

The transcription factor FOXO3a is a crucial cellular target of gefitinib (Iressa) in breast cancer cells

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Abstract

Gefitinib is a specific inhibitor of the epidermal growth factor receptor (EGFR) that causes growth delay in cancer cell lines and human tumor xenografts expressing high levels of EGFR. An understanding of the downstream cellular targets of gefitinib will allow the discovery of biomarkers for predicting outcomes and monitoring anti-EGFR therapies and provide information for key targets for therapeutic intervention. In this study, we investigated the role of FOXO3a in gefitinib action and resistance. Using two gefitinib-sensitive (i.e., BT474 and SKBR3) as well as three other resistant breast carcinoma cell lines (i.e., MCF-7, MDA-MB-231, and MDA-MB-453), we showed that gefitinib targets the transcription factor FOXO3a to mediate cell cycle arrest and cell death in sensitive breast cancer cells. In the sensitive cells, gefitinib treatment causes cell cycle arrest predominantly at the G₀-G₁ phase and apoptosis, which is associated with FOXO3a dephosphorylation at Akt sites and nuclear translocation, whereas in the resistant cells, FOXO3a stays phosphorylated and remains in the cytoplasm. The nuclear accumulation of FOXO3a in response to gefitinib was confirmed in tumor tissue sections from breast cancer patients presurgically treated with gefitinib as monotherapy. We also showed that knockdown of FOXO3a expression using small interfering RNA (siRNA) can rescue sensitive BT474 cells from gefitinib-induced cell-proliferative arrest, where-

as reintroduction of active FOXO3a in resistant MDA-MB-231 cells can at least partially restore cell-proliferative arrest and sensitivity to gefitinib. These results suggest that the FOXO3a dephosphorylation and nuclear localization have a direct role in mediating the gefitinib-induced proliferative arrest and in determining sensitivity to gefitinib. [Mol Cancer Ther 2007;6(12):3169–79]

Introduction

Breast cancer is the most common cancer in women in industrialized countries and is the second biggest killer after lung cancer (1). Research has shown that the inappropriate activation of growth factor–signaling cascades can support hormone-independent tumor cell growth and promote endocrine therapy resistance in breast cancer. Increased growth factor signaling has also been associated with estrogen-receptor–negative diseases and, hence, poor prognosis (2–5).

The recent availability of tyrosine kinase inhibitors (TKI) that specifically block the phosphorylation and function of epidermal growth factor receptor (EGFR) and HER2 has provided a novel therapeutic strategy. One such inhibitor is ZD1839/gefitinib (Iressa), a selective EGFR-TKI, and another is GW572016/lapatinib, a dual inhibitor of EGFR and HER2. These inhibitors act through competitive inhibition of ATP binding to the receptor tyrosine kinase domain (2, 4, 6). Both gefitinib and lapatinib can cause growth delay in cancer cell lines and human tumor xenografts expressing high levels of EGFR or HER2 and can significantly potentiate the antiproliferative effects of anti-estrogens and conventional chemotherapeutic drugs when used in combination (7, 8). Recent phase II/III clinical studies have shown that both gefitinib and lapatinib were well tolerated and provided antitumor activity in patients with breast cancer as well as with other types of cancer when used as a monotherapy or in combination with other therapies (7–10). However, the clinical data also showed that not all patients respond to the inhibitors, indicating the existence of an intrinsic or *de novo* resistance to the drug (5, 10, 11). In some clinical studies, the absence of direct association between EGFR expression levels and response to the therapy with gefitinib and lapatinib was noted (10, 12, 13). Indeed, even high EGFR expression *per se* was not sufficient to determine sensitivity to gefitinib because molecular defects in downstream signaling pathways might impair transmission of the signal and, thus, the action of the drug (14).

The activation of EGFR/erbB2 tyrosine kinases results in the activation of extracellular signal-regulated kinase 1/2 (ERK 1/2) mitogen-activated protein (MAP) kinases and phosphoinositide-3-kinase (PI3K)/protein kinase B (PKB, also called Akt) signaling pathway (10, 14, 15). The PI3K/Akt tumor suppressor signaling pathway plays an

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important role in the growth, survival, and development of normal and malignant cells (16, 17). One of the downstream targets of the PI3K/Akt signaling pathway is the Forkhead/winged helix box class O protein FOXO3a (formerly termed FKHL-1; ref. 18). Activated Akt directly controls the activity of FOXO3a through phosphorylation, leading to its translocation to the cytoplasm, where it is sequestered by the 14-3-3 chaperone protein (18). Inhibition of Akt leads to dephosphorylation and nuclear localization of FOXO3a, resulting in its activation. Studies in mammalian cells have shown that the activation of FOXO3a induces cell cycle arrest and/or apoptosis through the up-regulation of its key target genes such as p27^{Kip1} and Bim (19–22). An understanding of the downstream cellular targets of gefitinib will allow the discovery of markers that can serve to identify the subset of patients likely to benefit from the treatment.

Materials and Methods

Cell Culture

The human breast carcinoma cell lines BT474, SKBR3, MCF-7, MDA-MB-231, and MDA-MB-453 were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, and 100 units/mL penicillin-streptomycin at 37°C. Gefitinib (Iressa) was obtained from AstraZeneca and dissolved in DMSO to give 30 mmol/L stock solution for cell culture work.

Proliferation Assay

Cells were counted and plated at the same initial density on 24-well plates. They were then treated with 5 μmol/L gefitinib or the vehicle only and incubated for time periods ranging from 0 to 72 h. At each time point, cells were trypsinized and counted using a Neubauer hemocytometer under trypan blue exclusion.

Cell Cycle Analysis

Cell cycle analysis was done using propidium iodide staining. For this, cells were trypsinized, washed in PBS, and then fixed in 90% ethanol. Fixed cells were then washed twice in PBS and stained with 50 μmol/L propidium iodide in PBS containing 5 μg/mL DNase-free RNase for 1 h and then analyzed by flow cytometry using a FACS canto (BD Biosciences) and FACSDiva software (BD Biosciences).

Western Blotting

Western blotting was done on whole cell extracts prepared by lysing cells in NP40 lysis buffer [1% NP40, 100 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.4), 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 30 mmol/L Na β-glycerophosphate, and protease inhibitors ("Complete" protease inhibitor mixture, as instructed by the manufacturer, Roche Applied Science)] on ice for 15 min. Insoluble material was removed by centrifugation, and protein concentration was determined by Bio-Rad Dc protein assay. Twenty micrograms of protein was size fractionated using SDS-PAGE and electro-transferred onto Protran nitrocellulose membranes (Schliecher and Schuell). Membranes were blocked in 5% bovine serum albumin (BSA) or 5% milk in TBS plus 0.5% Tween for 30 min at room temperature

and then incubated with specific antibodies. Antibodies recognizing total FOXO3a were purchased from Upstate. Antibodies specific for FOXO3a (Thr²⁴)/FOXO3a phosphorylated at Thr³², Akt phosphorylated at the Ser⁴⁷³, total Akt, ERK 1/2 phosphorylated at Thr^{202/204}, total ERK 1/2, were purchased from Cell Signaling Technologies. Antibody to Bin was purchased from Calbiochem (Merck), anti-actin was from Santa Cruz Biotechnology (Autogen Bioclear), and anti-lamin was from Abcam. The mouse monoclonal antibody recognizing human p27^{Kip1} has previously been described (23). Primary antibodies were detected using horseradish peroxidase-linked anti-mouse or anti-rabbit conjugates as appropriate (DAKO) and visualized using the enhanced chemiluminescence detection system (Amersham Biosciences). Quantitation of phospho (P)-FOXO3a, P-Akt, and P-ERK 1/2 protein expression was done using ImageJ software (Image Processing and Analysis in Java).

