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 demonstrated that overexpression of HER2 leads to activation of PI3K/Akt pathway and subsequent inactivation of FOXO1A in HER2 overexpressing breast cancer cells, SKBR3, BT474 and MCF7-HER2. In wildtype SKBR3 and BT474 cells, Trastuzumab downregulates active Akt and increases FOXO1A expression that leads to increase in p27kip1, 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 down-regulation of FOXO1A resulted in nuclear export of p27kip1. 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 siRNA resulted in reducing Trastuzumab induced growth inhibition. In summary, Trastuzumab can inhibit proliferation of HER2 overexpressing breast cancer cells by re-activating FOXO1A through inhibition of the PI3K/Akt pathway. FOXO1A may therefore serve as a target for HER2 overexpressing breast tumors.

Keywords
FOXO1A; HER2/neu; Herceptin; Akt1; Breast Cancer

Introduction

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

Trastuzumab (Herceptin) is designed to target the extracellular domain of the HER2 receptor and block its function (9). In patients with metastatic breast cancer that 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). Even though current treatment regimens combining Trastuzumab with the taxane paclitaxel (13–14) or docetaxel (15) increase response rates, greater than 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 (PI-3K) and its associated protein kinase B (Akt) pathway has been demonstrated to be one of the important downstream signaling pathways that play a critical role toward anti-apoptosis and pathogenesis of cancer (21). The activation of Akt results in the downstream regulation of target molecules: glycogen synthase kinase-3 (GSK-3) (22); caspase-9 (23); pro-apoptotic Bcl-2 family member Bad (24); and FOXO (forkhead box O; forkhead members of the O subclass) family or transcription factors (21). The final outcome may result in cellular proliferation or anti-apoptosis (25,26). FOXO family of transcription factors, consisting of FOXO1, FOXO3a, FOXO4, and FOXO6, are direct phosphorylation targets of the protein kinase Akt (27–28).

The cell lines derived from patients who were resistant to Trastuzumab treatment has 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 down-regulation (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**

PI-3 kinase inhibitor, LY294002 (#9901), was obtained from Cell Signaling Technology; Heregulin β-1 (RP-318-PIA) was bought from Neo Markers and Trastuzumab was received as a gift from Genentech. The following antibodies were utilized and their source is indicated: anti-phospho-Akt Ser 473 (pAkt ser473) (#9271 and #9277), anti-Akt (#9272), anti-phospho-GSK-3β (#9336) and p27kip1 (#3686) from Cell Signaling Technology; anti-phosphor-Tyr (PT03), anti-cyclinD1 (CC12), anti-ErbB2-Neu (OP3P) and antic-erbB3 (PC27) from Oncogene Science; FOXO1A antibody (ab34406) from Abcam; and anti-β-actin from Sigma.
Cell lines and cell culture

Human Breast cancer cell lines SKBR3 and BT474 were obtained from American Type Culture Collection. Unless otherwise stated, monolayer cultures of SKBR3 and BT474 cells were maintained in DMEM/F12 media with 10% FBS. MCF7-HER2 and MCF7-neo cells (MCF-7 cells transfected with HER2 and empty vector) were kind gift from Dr. Kent Osborne (Baylor College of Medicine, Texas).

SKBR3/AA28 and BT474/AA9 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 was generated by transfection of pUSEamp(+) vector in SKBR3 and BT474 (Supplement 1 and Supplement 2: figure S2-1).

The transfected cells were maintained in growth media with 400μg/ml G418.

Quantitative real-time RT-PCR

Quantitative real-time PCR was performed with iCycle iQ real-time PCR detection system (Bio-Rad Lab, Hercules, CA) using SYBR Green Master Mix (#204143, QIAGEN). The mRNA levels of Akt1, HER2/neu (HER2) and FOXO1A were quantified by measuring the threshold cycle (Ct) and were adjusted with the level of 18S for each sample.

Overexpressing FOXO1A

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

shRNA/siRNA transfection

The HER2 shRNA (short hairpin RNA) and negative control shRNA were expression ArrestTM short hairpin RNA constructs, cloned into pSHAG-MAGIC2c (pSM2c) retroviral vector and were purchased from Open Biosystems (Huntsville, Al) (Supplement 1). The plasmid DNA was prepared by using a kit for plasmid DNA extraction (QIAGEN, UHraPure) according to manufacturer’s protocol. The transfection was performed by using Arrest-in™ transfection reagent (Open Biosystems) following the manufacture’s instructions.

