Gefitinib (Iressa) represses FOXM1 expression via FOXO3a in breast cancer


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Abstract

Gefitinib (Iressa) is a specific and effective epidermal growth factor receptor inhibitor. An understanding of the downstream cellular targets of gefitinib will allow the discovery of biomarkers for predicting outcomes and monitoring anti-epidermal growth factor receptor therapies and provide information for overcoming gefitinib resistance. In this study, we investigated the role and regulation of FOXM1 in response to gefitinib treatment in breast cancer. Using the gefitinib-sensitive breast carcinoma cell lines BT474 and SKBR3 as well as the resistant lines MCF-7, MDA-MB-231, and MDA-MB-453, we showed that gefitinib represses the expression of the transcription factor FOXM1 in sensitive, but not resistant, cells. FOXM1 repression by gefitinib is associated with FOXO3a activation and is mediated at the transcriptional level and gene promoter level. These results were verified by immuno-histochemical staining of biopsy samples from primary breast cancer patients obtained from a gefitinib neoadjuvant study. We also showed that ectopic expression of an active FOXO3a represses FOXM1 expression, whereas knockdown of FOXO3A expression using small interfering RNA can up-regulate FOXM1 and its downstream targets polo-like kinase, cyclin B1, and CDC25B and rescue sensitive BT474 cells from gefitinib-induced cell proliferative arrest. These results suggest that gefitinib represses FOXM1 expression via FOXO3a in breast cancer. We further showed that overexpression of a wild-type FOXM1 or a constitutively active FOXM1, ΔN-FOXM1, abrogates the cell death induced by gefitinib, indicating that FOXM1 has a functional role in mediating the gefitinib-induced proliferative arrest and in determining sensitivity to gefitinib. In summary, our study defined FOXM1 as a cellular target and marker of gefitinib activity in breast cancer.

Introduction

Breast cancer is the most prevalent women’s cancer in industrialized countries and the second biggest killer behind lung cancer (1). Recent research has suggested that overactivation of growth factor signaling cascades can support hormone-independent tumor cell growth and promote endocrine resistance in breast cancer (2, 3). The epidermal growth factor receptor (EGFR or ErbB1) was one of the first growth factor receptors to be characterized and is the founding member of a family of receptor tyrosine kinases, which also include HER-2 (ErbB2, neu), HER-3 (ErbB3), and HER-4 (ErbB4; refs. 4, 5). Like other family members, EGFR relays extracellular growth factor signals to the intracellular compartments, regulating cell proliferation, differentiation, survival, angiogenesis, and metastasis (6, 7). Aberrant EGFR signaling, whether due to EGFR overexpression, gene amplification, mutation, or rearrangement, has been reported in breast cancer as well as cancers of the lung, colon, head and neck, bladder, brain, prostate, and esophagus. A study combining the data from 40 studies of a total of 5,232 breast cancer cases has found EGFR to be positive in 48% of all breast cancers (8). In fact, EGFR plays an important role in breast carcinogenesis, and consistently, ectopic expression of EGFR has also been shown to induce hormone independence in human breast cancer cells (8).

The critical role of EGFR in cancer development and progression has made the receptor a highly promising target for the development of more specific, molecularly targeted cancer therapies. Gefitinib, also called Iressa or ZD1839, is a selective EGFR tyrosine kinase inhibitor that acts through competitive inhibition of ATP binding to the intracellular catalytic domain of EGFR tyrosine kinase, thus blocking EGFR autophosphorylation and downstream signaling (4, 6, 9, 10). Gefitinib can cause growth delay in breast cancer cell lines and xenografts expressing high levels of EGFR (6, 11). Recent phase II clinical studies showed that gefitinib shows antitumor activity in patients with breast cancer when used as a monotherapy or in combination with other drugs, such as docetaxel or anastrozole.
indicating that gefitinib can potentiate the antiproliferative effects of antiestrogens and conventional chemotherapeutic agents. However, the clinical data also revealed that not all patients respond to gefitinib (13–15) and that even high levels of EGFR expression do not necessarily correlate with clinical response to gefitinib treatment (15–18). Thus, a better comprehension of the downstream cellular targets of gefitinib is required for the identification of molecular markers, which may allow the selection of patients more likely to benefit from treatment as well as for monitoring anti-EGFR therapies and the development of novel treatment strategies for patients positive for EGFR but resistant to gefitinib and other anti-EGFR therapies.