Preparation of Nuclear and Cytoplasmic Extracts

Cells were lysed on ice for 20 min in cytosolic buffer (10 mmol/L HEPES, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 2 mmol/L DTT), after which NP40 was added to a final concentration of 1%, and then the cells were vortexed for 10 s. Nuclei were sedimented by 30 s centrifugation at 13,000 × *g*, and the supernatant containing the cytoplasmic fraction was snap frozen. The pellet, containing the nuclei, was resuspended in cytosolic buffer with 400 mmol/L NaCl and 1% NP40 and rotated at maximum speed on a wheel at 4°C for 15 min. The samples were then centrifuged at 4°C for 5 min at 13,000 × *g*. The supernatant containing the nuclear fraction was snap frozen and kept at -70°C.

Immunofluorescence

Cells were grown on sterile, 13-mm-diameter coverslips and fixed in 4% formaldehyde before being permeabilized in 0.01% v/v Triton X-100. Coverslips were blocked in PBS containing 3% BSA, and antibody recognizing FOXO3a (Upstate) was added at 50 μg/mL. Specific staining was visualized with a secondary antibody conjugated to Alexa 488 (anti-rabbit; Molecular Probes) and analyzed on a Zeiss confocal microscope with LSM meta 510 software.

Gene Silencing with Small Interfering RNAs

For gene silencing, cells were transiently transfected with 50 nmol/L of the following small interfering RNA (siRNA) reagents purchased from Dharmacon: FOXO3a siGENOME SMARTpool or Non-Targeting siRNA Pool. BT474 cells were cultured in six-well plates until 60% confluent and were transfected with 100 pmol/L of annealed oligonucleotides using OligofectAMINE (Invitrogen) according to manufacturer's instructions. Twenty-four hours after transfection, the cells were treated with 5 μmol/L gefitinib or DMSO vehicle. After 0, 12, 24, 48, and 72 h of treatment, both the suspension and the adherent cells were collected for Western blot analysis, proliferation assay, and flow-cytometric analysis.

Generation of MDA-MB-231-FOXO3a:ER Cell Lines

For the generation of MDA-MB-231 cells stably expressing FOXO3a(A3):ER* (24, 25), 10 μg of pBabe-puromycin-FOXO3a(A3):ER vector or empty vector was transfected

into MDA-MB-231 cells using calcium phosphate precipitation method (26). Cells were selected and maintained in the presence of 1 $\mu\text{g}/\text{mL}$ puromycin (InvivoGen), and clonal cell lines were generated.

Immunohistochemistry

Immunostaining was done on 4- μm sections of formalin-fixed, paraffin-embedded blocks from patient's tumor samples obtained before and after 4 weeks of treatment with 250 mg gefitinib per day (Iressa, AstraZeneca) as part of a neoadjuvant clinical trial with patients with early-stage

breast cancer (9). The control group consisted of the patients that had no treatment between biopsy and tumor resection. Antigen retrieval was done by microwaving the slides in the citrate buffer (pH 6.0). Primary antibody and FOXO3a 1:150 (Upstate) were applied overnight, and DAKO REAL EnVision Detection System, Peroxidase/DAB+ (DAKO) was used to visualize the staining. Intensity of FOXO3a staining for nucleus and cytoplasm was evaluated separately using 0 to 3 score system. A score of zero indicated no staining relative to background, 1+ = weak staining,

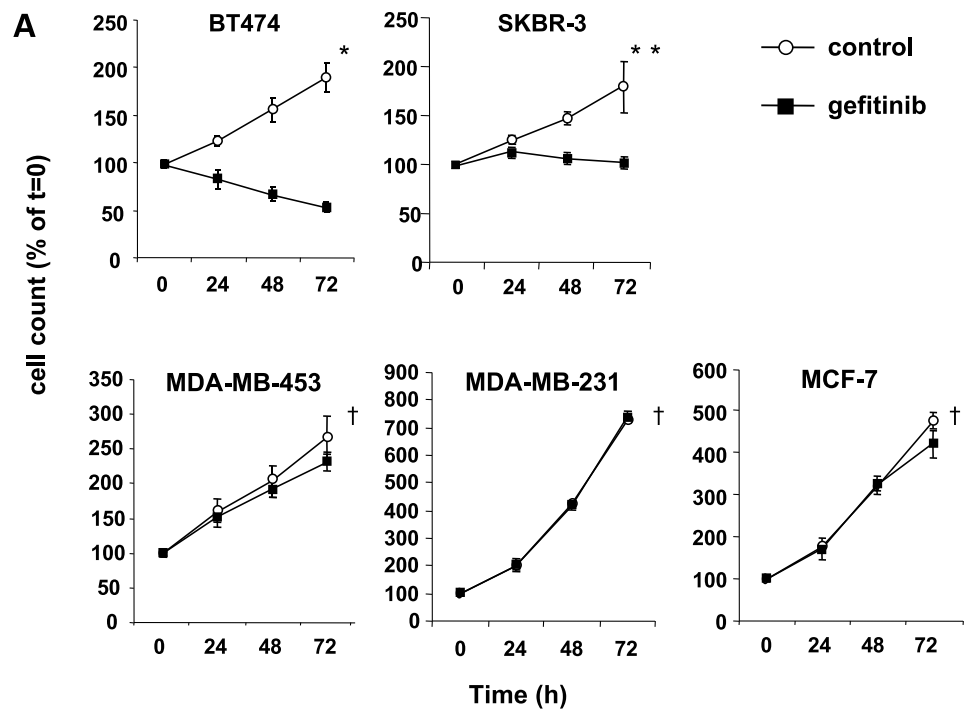
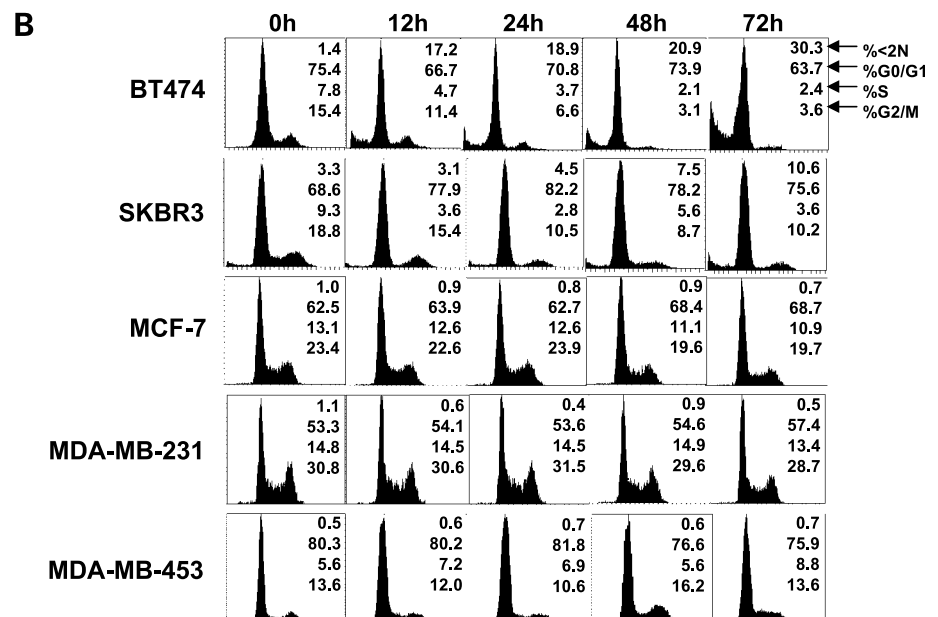