The FOXO1A was knocked down by transfecting chemically synthesized small interfering RNA of FOXO1A (ID# s5259; Ambion, Austin, TX) into the cells. The siPORT NeoFX reagent (Ambion, Austin, TX) was used for transfection following the manufacturer’s instructions. The HER2 and FOXO1A mRNA expression after shRNA/siRNA knock down were determined by Quantitative RT-PCR at 48hrs to 72hrs transfection.

Immunobloting and Immunoprecipitation

Cells were either treated with Trastuzumab at 10 μg/ml for 72 hrs or pre-incubated with different PI3K or AKT inhibitors for 16 hrs and then treated with Trastuzumab. The growth medium containing Trastuzumab were refreshed every 24hrs. For immunoblot analysis, total protein (30μg to 50μg) from cell lysates was used and for immunoprecipitations, 250μg of protein from whole cell lysates was used.

Immunohistochemistry (IHC) and Immunofluorescence (IF)

The IF was performed by incubating the slides with FITC or Tex Red fluorescence-conjugated secondary antibodies for 30min 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, then by Student t test. P-value ≤ 0.05 was considered significant for ANOVA test. P-Value ≤ 0.01 was considered significant for Student t test.

**Results**

FOXO1A expression is regulated by PI3K/Akt pathway

We first examined the constitutive expression of FOXO1A in HER2 overexpressing cells, SKBR3 (ER negative), BT474 (ER positive) and MCF7-HER2 (ER positive); HER2 moderate expressing cells, MCF-7 (ER positive) and non-tumorigenic breast cells, MCF12A. The constitutive expression of FOXO1A was lower in all breast cancer cells than that in non-tumorigenic breast cells, MCF12A. HER2 overexpressing cells, SKBR3, BT474 and MCF7-HER2 had lower FOXO1A compared to HER2 moderately expressing cells, MCF-7 (figure 1A). Conversely, the pAkt was higher in the HER2 overexpressing cells than that in MCF-7 (figure 1A bottom panel). 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 (HGR) treatment. Similarly, EGF also reduced FOXO1A expression compared to untreated cells (figure 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 IGF-I. Similarly, figure 1C demonstrates that HRG induces phosphorylation of HER3 receptor, formation of HER3/HER2 protein complex, and further phosphorylates HER2 receptor in SKBR3 cells. This results in activation of pAkt, while total AKT levels did not change (figure 1C). The PI3K inhibitor, Wortmannin increased FOXO1A expression significantly (figure 1A). Surprisingly, LY29004 (40 μM), 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 (figure 1D).

Effect of downregulating HER2 gene on pAkt and FOXO1A expression

Faltus et al (35) demonstrated that silencing the HER2 gene by siRNA/shRNA inhibits proliferation of HER2 overexpressing breast cancer cells. Using a similar approach we transfected the 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 hrs of shHER2 transfection, HER2 mRNA level decreased in SKBR3 cells and this was associated with decrease in HER2 extracellular membrane receptor expression (figure 2A). SKBR3 cells transfected with shHER2 showed a decrease in pAkt, but not total Akt (figure 2B). Non-transfected SKBR3 cells had FOXO1A expression mainly in the cytoplasmic and membrane regions (figure 2C left panel a–c). However, upon shHER2 transfection, there was a significant increase in nuclear uptake of FOXO1A (figure 2D left panel d–f). FOXO1A nuclear protein expression increased by almost 25% after silencing HER2 gene with shHER2 (figure 2C right panel).

To further confirm these results, pAkt and FOXO1A expression was examined in MCF-7 overexpressing HER2 cells, MCF7-HER2 and compared to MCF-7 transfected with empty vector, MCF7-neo cells. Figure 2D showed that MCF7-HER2 cells had significant increase in HER2 receptor and upregulated pAkt expression than that in MCF7-neo. The phosphorylated
FOXO1 (pFOXO1A) was increased and total FOXO1A was decreased in MCF7-HER2 compared to MCF7-neo (figure 2D).

Data from figure 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 employed either transient Akt transfection (36) or stably transfected Akt cell models. In order 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 data S2-1 A–B). 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 myr-Akt1 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 data S2-1C). Similar to investigations on wild type SKBR3 cells (figure 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 to SKBR3 and SKBR3/V33 cells. Immunohistochemistry analysis further confirmed loss of nuclear FOXO1A in SKBR3/AA28 (figure 3A bottom panel).