FOXM1 is a member of the forkhead box superfamily of transcription factors and a crucial transcriptional regulator of cell cycle progression (19–21). Consistently, depletion of FOXM1 expression results in cell cycle arrest, mitotic spindle defects, chromosome misaggregation, and mitotic catastrophe (19, 22, 23). FOXM1 controls these cellular functions through regulating the expression of several cell cycle regulators, including p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, cyclin B, Cks1, CDC25B, Aurora B kinase, survivin, polo-like kinase-1 (PLK1), centromere proteins A and B, Nek-2, and KIF20A (19, 20). Besides cell proliferation, FOXM1 has also been implicated in other processes involved in tumorigenesis and cancer progression, such as angiogenesis and the development of metastases (21). Previous studies have shown that FOXM1 is overexpressed in breast cancer and that elevated FOXM1 promotes tumor progression in malignancies such as gliomas, colorectal cancers, hepatocellular and prostate carcinomas, and lung adenocarcinomas (22–28). We have shown previously that 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 was obtained from AstraZeneca and dissolved in DMSO to give 30 mmol/L stock solution for cell culture work.

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 was obtained from AstraZeneca and dissolved in DMSO to give 30 mmol/L stock solution for cell culture work.

Plasmids and Transfections

The human FOXM1 promoter construct was a gift from Prof. Rene H. Medema (University Medical Center Utrecht) and has been described previously (30). The human EGFR expression vector pcDNA3.1-EGFR cDNA was a generous gift from Prof. William J. Gulick (University of Kent). For transfections, cells were seeded to a confluence of ~50% and incubated for 6 h with a master mix of transfection reagents containing Fugene-6 (Qiagen) and the plasmid DNA in a ratio of 3:1 diluted in Opti-MEM. Fresh medium was then added to replace the transfection reagent and cells were allowed to grow overnight before use.

Luciferase Reporter Assay

Cells were transfected with the human FOXM1 promoter and <i>Renilla</i> (pRL-TK; Promega) as internal transfection control using Fugene-6 (Qiagen). For promoter analysis, 24 h after transfection, cells were then collected, washed twice in PBS, and harvested for firefly/<i>Renilla</i> luciferase assays using the Dual-Glo Luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Luminescence was then read using the 9604 TopCount Perkin-Elmer plate reader.

Western Blotting

Western blotting was done on whole-cell extracts as described previously (29). Antibodies used were p-HER-2 (PN2A; Biosource, Invitrogen), EGFR (610016; Becton Dickinson UK), and p-EGFR and CDC25B (Abcam). Other antibodies, including HER-2 (C-18), cyclin B (H-433), PLK (F8), tubulin (H235), FOXM1 (C-20), and actin (I-19) were obtained from Santa Cruz Biotechnology (AutoGen Bioclear). 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 protein expression was done using ImageJ software (Image Processing and Analysis in Java).

Real-time Quantitative PCR

Total RNA was isolated using the RNeasy kit (Qiagen). Total RNA (1 μg) was reverse transcribed using the SuperScript first-strand synthesis system for real-time quantitative PCR (Invitrogen) and the resulting first-strand cDNA was used as the template in the real-time quantitative PCR analysis. All samples were done in triplicates. The following gene-specific primer pairs were designed using the ABI Primer Express software: FOXM1-sense 5′-TGCAAGCTAGGATGTGATCTTC-3′ and FOXM1-antisense 5′-GGAGCCCATCTCCATCAGA-3′ and L19-sense 5′-GGCGAAGGGTACAGCAAT-3′ and L19-antisense GCAGCAGCGCGCAA-3′. Specificity of each primer was determined using National Center for Biotechnology Information BLAST module. Real-time PCR was done with ABI PRISM 7700 Sequence Detection System using SYBR Green Mastermix (Applied Biosystems). FOXM1 and L19 transcript levels were quantified using the standard curve method. L19, a nonregulated ribosomal housekeeping gene, was used as an internal control to normalize input cDNA.