Figure 1. Effect of gefitinib on proliferation and cell cycle distribution of a panel of breast cancer cell lines. **A**, BT474, SKBR3, MCF7, MDA-MB-453, and MDA-MB-231 cells were treated with 5 $\mu\text{mol}/\text{L}$ gefitinib for 0, 24, 48, and 72 h, collected, and counted using Neubauer hemocytometer under trypan blue exclusion. Points, mean of three independent experiments, each at least in duplicate; bars, SD. Statistical analysis has been done using Student's *t* test. *, $P < 0.0001$; **, $P < 0.001$; †, $P = \text{ns}$. **B**, BT474, SKBR3, MCF7, MDA-MB-453, and MDA-MB-231 cells were treated with 5 $\mu\text{mol}/\text{L}$ gefitinib for 0, 12, 24, 48, and 72 h, fixed in ethanol, and stained with propidium iodide, and DNA content was determined by flow cytometry. The percentage of cells in each phase of the cell cycle (sub-G₁, G₀-G₁, S, and G₂-M) is indicated. Representative data from two independent experiments are shown.



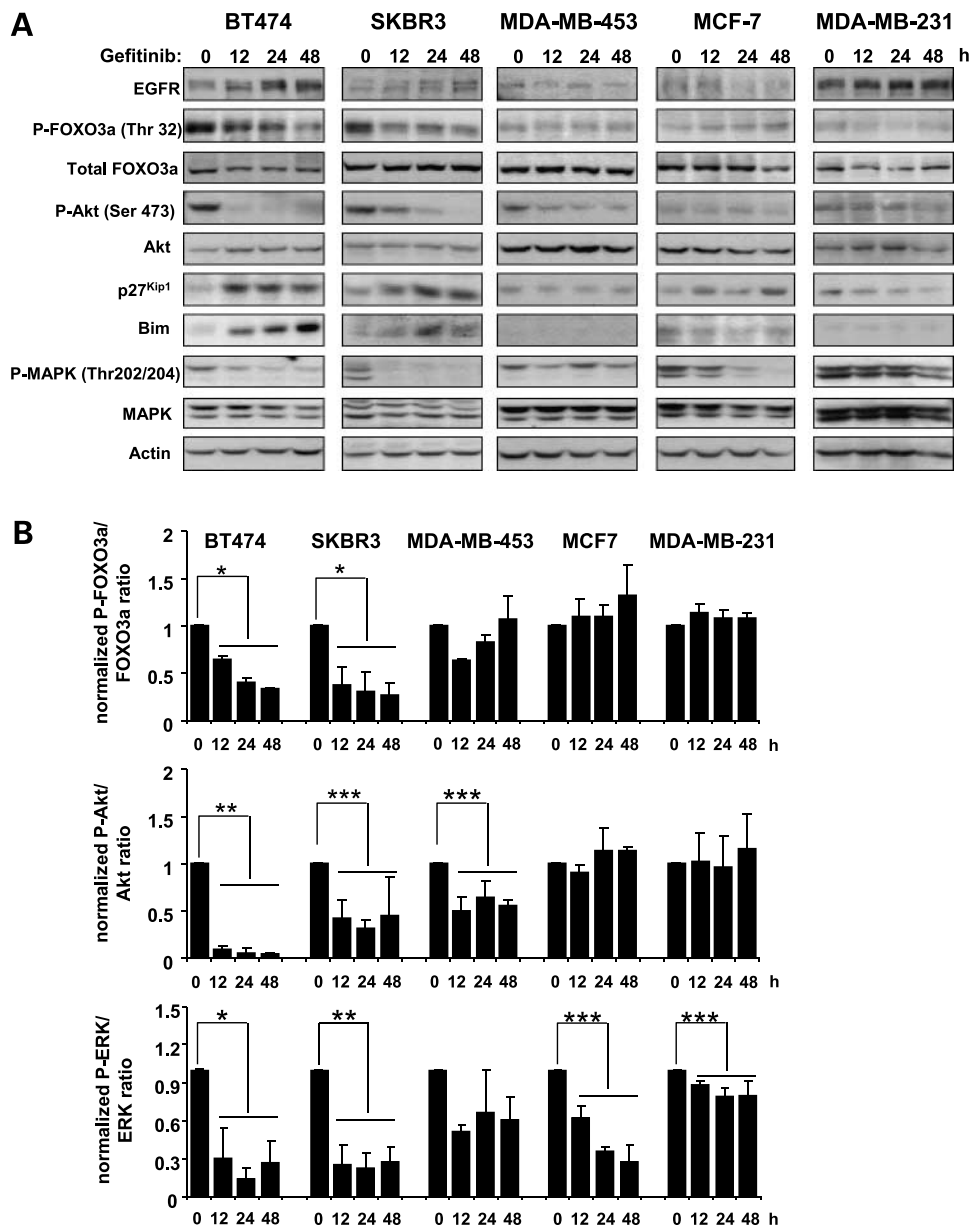


Figure 2. Changes in signal transduction pathway induced by gefitinib. **A**, protein lysates were prepared from breast cancer cells after treatment with 5 $\mu\text{mol/L}$ gefitinib at the time points indicated, and protein expression levels were analyzed by Western blotting using specific antibodies. The Western blot results shown represent the results from a number of attempts, which showed similar trends. Inhibition of phospho-Akt (*P-Akt*) correlates with inhibition of phospho-FOXO3a (*P-FOXO3a*) and induction of FOXO3a targets, Bim and p27^{Kip1} in cells that are sensitive to gefitinib but not in nonsensitive cells. It should be noted that these experiments are designed to follow changes in protein expression during treatment, and because different exposures were used for different cell lines, no comparison between cell lines can be made from these blots. **B**, quantitative analysis of P-FOXO3a, P-Akt, and P-ERK 1/2 protein expression. *Columns*, mean results of two independent experiments after normalizing the data to $t = 0$ h; *bars*, SD. Statistical analysis has been done using Student's *t* test. *, $P < 0.005$; **, $P < 0.001$; ***, $P < 0.05$.

2+ = moderate staining, 3+ = strong staining. For comparison of staining, the results were quantified by calculation of separate *H* scores for nucleus and cytoplasm that consider both staining intensity and the percentage of >cells stained at a specific range of intensities. A complete *H* score was calculated by summing the products of the percentage cells stained at a given staining intensity (0–100) and the staining intensity (0–3).