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

The p27kip1, a member of the Kip family of cyclin-dependent kinase (Cdk) inhibitors, plays an important role in cell cycle arrest (37). Its promoter activity has been associated with FOXO1A 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 demonstrate that total p27kip1 protein expression was significantly decreased in SKBR3/AA28 cells (figure 3B). As shown in figure 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 (figure 3B).

To further confirm if FOXO1A influences the expression of p27kip1, the full-length FOXO1A gene was transfected into MCF7-HER2 cells. The FOXO1A mRNA level was significantly increased in the FOXO1A transfected MCF7-HER2 cells (MCF7-HER2/FOXO1A) and the FOXO1A in MCF7-HER2/FOXO1A cells was exclusively located in nucleus (figure 3C). Figure 3C showed that the p27kip1 protein was mainly expressed in nucleus in MCF-7 cells however, it was translocated in both nuclear and cytoplasmic space in MCF7-HER2 cells. Overexpressing FOXO1A in the MCF7-HER2 cells (MCF7-HER2/FOXO1A) restored the nuclear expression of p27kip1 protein (figure 3C).

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

Our data from figure 3D confirm downregulation of HER2 mRNA and HER2 receptor protein by shHER2 treatment. However, in contrast to the SKBR3 cells (figure 2B) pAkt expression in SKBR3/AA28 cells (figure 3D) did not decrease significantly.
Sensitivity to Trastuzumab was affected by the reduction of FOXO1A and activation of Akt

SKBR3 cells with pAkt overexpression (SKBR3/AA28) demonstrated activated levels of pGSK-3β and Cyclin D1 (figure 4A). These cells did not respond to Trastuzumab. In contrast, wildtype 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 (figure 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 (figure 4A right panel). Similar results were observed in BT474 cells transfected with myr-Akt1 (BT474/AA9) and dominant negative Akt1 (BT474/DN5). As shown in figure 4B, BT474/AA9 demonstrated less response to Trastuzumab treatment with respect to downregulation of pAkt and cell growth inhibition compared to 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 FOXO1A mRNA and protein expression upon Trastuzumab treatment. In contrast, wildtype SKBR3 and BT474 cells responded to Trastuzumab with a significant increase in both mRNA and protein expression of FOXO1A. Next we examined if p27kip1, a universal cell cycle inhibitor was associated with FOXO1A expression in Trastuzumab responsive and resistant SKBR3 cells. Figure 4D, demonstrates that in wild type SKBR3 cells, p27kip1 was upregulated together with FOXO1A in response to Trastuzumab treatment. In contrast, p27kip1 expression did not change in Trastuzumab resistant SKBR3/AA28 cells.

Effect of Trastuzumab after inhibiting the PI3/Akt pathway

Figure 5A demonstrated that pAkt expression was blocked completely in wild type SKBR3 cells treated with PI3K inhibitor LY294002, or API-2. The 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 LY290042, the pAkt expression was completely blocked. Similarly alone or in combination with API-2 the pAkt expression was completely blocked. However, neither LY294002 (figure 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 (figure 5B). Addition of Trastuzumab with API-2 completely inhibited pAkt and pGSK-3β. Figure 5C demonstrates that API-2 treatment, can significantly upregulate FOXO1A mRNA levels in both wildtype SKBR3 and Trastuzumab resistant SKBR3/AA28 cells. These observations provide further evidence that by inhibiting pAkt, FOXO1A gets upregulated and makes these cells more responsive to Trastuzumab (figure 5D).

Overexpressing FOXO1A restored the sensitivity of Trastuzumab in HER2 and pAkt overexpressing cells