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: EGFR or FOXO3a siGENOME SMARTpool or Nontargeting siRNA Pool. BT474 and SKBR3 cells were cultured in six-well plates until 60% confluent and transfected with 100 pmol/L annealed oligonucleotides using OligofectAMINE (Invitrogen) according to the manufacturer’s instructions. Both the
Gefitinib Represses FOXM1 via FOXO3a in Breast Cancer

Tyr845 (2231; Cell Signaling), FOXO3a (1:150; Upstate), citrate buffer (pH 6.0). Primary antibodies, p-EGFR at between biopsy and tumor resection. Patients in tumor characteristics but received no treatment reasons. These control patients therefore matched the trial the final screening due to either personal choice or timing positive primary breast cancers but were not enrolled after the final screening due to either personal choice or timing reasons. These control patients therefore matched the trial patients in tumor characteristics but received no treatment between biopsy and tumor resection.

Antigen retrieval was done by microwaving the slides in citrate buffer (pH 6.0). Primary antibodies, p-EGFR at Tyr2231 (Cell Signaling), FOXO3a (1:150; Upstate), FOXM1 (C-20; dilution 1:450; Santa Cruz Biotechnology), and Ki-67 (Novocastra), were applied overnight and EGF positive primary breast cancers but were not enrolled after the final screening due to either personal choice or timing reasons. These control patients therefore matched the trial patients in tumor characteristics but received no treatment between biopsy and tumor resection.

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 treatment with 250 mg/d gefitinib as part of a neoadjuvant clinical trial with patients with early-stage breast cancer (12). The control group consisted of patients who were initially scanned for the trial. These were eligible patients who were postmenopausal and had estrogen receptor and EGFR positive primary breast cancers but were not enrolled after the final screening due to either personal choice or timing reasons. These control patients therefore matched the trial patients in tumor characteristics but received no treatment between biopsy and tumor resection.

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To investigate whether FOXM1 is a downstream signaling target of gefitinib in breast cancer, we first studied the effects of the anti-EGFR tyrosine kinase inhibitor gefitinib on FOXM1 expression in sensitive and resistant breast carcinoma cell lines. Initially, a dose titration of gefitinib in the sensitive cell line SKBR3 was done to show the effects of gefitinib on EGFR, FOXM1, and their downstream targets (data not shown; also see Fig. 1D). A dose of 5 μmol/L was selected for subsequent experiments, and this dose is consistent with what others used in previous similar tissue culture studies (32). The gefitinib-sensitive cell lines SKBR3 and BT474 as well as the resistant MDA-MB-453, MCF-7, and MDA-MB-231 cells were then treated with 5 μmol/L gefitinib for 0, 12, 24, and 48 h before protein lysate was subjected to Western blotting (Fig. 1A). Treatment with gefitinib led to a reduction in EGFR phosphorylation, indicating a decrease in EGFR activity by 12 h, in the sensitive SKBR3 and BT474 cells. In SKBR3 cells, the EGFR phosphorylation level recovered by the end of the time course (48 h), whereas EGFR phosphorylation remained low during the remaining time course in BT474 cells. Notably, over the same time course of gefitinib treatment, we detected an increase in total EGFR protein levels possibly due to a feedback mechanism. Interestingly, the pattern of HER-2 phosphorylation followed that of EGFR in the sensitive SKBR3 and BT474 cell lines, indicating that HER-2 also has a role in mediating gefitinib signaling. The apparent recovery of HER-2/EGFR phosphorylation and FOXM1 levels after 48 h gefitinib in SKBR3 may be due to the selection of resistant cells as a result of death of sensitive cells or a negative feedback signaling mechanism. Western blotting also showed that treatment with gefitinib led to a decrease in FOXO3a phosphorylation and a corresponding reduction in FOXM1 level, which coincided with a decrease in the FOXM1 targets CDC25B, PLK1, and cyclin B1. Although levels of phospho-HER2 decrease in gefitinib-treated MDA-MB-453, these cells do not have

Statistical Analysis

To test for differences between Ki-67, nuclear FOXO3a, and FOXM1 expression before and after gefitinib treatment and FOXM1 expression in treated and control groups before and after treatment, Dunnett’s two-tailed t test was done and the mean difference was considered significant at the P < 0.05 level. The relationship between fold changes in H-score in FOXM1 and Ki-67 or nuclear FOXO3a was statistically analyzed using Pearson’s correlation test based on their H-scores and was considered significant at P < 0.05. All statistical analysis was done with SPSS version 15 (SPSS).

