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Gefitinib Induces Apoptosis and Decreases Telomerase Activity in MDA-MB-231 Human Breast Cancer Cells

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Gefitinib is an anti-cancer drug that selectively inhibits epithelial growth factor receptor (EGFR) tyrosine kinase activity and induces apoptosis in many cancer cells. Cancer cells are often protected from apoptotic cell death by telomerase, however the gefitinib-induced telomerase inhibition remains unknown. Here we investigated the effects of gefitinib on telomerase activity in two different breast cancer lines, MCF-7 (low expression of EGFR) and MDA-MB-231 (high expression of EGFR). We observed the inhibition of EGFR phosphorylation that occurred only MDA-MB-231 cells cultured in media containing 10% FBS. Direct cytotoxicity was observed in MDA-MB-231 cells, but not MCF-7 cells when treated with concentrations of gefitinib ranging from 15 to 20 μM. This cytotoxicity was associated with decreased telomerase activity and downregulation of the telomerase subunit, hTERT. c-Myc has previously been shown to activate telomerase activity through transcriptional regulation of hTERT. A decrease in c-myc expression and DNA-binding activity following treatment with gefitinib was observed exclusively in MDA-MB-231 cells. We also demonstrated that gefitinib downregulates the activation of Akt and subsequent hTERT phosphorylation and translocation into the nucleus in MDA-MB-231 cells. These results indicate that gefitinib induces loss of telomerase activity through dephosphorylation of EGFR in MDA-MB-231 cells.

Key words: Gefitinib, Telomerase, Telomerase reverse transcriptase, Apoptosis

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INTRODUCTION

Telomerase is a ribonucleoprotein complex comprised of a telomerase reverse transcriptase (hTERT) catalytic subunit (Nakamura et al., 1997; Meyerson et al., 1997), telomerase RNA (TR) template (Feng et al., 1995), telomerase-associated protein (TEP-1) (Harrington et al., 1997), and chaperone proteins (p23 and Hsp90) (Holt et al., 1999). This complex possesses specialized reverse transcriptase activity that adds hexameric repetitive sequences (TTAGGG) onto the ends of eukaryotic chromosomes using a segment of its integral RNA component as a template (Greider and Blackburn, 1985 and 1989). The relationship between apoptosis and telomerase activity was firmly established by reports that many anti-cancer agents induce apoptosis through downregulation of telomerase activity. These reports have further demonstrated that this induction of apoptosis can involve downregulation of hTERT, hTR, or TEP-1 expression (Jagadeesh et al., 2006; Yeo et al., 2005; Ghosh and Bhattacharyya, 2005). Activation of telomerase has also been proven to be an especially important step in human carcinogenesis and repression of this enzyme's activity has a major tumor suppressive role in normal human somatic cells (Sebastian et al., 2005). Therefore, abnormally active telomerase has evolved into one of the most promising targets for cancer therapy.

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Many factors are known to regulate telomerase activity. hTERT appears to be especially important for upregulation of telomerase activity in many human cancer cells. The hTERT promoter contains numerous c-myc-binding sites that directly mediated hTERT transcriptional activation (Wu et al., 1999). This finding is consistent with the recently reported association between c-myc overexpression and induction of telomerase activity (Wang et al., 1998). In addition, hTERT may also be regulated by other posttranslational modifications. The expression of an hTERT subunit containing two putative Akt phosphorylation sites is upregulated in many cancer cells. Furthermore, Akt kinase is known to enhance telomerase activity through phosphorylation of hTERT at Ser^{824} (Kang et al., 1999).

The epidermal growth factor receptor (EGFR) is another therapeutic target that is highly expressed in many types of human cancer and is a strong prognostic indicator for breast, ovarian, cervical and esophageal cancers (Nicholson et al., 2001; Rho et al., 2007). Gefitinib (Iressa) is a quinazoline derivative that inhibits EGFR tyrosine kinase activity by binding to the adenosine triphosphate pocket within the EGFR catalytic domain (Wakeling et al., 2007). Previous reports have demonstrated that this drug can effectively induce apoptosis in many different tumor cell types through inhibition of intracellular signaling pathways involving phosphorylation of MAPK and PI3K/Akt (Anderson et al., 2001; Janmaat et al., 2003; Moasser et al., 2003; Moulder et al., 2001). Furthermore, gefitinib is able to induce apoptosis via a variety of cell-type specific mechanisms, including bad activation (Ciardiello et al., 2002; Gilmore et al., 2002), FAS upregulation (Chang et al., 2004), and G_1 arrest through activation of p21 and p27 (Magne et al., 2003). However, the underlying mechanisms responsible for gefitinib-induced inhibition of cell growth and stimulation of apoptosis are poorly understood. In particular, the involvement between telomerase activity and gefitinib in human breast cancer cells remains completely unknown.