Statistical Analysis

For statistical evaluation, data were first analyzed for normality of distribution using the Shapiro-Wilk and Kolmogorov-Smirnov test. The equality of variances was tested by using the Levene's test. Significance of the differences between two independent samples has been estimated using Student's *t* test or Mann-Whitney *U* test. Changes in

staining intensity from pre- to posttreatment in control and treatment groups were analyzed using mixed-design ANOVA with post hoc Bonferroni test. The ANOVA design was composed of one between-subjects factor (treatment art: no treatment or gefitinib) and two within-subjects factors (time: pretreatment/posttreatment; cellular localization: nucleus/cytoplasm). Statistical tests were two sided, with the effects reported as significant at $P < 0.05$. Statistical analysis was done with Statistica 6.1 (StatSoft) software.

Results

Effect of Gefitinib on Proliferation of Human Breast Cancer Cell Lines

The effect of gefitinib was evaluated on a panel of five breast cancer cell lines, including BT474, SKBR3, MCF-7,

MDA-MB-231, and MDA-MB-453. Cells were treated with 5 $\mu\text{mol/L}$ gefitinib for 0, 24, 48, and 72 h, and then the cell number was counted using a Neubauer hemocytometer under trypan blue exclusion. Treatment of BT474 and SKBR3 cells with gefitinib resulted in a significant reduction of the number of growing cells compared with nontreated cells ($P < 0.0001$ and $P < 0.001$, respectively; Fig. 1A). Gefitinib had no significant effect on the growth of MDA-MB-453, MDA-MB-231, and MCF-7 at any time point tested (Fig. 1A).

Treatment with Gefitinib Causes G₀-G₁ Arrest and Apoptosis in Sensitive but Not Resistant Cell Lines

To further examine the effect of gefitinib on cell proliferation and survival, we analyzed the cell cycle phase distribution by flow-cytometric analysis of propidium iodide-stained cells after treatment with 5 $\mu\text{mol/L}$ gefitinib for 0, 12, 24, 48, and 72 h. The data shown in Fig. 1B clearly shows that in the sensitive cell lines, BT474 and SKBR3, gefitinib induced G₀-G₁ arrest followed by apoptosis, whereas treatment of MDA-MB-231, MDA-MB-453, and MCF-7 cells with gefitinib did not elicit a cell death response or induce discernible changes in cell-cycle distribution at any time point (Fig. 1B).

EGFR Inhibition by Gefitinib Results in FOXO3a Dephosphorylation in Sensitive and Nonresistant Breast Cancer Cell Lines

To investigate the potential role of FOXO3a in mediating cell cycle arrest and apoptotic response to the EGFR

inhibitor, we compared the changes in protein expression of P-FOXO3a as well as P-Akt and P-ERK 1/2 in the five breast carcinoma lines following treatment with 5 $\mu\text{mol/L}$ gefitinib for 0, 12, 24, and 48 h. In the sensitive cell lines, BT474 and SKBR3, EGFR inhibition was associated with a significant reduction in P-Akt ($P < 0.001$ and $P < 0.05$, respectively) and significant decrease in P-FOXO3a protein levels ($P < 0.005$), whereas in the gefitinib-insensitive cell lines MDA-MB-231 and MCF-7, no significant decrease in the level of P-Akt and P-FOXO3a could be detected (Fig. 2A and B). For the other refractory line MDA-MB-453, gefitinib significantly reduced P-Akt level ($P < 0.05$), but there was no significant change in P-FOXO3a level. This finding suggests that FOXO3a could be, in some cases, a better indicator for gefitinib activity and response than its direct upstream regulator Akt. There were also no significant changes in total FOXO3a, Akt, and ERK levels in response to gefitinib, indicating that gefitinib modulates the activity of these signaling intermediates predominantly at post-translational levels. Notably, in both the sensitive and resistant cell lines, gefitinib treatment caused ERK dephosphorylation, suggesting that the ERK MAP kinase might not be essential for the cytotoxic effects of gefitinib in breast cancer cells (Fig. 2A and B). Furthermore, the ability of gefitinib to inhibit ERK signaling in the insensitive cells also implies that gefitinib resistance in breast cancer cell lines is due to deregulation of downstream signals rather

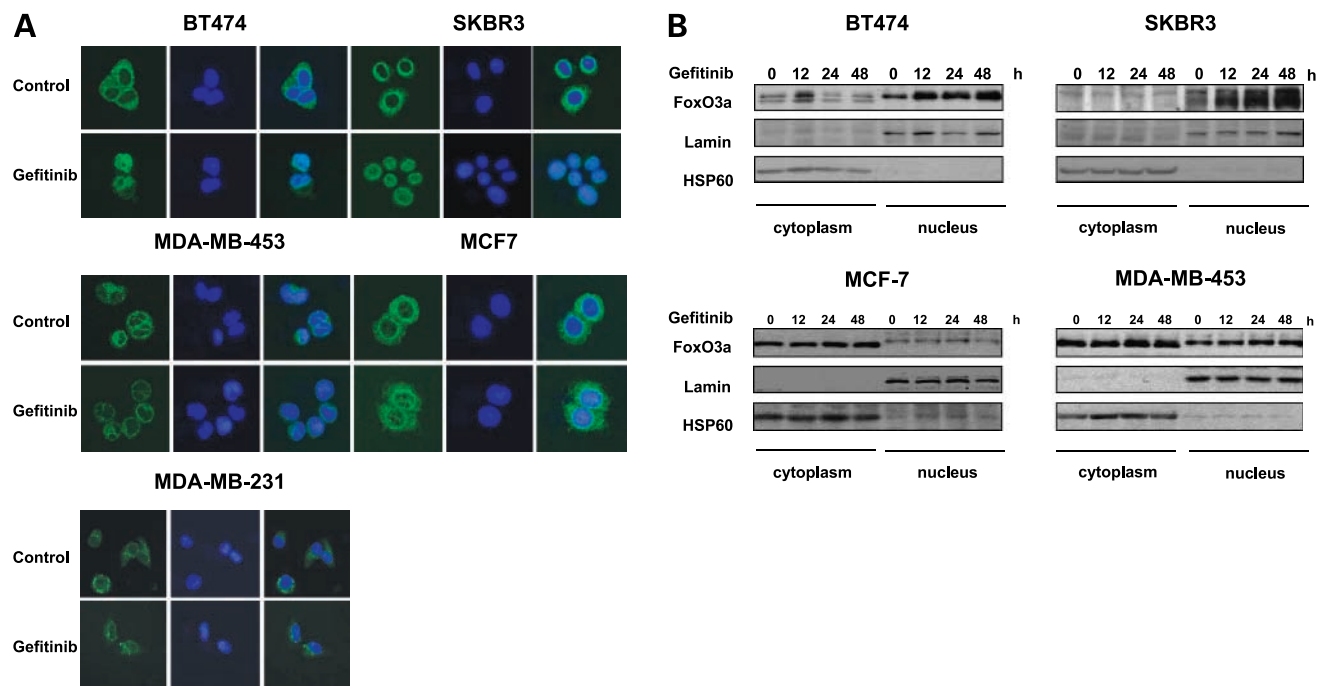


Figure 3. Gefitinib causes nuclear translocation of FOXO3a in sensitive BT474 and SKBR3 cells, but not in resistant MCF7, MDA-MB-453, and MDA-MB-231 cells as detected by confocal microscopy and Western blot analysis. **A**, BT474, SKBR3, MCF-7, MDA-MB-453, and MDA-MB-231 cells were cultured on sterile coverslips and treated for 24 h with 5 $\mu\text{mol/L}$ gefitinib or vehicle, before being fixed in 4% formaldehyde. FOXO3a was visualized with a rabbit polyclonal antibody followed by the addition of Alexa 488 (green)-labeled anti-rabbit antisera. 4',6-Diamidino-2-phenylindole (blue) was also applied to visualize the nuclei. **B**, nuclear and cytoplasmic lysates were prepared after treatment with 5 $\mu\text{mol/L}$ gefitinib at the times indicated, and changes in FOXO3a protein level were analyzed by Western blotting.