Cell Cycle Analysis

Cell cycle analysis was done by combined propidium iodide and bromodeoxyuridine staining or propidium iodide staining alone as described previously (31). Subconfluent cells with or without drug treatment were labeled for 2 h with 10 μmol/L bromodeoxyuridine (Sigma UK). Cells were trypsinized, collected by centrifugation, and resuspended in PBS before fixing in 90% ethanol. The fixed cells were incubated first with 2 N HCl and then with 0.5% Triton X-100 for 30 min at room temperature and with FITC-conjugated anti-bromodeoxyuridine antibodies (Becton Dickinson UK) at 1:3 dilution in 0.1 mol/L sodium borate (pH 8.5) for 30 min, with PBS washes between each treatment. The cells were incubated with 5 μg/mL propidium iodide, 0.1 mg/mL RNase A, 0.1% NP-40, and 0.1% trisodium citrate for 30 min before analysis using a Becton Dickinson FACScan® analyzer. The cell cycle profile was analyzed using Cell Diva software (Becton Dickinson UK).

Results

EGFR Inhibitor Gefitinib Reduces FOXM1 Expression in Sensitive but Not Resistant Breast Cancer Cell Lines

Statistical Analysis

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changes in FOXO3a phosphorylation or FOXM1 levels. This may be due to molecular alterations in these cells, which may perturb signaling from EGFR. Previously, it has been found that MDA-MB-453 cells have genetic changes in phosphatidylinositol 3-kinase and PTEN, critical regulators of the EGFR/HER-2 to FOXO3a signaling cascade, which may affect signaling from the receptor tyrosine kinases (33). For the resistant cells, the expression levels of FOXM1, CDC25B, PLK1, and cyclin B1 remained largely unchanged following gefitinib treatment. Furthermore, real-time quantitative PCR analysis showed that gefitinib reduced FOXM1 mRNA transcripts in the sensitive SKBR3 and BT474 but not in the resistant MDA-MB-453 cell lines (Fig. 1B). Transient transfection of the sensitive cell lines with a full-length FOXM1 promoter reporter construct showed a decrease in promoter activity when treated with gefitinib, whereas there was no change in promoter activity in the resistant MDA-MB-453 cells (Fig. 1C). We next treated the

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Effect of gefitinib on expression of FOXM1 and related signal transduction pathway components in a panel of breast cancer cell lines. 

<table>
<thead>
<tr>
<th>A</th>
<th>BT474</th>
<th>SKBR3</th>
<th>MDA-MB-453</th>
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<th>MDA-MB-231</th>
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**B**

![Graphs](https://example.com/graphs.png)

**C**

![Graphs](https://example.com/graphs.png)

![Graphs](https://example.com/graphs.png)

**D**

![Graphs](https://example.com/graphs.png)
gefitinib-sensitive SKBR3 cells with various doses (0, 0.1, 0.5, 1, 5, and 10 μmol/L) of gefitinib and the cell lysates prepared after 24 h of treatment were analyzed by Western blotting to investigate the effects of gefitinib on EGFR, FOXM1, FOXO3a, and their downstream targets (Fig. 1D). The results showed a dose-dependent decrease in EGFR phosphorylation that paralleled the reduction of FOXM1 expression and the expression of its downstream targets, PLK and cyclin B. This gefitinib-dependent FOXM1 decrease was again associated with a corresponding induction of FOXO3a activity as revealed by a reduction in FOXO3a phosphorylation but not protein level and confirmed by the induction of the FOXO3a targets, Bim and p27Kip1. Collectively, these results suggested that FOXM1 is a cellular target of gefitinib in breast cancer cells and that gefitinib represses FOXM1 expression at least partially at the transcriptional and gene promoter levels. These findings are also consistent with the hypothesis that gefitinib represses FOXM1 through inducing FOXO3a activity.