Figure 6A showed that FOXO1A mRNA level increased by 3.1 fold in SKBR3/AA28 cells transfected with full length FOXO1A gene for 3 days compared to cells transfected with empty vector only. MTT growth assay in figure 6A (left panel) 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 FOXO1A showed increased sensitivity to Trastuzumab and its sensitivity to Trastuzumab was at the same level as wildtype SKBR3 cells. We also investigated if transfecting FOXO1A 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, RT-Q-PCR was used to confirm the presence of FOXO1A. Figure 6A showed that FOXO1A mRNA levels increased by 3.0 fold in FOXO1A transfectant, SKBR3/FOXO1A-3 cells compared to 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 FOXO1A transfected SKBR3/FOXO1A-3 cells (figure 6A left panel). An interesting outcome of FOXO1A transfection in HER2 overexpressing SKBR3 cells was the significant downregulation of HER2 mRNA (supplement 2: figure S2-2). To further demonstrate the importance of FOXO1A regulation is not specific to SKBR3 cell line only, the full length FOXO1A gene was also transfected into the relatively resistant to Trastuzumab cells, BT474/AA9. Figure 6B demonstrated 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 knock-down BT474/DN5 cells (figure 6B). Similar experiments were performed in MCF7-HER2 cells. Figure 6C showed that cell growth inhibition was increased by Trastuzumab in MCF7-HER2 with full length FOXO1A transfection and decreased in the knock-down FOXO1A MCF7-HER2 cells (figure 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 demonstrated 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 demonstrate 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, CML, 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 demonstrates 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 p27\(^{kip1}\). Trastuzumab blocks HER2 receptor, down-regulates pAkt and downstream molecules. The down-regulation of pAkt by Trastuzumab results to re-activation of FOXO1A that leads to increase in p27\(^{kip1}\), culminates in decrease in cell cycle protein such as cyclin D1, and finally inhibits cell proliferation. Trastuzumab, however, was not able to completely down-regulate the activated pAkt in SKBR3/AA28 and BT474/AA9. Trastuzumab also failed to increase FOXO1A in those cells and consequently p27\(^{kip1}\) expression in SKBR3/AA28. With respect to cell proliferation, SKBR3/AA28 and BT474/AA9 cells demonstrated 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. The 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 down regulation of FOXO1A, resulting in inhibition of cell proliferation.

Our data demonstrate 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. To further confirm that the treatment with Trastuzumab leading to decreased HER2 mediated cell proliferation is associated with FOXO1 regulation, is not only specific to ER negative cell line SKBR3; we overexpressed FOXO1A into HER2 overexpressing cells with positive estrogen receptor, BT474/AA9 and MCF7-HER2 cells. A significant increase in sensitivity to Trastuzumab-induced growth inhibition was observed in both BT474/AA9 and MCF7-HER2 cells transfected with FOXO1A. In contrast the BT474/DN5 and MCF7-HER2 cells with siRNA knock-down FOXO1A became resistant to Trastuzumab. Furthermore, SKBR3 cells with stable FOXOA1 transfection showed a significant decrease in their HER2 mRNA levels compared to 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.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**References**


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 HGR, 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 non-treated cells (control) adjusted for 18S. Each bar represents the mean of three determinations. * P<0.05 compared to control. (C) Cells were induced with HGR for 20 min or treated with Wortmannin (200nM, 16hrs). (D) Cells were treated with or without HGR and Wortmannin for 3 days. MTT assay was performed at day 3. The bar graph indicates the percentage changes of cell growth in treated cells compared with untreated cells. Each bar indicates the mean of 6 determinations and standard deviation. The statistical significance was determined by ANOVA analysis. * P<0.05 compared to control.
Figure 2. shRNA silences HER2, decreases pAkt and increases nuclear expression of FOXO1A

