shHER2 treatment. However, in contrast to the SKBR3 cells (Fig. 2B), pAkt expression in SKBR3/AA28 cells (Fig. 3D) did not decrease significantly.

Sensitivity to trastuzumab was affected by the reduction of FOXOA1 and activation of Akt

SKBR3 cells with pAkt overexpression (SKBR3/AA28) showed activated levels of pGSK-3β and cyclin D1 (Fig. 4A). These cells did not respond to trastuzumab. In contrast, wild-type SKBR3, vector-transfected SKBR3 (V33), and dominant-negative Akt (DN9) cells responded favorably to trastuzumab, with respect to downregulation of pAkt, pGSK-3β, and cyclin D1 (Fig. 4A). Cell growth measured by MTT assay further confirmed that unlike vector-transfected (SKBR3/V33) and dominant-negative Akt-transfected (DN9) cells, SKBR3/AA28 cells were relatively resistant to time-dependent trastuzumab treatment (Fig. 4A, right). Similar results were observed in BT474 cells transfected with myrAkt1 (BT474/AA9) and dominant-negative Akt1 (BT474/DN5). As shown in Fig. 4B, BT474/AA9 showed less response to trastuzumab treatment with respect to downregulation of pAkt and cell growth inhibition compared with BT474/V (vector transfection only) and BT474/DN5.

Figure 4C confirms that trastuzumab-resistant (SKBR3/AA28 and BT474/AA9) cells did not show a difference in FOXOA1 mRNA and protein expression on trastuzumab treatment. In contrast, wild-type SKBR3 and BT474 cells responded to trastuzumab with a significant increase in both mRNA and protein expression of FOXOA1. Next, we examined if p27kip1, a universal cell cycle inhibitor, was associated with FOXOA1 expression in trastuzumab-responsive and trastuzumab-resistant SKBR3 cells. Figure 4D shows that in wild-type SKBR3 cells, p27kip1 was upregulated together with FOXOA1 in response to trastuzumab treatment. In contrast, p27kip1 expression did not change in trastuzumab-resistant SKBR3/AA28 cells.

Effect of trastuzumab after inhibiting the PI3K/Akt pathway

Figure 5A shows that pAkt expression was blocked completely in wild-type SKBR3 cells treated with the PI3 inhibitor LY294002 or API-2. API-2 has been reported to be a highly selective inhibitor of Akt signaling in human tumor cells with aberrant Akt, leading to decrease in cell growth and induction of apoptosis (41). Trastuzumab partially downregulated pAkt. However, in combination with LY294002, the pAkt expression was completely blocked. Similarly, alone or in combination with API-2, the pAkt expression was completely blocked. However, neither LY294002 (Fig. 5B) nor wortmannin (data not shown) caused significant inhibition of pAkt and pGSK-3β in SKBR3/AA28 cells. Subsequently, addition of trastuzumab in combination with LY294002 also had no effect on pAkt and pGSK-3β. However, treatment with API-2 caused a partial downregulation of both pAkt and pGSK-3β (Fig. 5B). Addition of trastuzumab with API-2 completely inhibited pAkt and pGSK-3β. Figure 5C shows that API-2 treatment can significantly upregulate FOXOA1 mRNA levels in both wild-type SKBR3 and trastuzumab-resistant SKBR3/AA28 cells. These observations provide further evidence that by inhibiting pAkt, FOXOA1 gets upregulated and makes these cells more responsive to trastuzumab (Fig. 5D).

Overexpressing FOXOA1 restored the sensitivity of trastuzumab in HER2- and pAkt-overexpressing cells

Figure 6A shows that FOXOA1 mRNA level increased by 3.1-fold in SKBR3/AA28 cells transfected with full-length FOXOA1 gene for 3 days compared with cells transfected with empty vector only. MTT growth assay in Fig. 6A (left) showed that SKBR3/AA28 cells transfected with pcDNA3 vector alone did not change the cell growth between treated and untreated cells. However, cells transfected with FOXOA1 showed increased sensitivity to trastuzumab, and its sensitivity to trastuzumab was at the same level as wild-type SKBR3 cells. We also investigated if transfecting FOXOA1 gene in wild-type SKBR3 (HER2-overexpressing cells) would have any additional benefit with reference to trastuzumab sensitivity and HER2 mRNA levels. After clonal selection, quantitative RT-PCR was used to confirm the presence of FOXOA1. Figure 6A shows that FOXOA1 mRNA levels increased by 3.0-fold in FOXOA1 transfectant SKBR3/FOXOA1-3 cells.
compared with vector only–transfected cells (SKBR3/pcDNA3). Furthermore, MTT assay showed that after 3 days of trastuzumab treatment, cell growth was inhibited by 18% in SKBR3; however, this inhibition increased to 31% in FOXOA1-transfected SKBR3/FOXO1A-3 cells (Fig. 6A, left). An interesting outcome of FOXOA1 transfection in HER2-overexpressing SKBR3 cells was the significant downregulation of HER2 mRNA (Supplementary Fig. S2). To further show that the importance of FOXOA regulation is not specific to SKBR3 cell line only, the full-length FOXO1A gene was also transfected into the relatively resistant trastuzumab cells, BT474/AA9. Figure 6B shows that cell growth was significantly inhibited by trastuzumab, consistent with increase in FOXO1A mRNA expression in the BT474/AA9 cells transfected with FOXO1A. In contrast to BT474/AA9 cells, the BT474/DN5 cells were more sensitive to trastuzumab. However, the BT474/DN5 cells became less responsive to trastuzumab after FOXO1A knockdown. The FOXO1A expression was significantly decreased in BT474/DN5 cells transfected with siRNA-FOXO1A, and trastuzumab was not able to inhibit the cell growth in the FOXO1A knockdown BT474/DN5 cells (Fig. 6B). Similar experiments were performed in MCF7-HER2 cells. Figure 6C shows that cell growth inhibition was increased by trastuzumab in MCF7-HER2 cells transfected with full-length FOXO1A transfection and decreased in the knockdown FOXO1A MCF7-HER2 cells (Fig. 6C).
Discussion