Gefitinib Induces FOXO3a Nuclear Accumulation and FOXM1 Down-Regulation in Breast Cancer Patient Samples

To further confirm our cell culture findings, we analyzed by immunohistochemical staining the expression of FOXM1 in cancer patient samples obtained before and after 4 weeks of gefitinib treatment in a neoadjuvant study in patients with early breast cancer (12). Staining was done on 11 cases where patients with tumor size reduction >1 cm after gefitinib treatment as measured by ultrasonography (Supplementary Fig. S1).1 Tumor tissue sections from 11 patients that had no treatment between biopsy and surgical tumor excision were used as controls. After estimating staining intensity using H-scores for nuclear FOXO3a, FOXM1, and Ki-67 expression (Fig. 2A and B; Supplementary Fig. S1),1 FOXM1 expression was significantly down-regulated in response to gefitinib, concomitant with a reduction in Ki-67 staining and an increase in the level of nuclear FOXO3a (Fig. 2A; Supplementary Fig. S2).1 Test analysis also indicated a significant difference in FOXM1 staining between the treated group before and after gefitinib (P ≤ 0.05) and between the treated and control groups after treatment (P ≤ 0.05; Fig. 3A; Supplementary Fig. S3).1 However, there were no significant differences in FOXM1 expression between the treated and control groups before treatment and the control group before and after treatment (data not shown). The relationship between fold changes in FOXM1 and Ki-67 or nuclear FOXO3a was statistically analyzed using Pearson’s correlation test based on their H-scores and was considered significant at P ≤ 0.05 (t test). The results indicated a significant and strong positive correlation between FOXM1 and Ki-67 expression (n = 22; Pearson coefficient = 0.697; P ≤ 0.05) and a
significant inverse correlation between FOXM1 and nuclear FOXO3a expression \((n = 20;\) Pearson coefficient \(= -0.669;\) \(P \leq 0.05\)) in response to treatment (gefitinib or control). Together, these findings from the clinical samples support our hypothesis that gefitinib represses cell proliferation and FOXM1 expression through activating FOXO3a (Fig. 3B; Supplementary Fig. S3). 1

**Ectopic FOXO3a Expression Represses FOXM1 Expression in Gefitinib-Sensitive Breast Cancer Cells**

We have shown previously that gefitinib represses phosphatidylinositol 3-kinase-PKB/Akt to induce FOXO3a dephosphorylation and nuclear accumulation in sensitive breast cancer cells (29). The inverse correlation between nuclear FOXO3a and FOXM1 expression in response to gefitinib treatment in breast cancer cells suggested that gefitinib could activate FOXO3a to repress FOXM1 expression. To test this hypothesis, we transiently transfected into the gefitinib-sensitive breast cancer cell line SKBR3 a constitutively active FOXO3a, FOXO3a(A3), in which all three PKB/Akt phosphorylation sites were mutated to alanine. Overexpression of FOXO3a(A3) in cycling SKBR3 cells resulted in a 2-fold down-regulation of FOXM1 expression, which was not observed in nontransfected cells or SKBR3 cells transfected with the empty expression vector (Fig. 4A). Confocal microscopy showed that the inability of FOXO3a(A3) transfection to completely repress FOXM1 expression was due to the fact that less than half of the cells were overexpressing the FOXO3a(A3) construct under optimal transfection conditions in these cells (Fig. 4B). Because gefitinib has been shown to repress FOXM1 expression at the promoter level, we next examined if transiently overexpressing FOXO3a(A3) could also repress FOXM1 promoter activity in SKBR3 cells. The transfection results showed that FOXO3a(A3) could repress the FOXM1 promoter activity in a dose-dependent manner as an increase in levels of the FOXM1 target CDC25B, further supporting that FOXM1 is a cellular target of EGFR and its inhibitor gefitinib (Fig. 4D).