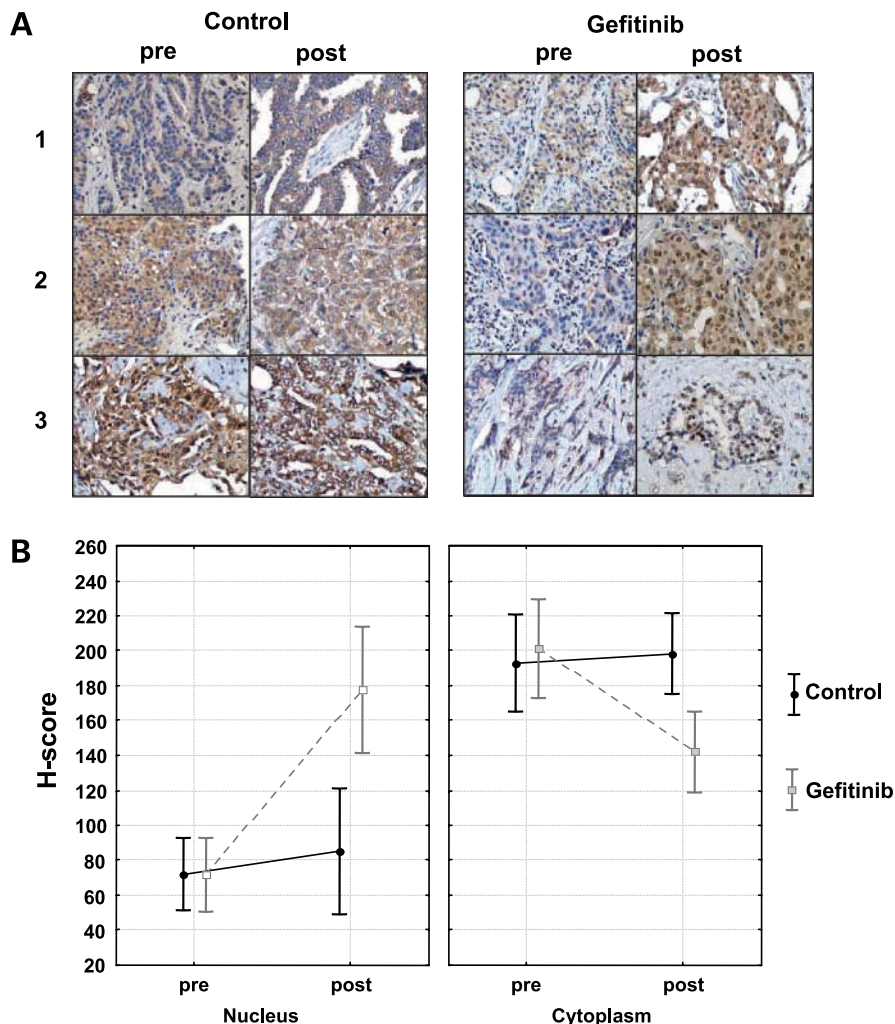


Figure 4. Immunohistochemical staining for FOXO3a of breast cancer tumor tissues. **A**, tumor tissue sample obtained from breast cancer patients before and 4 wk after treatment with gefitinib were stained with FOXO3a antibody. Control group of patients received no treatment between primary biopsy and subsequent surgical tumor resection. In the control group, cytoplasmic staining can be observed in both pre- and post-cases, whereas in the gefitinib-treated group, cytoplasmic staining in pretreatment cases changes to predominantly nuclear staining after treatment (*post*; magnification, $\times 200$). **B**, statistical analysis using a mixed-design ANOVA with post hoc Bonferroni test revealed significant increase in FOXO3a nuclear staining intensity with a concomitant decrease in cytoplasmic staining after gefitinib treatment, whereas no such changes were observed in the control group.

than EGFR itself. We also obtained data showing that similar results were obtained after treatment with $1 \mu\text{mol/L}$ lapatinib in these breast carcinoma cell lines (data not shown).

FOXO3a induces the expression of the cyclin-dependent kinase inhibitor $p27^{\text{Kip1}}$ and the BH3-only protein Bim in breast cancer cells to mediate cell cycle arrest and cell death, respectively (22, 27). To determine if decreased phosphorylation of FOXO3a in response to gefitinib was associated with its activation, we analyzed the expression of the FOXO3a targets, $p27^{\text{Kip1}}$ and Bim. As shown in Fig. 2, there was an increase in $p27^{\text{Kip1}}$ and Bim protein expression in the sensitive BT474 and SKBR3 cell lines, but not in the insensitive lines MDA-MB-231, MD-MB-453, and MCF-7 after gefitinib treatment.

Gefitinib Induces Nuclear Translocation of FOXO3a

It is well characterized that phosphorylation by Akt relocates FOXO3a to the cytoplasm and inhibits its transactivation activity (18). To investigate further the mechanism of action of gefitinib, we next studied the subcellular localization of FOXO3a in response to gefitinib treatment

by confocal microscopy following staining with a FOXO3a-specific antibody. In the untreated gefitinib-sensitive BT474 and SKBR3 cells, endogenous FOXO3a resided almost exclusively in the cytoplasm, whereas 24 h after gefitinib treatment, FOXO3a relocated predominantly to the nucleus (Fig. 3A). Conversely, in the resistant MDA-MB-453, MDA-MB-231, and MCF-7 cell lines, FOXO3a remained predominantly in the cytoplasm before and after gefitinib treatment (Fig. 3A). The change in FOXO3a localization was further confirmed by Western blot analysis of nuclear/cytoplasmic lysates. As shown in Fig. 3B, treatment of BT474 and SKBR3 cells with gefitinib resulted in a decrease in FOXO3a level in the cytoplasm and a parallel increase in FOXO3a in the nucleus. Consistent with the confocal microscopy findings, little or no changes in cytoplasmic and nuclear distribution of FOXO3a was detected in the insensitive cell lines after gefitinib treatment (Fig. 3B). Together, these results suggest that gefitinib induces FOXO3a dephosphorylation and nuclear translocation in sensitive breast carcinoma cells to mediate G_0 - G_1 cell cycle arrest and apoptosis through activating the expression of gene products.