Activation of PI3K/Akt pathway and subsequent inactivation of FOXO transcription factors have been observed in different cancers (27, 31). Recent clinical and translational studies from our laboratory and others have clearly shown that breast cancer patients with HER2-positive tumors have greater potential to increase pAkt expression in their tumors (42–44). An increase in tissue pAkt expression in HER2-overexpressing breast cancer patient leads to poor disease outcome with significant decrease in 5-year disease-free survival rate (42). Hence, therapeutic targeting of the PI3K/Akt pathway offers the opportunity to improve disease-free and overall survival in these HER2-overexpressing breast cancer patients. Current targets for PI3K/Akt are fairly effective in vitro but show significant toxicity in vivo. Hence, there is a need to identify additional or alternate targets associated with PI3K/Akt signaling cascade.

FOXO transcriptional factors, especially FOXO3 and FOXO1, are regulated by PI3K/Akt pathway (45). Deregulation of FOXO3 or FOXO1 has been found in different cancers, including breast, prostate, chronic myelogenous leukemia, glioblastoma, rhabdomyosarcoma, and leukemia (27, 45). The deregulated FOXOs have been reported to contribute to resistance to paclitaxel, doxorubicin, and cisplatin treatment (46–48).

Our current study clearly shows that FOXO1A is regulated by the PI3K/Akt pathway in HER2-overexpressing breast cancer cells. Overexpression of active Akt leads to decrease in FOXO1A expression and nuclear export of p27kip1. Trastuzumab blocks HER2 receptor and downregulates pAkt and downstream molecules. The downregulation of pAkt by trastuzumab results to reactivation of FOXO1A that leads to increase in p27kip1, culminates in decrease in cell cycle protein such as cyclin D1, and finally inhibits cell proliferation. Trastuzumab, however, was not able to completely downregulate the activated pAkt in SKBR3/AA28 and BT474/AA9. Trastuzumab also failed to increase FOXO1A in those cells and, consequently, p27kip1 expression in SKBR3/AA28. With respect to cell proliferation, SKBR3/AA28 and BT474/AA9 cells
showed lesser sensitivity to trastuzumab. In contrast, cells expressing dominant-negative Akt1 (SKBR3/DN9 and BT474/DN5) showed more sensitivity to growth inhibition by trastuzumab.

In addition to the PI3K inhibitors, we also examined the effect of Akt/protein kinase B inhibitor-2 (API-2) on inhibition of pAkt in HER2- and Akt1-overexpressing SKBR3 cells. API-2 was first identified to inhibit cell growth in Akt2-transformed but not empty vector LXSN-transfected NIH3T3 cells (49, 50). Later, it was shown to suppress pAkt level without inhibition of PI3K (41). Here, we report that API-2 inhibits pAkt expression significantly in SKBR3/AA28 and caused downregulation of FOXO1A, resulting in inhibition of cell proliferation.

Our data show that inactivation of FOXO1A by activated Akt increases breast cancer cell survival and inhibits the apoptotic properties of trastuzumab in HER2-overexpressing cells. To address the question that overexpression of FOXO1A can overcome resistance or increase sensitivity to trastuzumab, we used stable transfection as well as transient transfection strategies that overexpressed FOXO1A into both SKBR3 and SKBR3/AA28 cells. We observed significant increase in sensitivity to trastuzumab-induced growth inhibition in both SKBR3 and SKBR3/AA28 cells transfected with FOXO1A.
FOXO1A. To further confirm that the treatment with trastuzumab leading to decreased HER2-mediated cell proliferation is associated with FOXO1 regulation and is not only specific to ER-negative cell line SKBR3, we overexpressed FOXO1A into HER2-overexpressing cells with ER-positive BT474/A9 and MCF7-HER2 cells. A significant increase in sensitivity to trastuzumab-induced growth inhibition was observed in both BT474/A9 and MCF7-HER2 cells transfected with FOXO1A. In contrast, the BT474/DN5 and MCF7-HER2 cells with siRNA knockdown FOXO1A became resistant to trastuzumab. Furthermore, SKBR3 cells with stable FOXO1A transfection showed a significant decrease in their HER2 mRNA levels compared with vector only–transfected and parental SKBR3 cells. This observation suggests that FOXO1A may transcriptionally regulate HER2 gene expression in HER2-overexpressing breast cancer cells. The mechanism(s) by which FOXO1A transcription factor may regulate HER2 gene needs to be investigated further.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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