**Silencing of FOXO3a by siRNA Results in a Reduction of Gefitinib-Induced Repression of FOXM1, Cell Proliferative Arrest, and Cell Death**

To show that FOXO3a has a critical role in the down-regulation of FOXM1 by gefitinib treatment, we transfected the gefitinib-sensitive BT474 breast carcinoma cells expressing high levels of FOXO3a with either a FOXO3a-specific siRNA or a nonspecific control siRNA pool and studied the expression of FOXM1 and its downstream targets 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). As observed previously, gefitinib treatment led to a decrease in p-EGFR and an increase in total EGFR in both control and FOXO3a siRNA cells. Moreover, the FOXO3a-specific siRNA, but not the control siRNA, reduced induction of Bim by gefitinib treatment. In the FOXO3a siRNA-transfected cells, the basal expression levels of FOXM1 and downstream targets PLK1, cyclin B1, and CDC25B are higher compared with the untreated control cells. Moreover, silencing of FOXO3a by siRNA resulted in a reduction of gefitinib-induced proliferative arrest (Fig. 5C). Moreover, cell cycle analysis also showed that silencing of FOXO3a by siRNA

![Figure 3](https://mct.aacrjournals.org/figure/3/)

**Figure 3.** Analysis of the relationship among Ki-67, nuclear FOXO3a, and FOXM1 expression in breast cancer patient samples. **A**, to test for differences between FOXM1 expression before and after treatment in gefitinib-treated and control groups, Dunnett’s t test was done and the mean difference was considered significant at \(P \leq 0.05\). **B**, relationship between the fold of change in FOXM1 status and Ki-67 or nuclear FOXO3a expression was statistically analyzed using Pearson’s correlation test based on the H-scores and was considered significant at \(P \leq 0.05\).
can partially rescue cells from gefitinib-induced cell cycle arrest and cell death (Fig. 5C). This is evident from the increase in the fraction of cells in S and G2-M phases (at 48 h) and reduction in the apoptotic sub-G1 fraction (at 48 h) in FOXO3a siRNA-transfected BT474 cells compared with control cells (Fig. 5C). Consistently, there was also a significant increase in viable cells in response to gefitinib treatment in the FOXO3a siRNA-transfected cells compared with the controls (Fig. 5D). Together, these results suggested that gefitinib represses FOXM1 by activating FOXO3a to mediate cell death and proliferative arrest in sensitive breast carcinoma cells.

Expression of FOXM1 Overcomes the Antiproliferative Effects of Gefitinib in the Sensitive SKBR3 Cells

To show that FOXM1 is a central signaling target of gefitinib, SKBR3 cells expressing lower levels of FOXO3a were either transfected with the empty expression vector or an expression vector encoding for full-length FOXM1 or a constitutively active NH2-terminal truncated form of FOXM1, ΔN-FOXM1, and then treated with 5 μmol/L gefitinib. The expression of FOXM1 was analyzed by Western blotting (Fig. 4A). The results showed that the FOXM1 expression was significantly increased in cells transfected with the full-length FOXM1 expression vector compared with the control cells. The expression of FOXM1 was also analyzed by luciferase reporter assay (Fig. 4C). The results showed that the FOXM1 promoter activity was significantly increased in cells transfected with the full-length FOXM1 promoter/reporter construct compared with the control cells. The expression of FOXM1 was also analyzed by quantitative real-time PCR (Fig. 4D). The results showed that the FOXM1 mRNA level was significantly increased in cells transfected with the full-length FOXM1 expression vector compared with the control cells.