Gefitinib Induces FOXO3a Nuclear Accumulation in Breast Cancer Patients' Samples

To extend our *in vitro* findings, we analyzed by immunohistochemical staining the expression and subcellular localization of FOXO3a in cancer patient samples obtained before and after 4 weeks of gefitinib treatment, as part of a recently carried out presurgical study in patients with early breast cancer (9). Staining was done on 13 cases that included patients with tumor size reduction more than 1 cm measured by ultrasonography. As the control group, we used tumor tissue sections from 14 patients that had no treatment between biopsy and surgical tumor excision (Fig. 4A). After estimating staining intensity using *H* scores for nucleus and cytoplasm separately, the results were analyzed using a mixed-design ANOVA with a post hoc Bonferroni test (Fig. 4B). There was no significant difference in total (nuclear plus cytoplasmic) FOXO3a levels between treatment groups (i.e., control and treated groups, $P = 0.64$) and times of treatment (i.e., pre- and posttreatment, $P = 0.116$). The analysis also showed no evidence of interaction between treatment and time, indicating that there are no significant differences in total FOXO3a levels before and after treatment ($P = 0.58$). However, the analysis showed that there was a significantly higher intensity of FOXO3a

staining in the cytoplasm than the nucleus ($P < 0.001$). FOXO3a staining was stronger in the cytoplasm than the nucleus in control group, whereas no such differences were noted in the treated group, resulting in a significant interaction between subcellular localization and treatment groups ($P < 0.001$). When analyzing the interaction between time of treatment and subcellular localization, there was a significant increase in nuclear but not cytoplasmic FOXO3a staining ($P < 0.001$). Addition of treatment as a third variable to the analysis resulted in a significant triple interaction (time of treatment \times subcellular localization \times treatment groups; $P < 0.001$). Post hoc test was done to break down this interaction, and the analysis showed that before treatment, cytoplasmic staining was significantly stronger than nuclear staining in both the control and treated groups ($P < 0.001$ for both). Furthermore, after gefitinib treatment, there was a significant increase in nuclear staining intensity ($P < 0.001$), with a concomitant decrease in cytoplasmic staining intensity ($P = 0.005$), whereas in the control group, there were no such changes in staining intensity in both nucleus and cytoplasm. These findings suggested that gefitinib treatment causes subcellular translocation of FOXO3a from cytoplasm to nucleus in breast tumor cells of cancer patients sensitive to the drug, confirming our

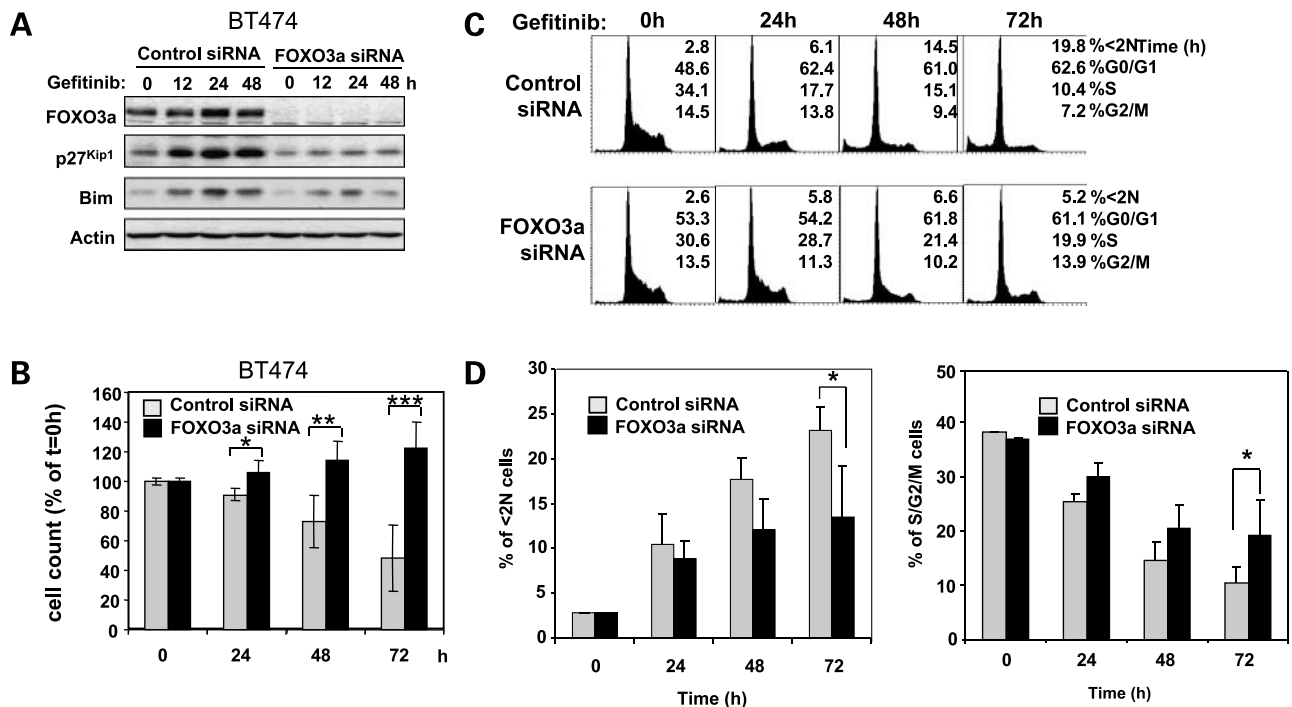


Figure 5. Effect of FOXO3a-specific siRNA on expression of FOXO3a, p27^{Kip1} protein level, and cell proliferation in response to gefitinib treatment in BT474 cells. BT474 cells transfected with control siRNA pool or FOXO3a siRNA pool were treated with gefitinib for the times indicated. **A**, protein lysates were prepared at the times indicated and analyzed by Western blotting. **B**, cell counts at times indicated following gefitinib treatment. Columns, mean of three independent experiments, each in triplicate; bars, SD. Statistical evaluation was done using Student's *t* test. *, $P < 0.005$; **, $P < 0.001$; ***, $P < 0.0001$. **C**, parallel cells were also fixed in ethanol, and the DNA content was analyzed after propidium iodide staining by flow cytometry. The percentage of cells in each phase of the cell cycle (sub-G₁, G₀-G₁, S, and G₂-M) are indicated. Representative data from three independent experiments are shown. **D**, comparison of proliferating cells, as judged by total cells in S-G₂-M phases, and apoptotic sub-G₁ cells between BT474 cells transfected with FOXO3a-specific or nonspecific siRNA during gefitinib treatment. Columns, mean of the three independent experiments; bars, SD. Statistical evaluation was done using the Mann-Whitney *U* test. *, $P < 0.05$, statistically significant differences.