(A) The SKBR3 cells were transfected with either shHER2 or negative sequence (neg) for 72 hrs. The HER2 mRNA levels were measured by RT-Q-PCR and adjusted with 18S. The bar indicates fold change of HER2 mRNA in the shHER2 transfected cells compared with non-transfected cells. Each bar indicates the mean of three determinations. * P<0.05 compared to control and negative sequence of HER2 transfected cells determined by ANOVA analysis (left panel). HER2 membrane receptor in SKBR3 (a), SKBR3 transfected with negative sequence (b), SKBR3 transfected with shHER2 (c), was determined by immunofluorescence analysis (IF) with FITC labeled anti-HER2 antibody (middle panel), and the cell nuclei were labeled by propidium iodide (red). The merged FITC (green) and propidium iodide (red) labeled cells were presented in top panel (a–c). The arrows indicate the FITC labeled HER2 membrane receptor (green). The bar graph in the right indicates number of HER2 positive cells and statistical significance was determined by ANOVA analysis. *p<0.05 compared to untransfected cells. (B) Shows protein levels of p-Akt and AKT in the same transfected cells. (C) FOXO1A and β-actin protein expression in SKBR3 and SKBR3 cells transfected with shHER2 were determined by IF (left panel). The white arrows indicate FITC (green) labeled FOXO1A protein located in cell nucleus (d) or cytoplasm (a) and yellow arrows indicate Texas Red (red) labeled β-actin located in cytoplasm (b, e). The merged FITC labeled FOXO1A (green) and Texas Red labeled β-actin (red) were presented in c and f. The cells with positive nuclear labeling FOXO1A were counted in five different areas and adjusted with positive β-actin labeling cells (right panel). (D) IF with FITC labeled anti-HER2 antibody indicated increased HER2 membrane receptor (green, indicated by white arrows) in MCF7-HER2 cells (left panel).
Figure 3. Myr-Akt1 inhibits FOXO1A and p27kip protein expression and FOXO1A overexpressing 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 panel) and IHC (bottom panel). (B) Shows 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 hrs. FOXO1A mRNA was determined by RT-Q-PCR. 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 hrs. The fold changes of HER2 mRNA level in the shHER2 transfected cells were compared with non-transfected cells and shown in bar graph (bottom left panel). HER2 membrane receptors in the same cells were determined by IF analysis with FITC labeled anti-HER2 antibody (green) and the cell nucleus were labeled by propidium iodide (red). The merged FITC (green) and propidium iodide (red) labeled cells were presented in a–c. The arrows indicate the FITC labeled HER2 membrane receptor (green) (top panel). p-Akt and total AKT were examined in the same transfected cells (bottom right panel).
Figure 4. Activation of Akt inhibits FOXO1A and affects sensitivity of Trastuzumab

The indicated cells were treated with or without Trastuzumab (10μg/ml) for 72hrs. (A) Western blot analysis was performed with indicated antibodies in SKBR3/V33, SKBR3/AA28, SKBR3/DN9 and SKBR3 parental cells (A) as well as in BT474, BT474/AA9 and BT474/DN5 cells (B). Cell growth was determined by MTT assay in SKBR3/V33, SKBR3/AA28, SKBR3/DN9 at each indicated days (A) and in BT474, BT474/AA9 and BT474/DN5 cells at day 3 (B). Each time point is mean of six determinations. * P<0.05 compared to untreated cells using ANOVA analysis. (C) mRNA level of FOXO1A in SKBR3, SKBR3/AA28, BT474 and BT474/AA9 treated with or without Trastuzumab was measured by RT-Q-PCR and adjusted with 18S (top panel). Each bar indicates the mean of three determinations plus standard deviation. * P<0.05 compared to untreated cells using ANOVA analysis. FOXO1A and (bottom panel) and p27 protein (D) was evaluated by western blot analysis in the indicated cells.
Figure 5. Inhibition of phosphorylation of Akt increases FOXO1A and enhances antitumor effect of Trastuzumab

SKBR3 (A) and SKBR3/AA28 (B) cells were treated with either Trastuzumab (72 hrs), LY294002 (16 hrs), API-2 (16 hrs) alone or Trastuzumab in combination with LY294002 or API-2 as indicated in A and B. Western blot analysis was performed with the indicated antibodies. (C) RNA from the API-2 treated and untreated SKBR3 and SKBR3/AA28 cells was used to perform RT-Q-PCR with FOXO1A and 18S primers. Each bar indicates mean of three determinations plus standard deviation and * P<0.05 compared to untreated cells using ANOVA analysis. (D) Cells were treated with either API-2 or API-2 in combination with Trastuzumab for 3 days. MTT assay was performed at day 3 as described in methods. Each bar indicates mean of six determinations plus standard deviation and * P<0.05 compared to untreated cells using ANOVA analysis.
Figure 6. Overexpressing FOXO1A restores the sensitivity of Trastuzumab

Full length FOXO1A gene was transient transfected into SKBR3/AA28 (A), BT474/AA9 (B) and MCF7-HER2 (C) cells for 72hrs and stably transfected into SKBR3 (A) cells. The FOXO1A was knocked down by transfected siRNA-FOXO1A into BT474/DN9 (B) and MCF7-HER2 (C) cells for 72 hrs. The mRNA level of FOXO1A in the transfectants was determined by RT-Q-PCR with FOXO1A primers and adjusted by 18S. Each bar was mean of three determinations and * P<0.05 compared to untransfected cells using ANOVA analysis. Cell growth in the same transfectants treated with and without trastuzumab was examined by MTT assay and compared to untreated cells. Each bar was mean of six determinations and * P<0.05 compared to untreated cells using ANOVA analysis.