Figure 4. Effect of ectopic expression of FOXO3a(A3) and EGFR on FOXM1 expression in SKBR3 cells. SKBR3 cells transfected with vector expressing the constitutively active FOXO3a(A3) or vector control were analyzed for the expression of FOXM1. A, cell lysates were prepared at the times indicated, and the expression of FOXO3a, FOXM1, and tubulin was analyzed by Western blotting. Bottom, quantitative analysis of FOXM1 protein expression. Columns, mean of three independent experiments after normalizing the data to t = 0 h; bars, SD. Statistical analysis has been done using Student’s t test. *, P < 0.05. B, parallel SKBR3 cells were cultured on sterile coverslips and transfected with FOXO3a(A3) in parallel before being fixed in 4% formaldehyde. FOXO3a was visualized with a rabbit polyclonal antibody followed by the addition of Alexa Fluor 488 (green)-labeled anti-rabbit antisera. 4',6-Diamidino-2-phenylindole (blue) was also applied to visualize the nuclei. C, SKBR3 cells were transiently transfected with 1 μg of the human full-length FOXM1 promoter/reporter construct together with increasing amounts (0, 5, 10, and 20 μg) of pcDNA-FOXO3a(A3). Cells were harvested 24 h after transfection and assayed for luciferase activity. D, SKBR3 cells were transfected with vector expressing the EGFR or vector control were analyzed for the expression of FOXM1. Cell lysates were prepared and the expression of EGFR, FOXM1, CDC25B, and tubulin was analyzed by Western blotting. Bottom, quantitative analysis of FOXM1 protein expression. Columns, mean of three independent experiments after normalizing the data to t = 0 h; bars, SD. Statistical analysis has been done using Student’s t test. *, P < 0.05.
gefitinib for 24, 48, and 72 h. Western blot analysis showed that the endogenous FOXM1 was expressed in the transfected SKBR3 cells, but its expression was repressed by gefitinib (Fig. 6A). However, the cytomegalovirus promoter-driven full-length FOXM1 or ΔN-FOXM1 was expressed constantly at high levels before and after gefitinib treatment (Fig. 6A). Cell cycle assays showed that gefitinib induced high levels of cell death in the empty vector-transfected SKBR3 cells as revealed by the gradual increase in sub-G₁ cells over time following gefitinib treatment. By comparison, the levels of cell death induced by gefitinib in the SKBR3 cells expressing the full-length FOXM1 or constitutively active ΔN-FOXM1 were much lower (Fig. 6B and C). Together, these results show that gefitinib activates FOXO3a to repress FOXM1 expression to mediate cell cycle arrest and cell death in sensitive breast cancer cells.

Discussion

The EGFR is frequently overexpressed or mutated in many cancers and EGFR has become an important molecular target in epithelial malignancies. Given the critical role played by EGFR in the tumorigenesis and progression of cancer, therapeutic agents, including monoclonal antibodies (e.g., cetuximab) and small-molecule tyrosine kinase inhibitors, have been developed to specifically block EGFR signaling (3, 6, 13). These EGFR antagonists have shown antitumor activity clinically in non-small cell lung, head and neck, colorectal, and pancreatic cancers; however, there is also a high number of patients who are resistant to these drugs (34). Gefitinib is a selective inhibitor of EGFR tyrosine kinase. Despite the fact that EGFR is frequently overexpressed or overactivated in human primary breast carcinoma, the majority of breast cancer patients do not have a significant response to treatment with gefitinib (2, 13, 35–37).

We showed previously that the forkhead transcription factor FOXO3a is an essential cellular target of gefitinib in breast cancer and that gefitinib inhibits Akt signaling to activate FOXO3a to induce cell cycle arrest and cell death in sensitive breast carcinoma cells (29). Furthermore, we also showed that reintroduction of active FOXO3a in resistant breast carcinoma cells can restore sensitivity to gefitinib (29). In the present study, we investigated the role and regulation of FOXM1 in response to gefitinib treatment in breast cancer. Using the gefitinib-sensitive breast carcinoma cell lines BT474 and SKBR3 as well as the resistant lines MCF-7, MDA-MB-231, and MDA-MB-453, we showed that...
gefitinib represses the expression of the transcription factor FOXM1 in sensitive but not resistant cells. The repression of FOXM1 by gefitinib was also associated with the down-regulation of FOXM1 activity as revealed by the concomitant decrease in expression of the FOXM1 downstream targets, PLK, cyclin B1, and CDC25B. We also showed that FOXM1 repression by gefitinib is mediated at the transcriptional and promoter levels, as a reduction in FOXM1 mRNA transcripts and a decrease in promoter activity were seen following treatment with gefitinib in sensitive cell lines.