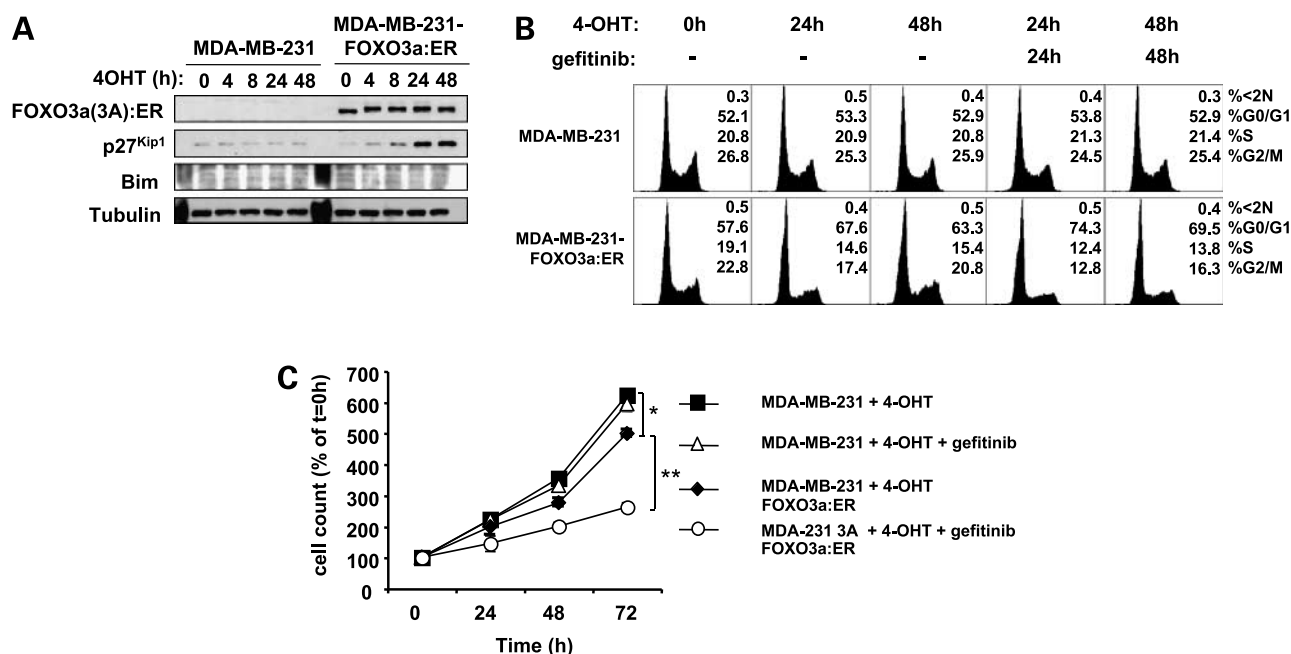


Figure 6. Effect of FOXO3a(A3):ER induction and gefitinib treatment on FOXO3a and p27^{Kip1} expression and cell proliferation in MDA-MB-231 cells. MDA-MB-231-FOXO3a(A3):ER and MDA-MB-231 wild-type cells were treated with 4OHT and/or gefitinib for the indicated times. **A**, cell lysates were prepared at the times indicated, and the expression of FOXO3a(A3):ER, p27^{Kip1}, Bim, and β -tubulin was analyzed by Western blotting. **B**, cells were fixed at 0, 24, and 48 h after treatment, and cell cycle phase distribution was analyzed by flow cytometry after propidium iodide staining. The percentages of cells in each phase of the cell cycle (sub-G₁, G₀-G₁, S, and G₂-M) are indicated. Representative data from three independent experiments are shown. **C**, cell counts at times indicated following gefitinib treatment. Points, mean of two independent experiments each in triplicate; bars, SD. Statistical analysis has been done using Student's *t* test. *, *P* < 0.05, for MDA-MB-231 versus MDA-MB-231 FOXO3a:ER; **, *P* < 0.01, for MDA-MB-231 FOXO3a:ER versus MDA-MB-231 FOXO3a:ER + gefitinib.

findings from the tissue culture cells. Nevertheless, we were unable to study the contribution of FOXO3a in gefitinib resistance in these samples because of the fact that almost all patients responded to gefitinib because these were primary breast cancer patients without prior drug treatments.

Silencing of FOXO3a by siRNA Results in a Reduction of Gefitinib-Induced Cell Death and Proliferative Arrest

To show that FOXO3a has an essential role in response to gefitinib treatment, we transfected the gefitinib-sensitive BT474 breast carcinoma cells with either a FOXO3a-specific siRNA or a nonspecific control siRNA pool and studied the proliferation of these cells after gefitinib treatment. Western blot analysis showed that the FOXO3a-specific siRNA, but not control siRNA, knocked down the expression of endogenous FOXO3a in the BT474 cells (Fig. 5A). Moreover, the FOXO3a-specific siRNA, but not the control siRNA, inhibited the induction of p27^{Kip1} and also reduced the induction of Bim by gefitinib treatment. Consistent with our earlier results (Fig. 1A), treatment of control siRNA-transfected BT474 cells with gefitinib resulted in a dramatic decrease in a number of growing cells (*P* < 0.001; Fig. 5B). Conversely, the gefitinib-induced decrease in cell proliferation was overcome in BT474 cells treated with FOXO3a targeting siRNA pool (*P* < 0.001), indicating that FOXO3a is responsible for the effect of gefitinib on cell growth (Fig. 5B). Consistent with this, cell cycle analysis showed that silencing of FOXO3a by siRNA can partially rescue cells

from gefitinib-induced proliferative arrest and cell death. This is evident from the increase in the fraction of cells in S and G₂-M phases (*P* < 0.05 at 72 h) and reduction in the apoptotic sub-G₁ fraction (*P* < 0.05 at 72 h) in siFOXO3a-transfected BT474 cells compared with control cells (Fig. 5C and D). These results suggested that gefitinib targets FOXO3a to mediate cell death and proliferative arrest in sensitive breast carcinoma cells.

Reintroduction of FOXO3a Restores Proliferative Arrest and Sensitivity in Gefitinib-Resistant Breast Cancer Cells

To test the hypothesis that FOXO3a dephosphorylation and nuclear translocation crucially determine sensitivity to gefitinib, we introduced an inducible form of active FOXO3a, FOXO3a(A3):ER*, in which all three Akt phosphorylation sites were mutated to alanine into the highly resistant ER-negative breast cancer cell line MDA-MB-231. Addition of 4-hydroxytamoxifen (4-OHT) to these MDA-MB-231-FOXO3a:ER cells results in the rapid nuclear translocation and induction of FOXO3a activation, recapitulating the FOXO3a dephosphorylation and nuclear accumulation following gefitinib treatment (Supplementary Fig. S1).³ Additionally, we found that although p27^{Kip1}

³ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

is up-regulated upon activation of FOXO3a in the MDA-MB-231-FOXO3a:ER cells, Bim expression is not induced (Fig. 6A). Cell cycle analysis showed that the reintroduction of an activated mutant of FOXO3a in MDA-MB-231 restored proliferative arrest, indicating that the failure of FOXO3a dephosphorylation and nuclear translocation in response to gefitinib treatment is essential for conferring resistance to MDA-MB-231 cells (Fig. 6B). More importantly, our results showed that despite gefitinib alone having no effect on the cell cycle progression of MDA-MB-231 cells, gefitinib further reduced cells in the S and G₂-M phase compared with FOXO3a induction alone, suggesting that FOXO3a activation resensitizes resistant cells to gefitinib (Fig. 6B). We also evaluated the effect of FOXO3a induction on the growth of MDA-MB-231 cells by cell counting. Consistent with our previous results (Fig. 1A), treatment of MDA-MB-231 cells with gefitinib did not affect cell growth (Fig. 6C). However, induction of FOXO3a activity by tamoxifen in MDA-MB-231-FOXO3a:ER cells significantly decreased cell proliferation starting from the 48-h time point ($P < 0.05$), and additional treatment with gefitinib further significantly reduced cell growth ($P < 0.01$; Fig. 6C). It is notable that FOXO3a induction in MDA-MB-231 cells activated cell cycle arrest but little apoptosis. The reason for this is unclear; however, one potential explanation could be that in MDA-MB-231 cells, expression of the proapoptotic FOXO3a target Bim is deregulated. Consistent with this idea, we have shown here and previously that Bim expression is undetectable in MDA-MB-231 cells in response to different apoptotic stimulations (22, 27). Taken together, these results confirmed that not only is FOXO3a an integral signaling molecule in the anti-mitogenic action of gefitinib, its function also determines the sensitivity to gefitinib in human breast cancer cells.

Discussion

EGFR plays a central role in the development and progression of a number of common malignancies of epithelial origins, including breast, colon, pancreatic, and lung cancers (28–30). As a consequence, EGFR has emerged as an obvious molecular target for cancer therapy, with monoclonal antibodies (e.g., Cetuximab) and small-molecule TKIs, such as gefitinib (Iressa) being developed to specifically block EGFR signaling (5, 31). These anti-EGFR agents have shown promising outcomes in initial preclinical and clinical settings; however, there is also a high rate of *de novo* and acquired resistance, which limits the efficacy of such treatments (32). Nevertheless, lapatinib, the dual EGFR and HER2 inhibitor, has been shown to improve disease-free survival in advanced breast cancer patients (33).

In pulmonary adenocarcinoma, resistance-related secondary mutations (e.g., T790M) in EGFR have been shown to occur after initial gefitinib treatment, but these mutations are uncommon and, therefore, unlikely to be the major cause of acquired resistance in breast cancer. Consistent with this, the immediate signaling is intact in most

gefitinib- and lapatinib-resistant breast cancer cells, indicating that signaling events further downstream of EGFR determine the sensitivity to these anti-EGFR agents. We have screened a panel of breast carcinoma cell lines (data not shown) and identified two cell lines, BT474 and SKBR3, which are sensitive to gefitinib treatment. Using these two gefitinib-sensitive as well as three other resistant breast carcinoma cell lines (i.e., MCF7, MDA-MB-231, and MDA-MB-453), we have shown that gefitinib targets the transcription factor FOXO3a to mediate cell cycle arrest and cell death in sensitive breast cancer cells, BT474 and SKBR3. In the sensitive cells, gefitinib or lapatinib treatment causes cell cycle arrest predominantly at the G₀-G₁ phase and apoptosis, which is associated with FOXO3a dephosphorylation at Akt sites and nuclear translocation, whereas in the resistant cells, FOXO3a stays phosphorylated and remains in the cytoplasm. These results were verified by immunohistochemical staining of breast cancer patient biopsy samples. Although this pilot *in vivo* study is limited by a relatively small sample size, the results show that nuclear expression of FOXO3a is significantly increased in patients treated with gefitinib compared with those untreated. Furthermore, silencing of the endogenous FOXO3a expression by siRNA at least partially rescued the highly sensitive carcinoma cell line BT474 from undergoing proliferative arrest and apoptosis in response to gefitinib, suggesting that the induction of FOXO3a expression has a direct role in mediating the effect of gefitinib on cell proliferation and survival. A number of other findings also suggested that activation of FOXO3a plays an important role in determining sensitivity to gefitinib. First, gefitinib induced FOXO3a dephosphorylation and nuclear translocation only in the sensitive cells, whereas other signaling events, such as ERK dephosphorylation, proceeded normally in both sensitive and resistant cells. Second, expression of an inducible, constitutively dephosphorylated FOXO3a mutant was sufficient to partially restore sensitivity to gefitinib in the highly resistant MDA-MB-231 cells. These results are consistent with previous published data showing that lapatinib also targets FOXO3a in breast cancer cells to induce apoptosis (34, 35). Furthermore, blockage of EGFR and HER2 signaling using monoclonal antibodies cetuximab and trastuzumab also induces nuclear translocation of FOXO3a (36). Taken together, our data and those of others showed that FOXO3a is an essential target of anti-EGFR and HER2 therapies in breast cancer. However, because silencing of FOXO3a expression in BT474 cells does not completely rescue the cells from apoptosis, it is reasonable to conclude that other mechanisms can also be involved in determining response to gefitinib treatment.

A previous study using BT474 as cell culture model has attributed the cytotoxic effect of lapatinib solely to their ability to inhibit ERB2 and not EGFR, based on the observation that EGFR could not be detected in these cells (34). Despite this report, we show that BT474 is sensitive to gefitinib, which only targets EGFR and not ERB2. In support of our findings, others have previously established

that EGFR is expressed in BT474 breast carcinoma cells (37). Overexpression of EGFR is only one of the mechanisms responsible for the activation of EGFR signaling pathway. The absence of direct correlation between EGFR expression levels and response to the treatment with EGFR inhibitors has been previously reported (38). Consistent with this, our data show that MDA-MB-231 cells express high EGFR levels and are resistant to gefitinib treatment, whereas BT474 cells have low EGFR expression, but are highly sensitive to gefitinib. Similarly, neither ERK 1/2 nor Akt activity has been proven to be a reliable predictor of gefitinib response (39). These studies are in accordance with our results showing that a decrease in phospho-ERK 1/2 and phospho-Akt does not necessarily associate with response to gefitinib. Although Akt plays an important role in the regulation of FOXO3a activity, it is not the only kinase that can phosphorylate and deactivate FOXO3a. For example, serum- and glucocorticoid-inducible kinase (SGK) can also phosphorylate FOXO3a at Thr³² (40). This can explain the failure of gefitinib to reduce FOXO3a phosphorylation in MDA-MB-453 cells despite an inhibitory effect on Akt.

Gefitinib is able to sensitize breast cancer cells to chemotherapy-induced apoptosis (31), which could be due to the fact that gefitinib and chemotherapeutic agents mediate cell death through overlapping signaling pathways. Consistent with this idea, we have previously shown that chemotherapeutic drugs, such as paclitaxel, mediate cell cycle arrest and apoptosis through enhancing FOXO3a activity in breast cancer cells (22, 27). Because gefitinib and lapatinib are, in general, effective, safe, and well-tolerated therapeutic agents (41), it may be beneficial to concurrent chemotherapy and gefitinib or lapatinib treatments.

In summary, the present study helps to define the mechanism by which gefitinib mediates cell cycle arrest and apoptosis in breast cancer. It also reveals that the transcription factor FOXO3a could be a useful early biomarker for predicting outcomes and for monitoring anti-EGFR and HER2 therapies as well as a biomarker for the clinical course of anti-EGFR/HER2 agents. Moreover, our results suggest that targeting FOXO3a in breast cancer could potentially improve the effectiveness of anti-EGFR/HER2 agents, such as gefitinib and lapatinib.

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