The physiological significance of these findings was verified by immunohistochemical staining in breast cancer patient biopsy samples derived from a recently conducted gefitinib neoadjuvant study (12). Although the original clinical trial was designed to address the clinical question regarding the combination of gefitinib treatment with anastrazole (12), this work is focused on trying to understand the cellular mechanism involved in the response to EGFR inhibition. Therefore, the patient samples analyzed were from the gefitinib-only arm of the study. In these patient samples, we found that gefitinib treatment caused dephosphorylation and inactivation of EGFR, leading to an increase in nuclear FOXO3a and a concomitant decrease in FOXM1 expression. The down-regulation of FOXM1 expression was associated with a decrease in cell proliferation as revealed by a reduction in levels of Ki-67, a marker for cell proliferation. These results from breast cancer cell lines and patient samples are consistent with our hypothesis that gefitinib targets FOXO3a to repress FOXM1 expression and cell proliferation in sensitive breast cancer. This is further supported by the observation that ectopic expression of a constitutively active FOXO3a represses FOXM1 expression, whereas knockdown of FOXO3a expression using siRNA can up-regulate FOXM1 and rescue sensitive breast cancers cells from gefitinib-induced cell proliferative arrest. The down-regulation of FOXM1 expression by gefitinib could be critical for the antitumor function of gefitinib, as FOXM1 is a crucial regulator of cell proliferation and it regulates the expression of a host of target genes important for cell cycle progression and survival. This notion is confirmed by the observations that overexpression of the full-length FOXM1 or a constitutively active FOXM1, ΔN-FOXM1, abrogates the proliferative arrest and apoptosis induced by gefitinib, indicating that FOXM1 has a functional role in mediating the gefitinib-induced proliferative arrest and in determining sensitivity to gefitinib. Interestingly, overexpression of wild-type FOXM1 or constitutively active FOXM1 did not efficiently overcome the antiproliferative effect of gefitinib, indicating that gefitinib may either have other cell cycle regulators as targets or also regulate FOXM1 activity through other post-transcriptional mechanisms. Indeed, phosphorylation of FOXM1 by cyclin E-CDK2 (38) or extracellular signal-regulated kinase 1/2-mitogen-activated protein kinase (39) has been shown previously to be able to relocate FOXM1 to the nucleus, where it is functional. Phosphorylation of the retinoblastoma protein pRB by cyclin D1-CDK4 has also been shown to relieve the repression of FOXM1 by pRB through disrupting their direct interaction (40). Consistently, gefitinib has been reported to inhibit the activity of cyclin D1-CDK4 and cyclin E-CDK2 complexes as well as extracellular signal-regulated kinase 1/2.

Because gefitinib targets FOXM1 through FOXO3a in breast cancer, it is therefore possible to increase the efficacy of gefitinib or treat gefitinib-resistant diseases through targeting FOXM1. In fact, FOXM1 has already been reported to be a valid target for the development of anticancer therapeutics (21). For example, a novel thiazole antibiotic thioestreon has recently been reported to selectively induce cell cycle arrest and cell death in breast cancer cells through the down-regulation of FOXM1 expression (41). Similarly, a related thiazole antibiotic, siomycin A, has also been found to repress the expression of FOXM1 and cell proliferation (42). Furthermore, a cell-penetrating

![Figure 6](image_url)

**Figure 6.** Effect of ectopic expression of FOXM1 on gefitinib treatment in SKBR3 cells in response to gefitinib treatment. SKBR3 cells were transfected with a vector expressing the full-length FOXM1 or constitutively active ΔN-FOXM1 or vector control were analyzed for the expression of FOXM1. A, cell lysates were prepared at the times indicated, and the expression of FOXM1 and tubulin was analyzed by Western blotting. B, cells were fixed at 0, 24, 48, and 72 h after treatment, and cell cycle phase distribution was analyzed by flow cytometry after propidium iodide staining. Percentage of cells in each phase of the cell cycle (sub-G1, G0-G1, S, and G2-M). C, sub-G1 population at times indicated following gefitinib treatment. Representative data from three independent experiments. Mean ± SD. Statistical analysis was done using t test.
ARF peptide inhibitor of FOXM1 has been shown to selectively induce apoptosis in human hepatocellular carcinoma cell lines and mouse models (26).

In summary, our study defined FOXM1 as a cellular target and marker for gefitinib action in breast cancer and showed that FOXM1 functions downstream of FOXO3α in response to gefitinib. These findings could have important implications for the development of therapeutic agents to increase the effectiveness of existing anti-EGFR therapeutics and to overcome resistance to EGFR antagonists.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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Gefitinib (Iressa) represses FOXM1 expression via FOXO3a in breast cancer


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