In the present study we first examined the effect of gefitinib on apoptosis and telomerase activity in human breast cancer cells. We report for the first time that gefitinib strongly induced apoptosis and repressed telomerase activity in MDA-MB-231 cells via transcriptional downregulation of hTERT through c-myc and posttranslational modification of hTERT protein via the inactivation of Akt. Our results also demonstrate phosphorylation and expression of EGFR is closely related to gefitinib-induced apoptosis and telomerase downregulation in MDA-MB-231 cells.

MATERIALS AND METHODS

Reagents

Gefitinib was kindly provided by Astra Zeneca Korea (Seoul). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide (MTT), 4,6-diamidino-2-phenylindole (DAPI), and propidium iodide (PI) were purchased form Sigma Chemical Co. (St. Louis, MO). Antibodies against EGFR, c-myc, PARP, hTERT, and capase-8 were purchased form Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho (p)-Akt, Akt, and p-serine were purchased from Cell Signaling (Beverly, MA) and antibody against β-actin was obtained from Sigma. Goat anti-rabbit or mouse IgG-Alexa fluor 488 and goat anti-mouse IgG-Texas Red were purchased from Molecular Probes (Eugene, OR). Lightshift EMSA Optimization kit was purchased form Pierce (Rockford, IL).

Cell culture and growth assay

The two human breast cancer cell lines used in this study, MCF-7 and MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin (Sigma) in 5% CO_2 at 37°C.

Cell viability and growth

Cells were seeded at 4×10^4 cells/mL and then treated with the indicated concentrations of gefitinib. After 72 h incubation, the cell number and viability were determined by trypan blue exclusion assay and MTT assay, respectively.

PI staining

Floating and adherent cells were pooled, washed with PBS, fixed in 70% ethanol for 20 min at -20°C, and incubated with 40 μg/mL PI and 100 μg/mL RNase A for 30 min at 37°C in the dark. Cells were analyzed using a FACSCalibur flow cytometer (Becton Dickenson; San Jose, NJ). The percentage of cells containing sub-G_1 DNA content was calculated as an indication of apoptosis.

RNA extraction and RT-PCR

Total RNA was isolated using the Trizol reagent (GIBCO-BRL; Gaithersburg, MD) according to the manufacturer's recommendations. Genes of interest were amplified from cDNA which was reverse transcribed from 1 μg of total RNA using the One-Step RT-PCR Premix (iNtRON Biotechnology). Primers and conditions for the amplification of hTERT and GAPDH

have been described previously (Jagadeesh et al., 2006).

Western blot analysis

Total cell extracts were prepared using PRO-PREP protein extraction solution (iNtRON Biotechnology). The preparation of cytoplasmic and nuclear extracts was conducted using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce; Rockford, IL). Total cell extracts were separated on 10% polyacrylamide gels, and then transferred to nitrocellulose membranes using standard procedures. The membranes were developed using an ECL reagent (Amersham; Arlington Heights, IL).

Telomerase activity assay

Telomerase activity was measured using a PCR-based telomeric repeat amplification protocol (TRAP) enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim; Mannheim) according to the manufacturer's instructions.

Immunofluorescence and nuclear staining

After treatment with gefitinib for 72 h, cells were harvested, washed in ice-cold PBS, fixed with 3.7% paraformaldehyde, and then permeabilized with 0.2% Triton-X 100. Fixed cells were washed with PBS and nuclei were stained with DAPI solution, and the cells were incubated with anti-EGFR antibody which was detected using anti-mouse IgG conjugated with Alexa Fluor 488. Nuclear morphology and EGFR were evaluated by fluorescence microscopy.

Electrophoretic mobility shift assays (EMSAs)

DNA-protein binding assays were carried out with nuclear extract. Synthetic complementary c-myc (5'-GGA AGC AGA CCA CGT GGT CTG CTT CC-3') binding oligonucleotides (Santa Cruz Biotechnology) were 3'-biotinylated using the biotin 3'-end DNA labeling kit (Pierce).

Phosphorylation of hTERT

After treatment with gefitinib, cells were harvested, washed in ice-cold PBS, fixed with 3.7% paraformaldehyde and then permeabilized with 0.2% Triton-X 100. Cells were then washed with PBS and blocked with 2% BSA for 30 min. Rabbit anti-hTERT and mouse anti-p-serine were used as primary antibodies and anti-rabbit IgG-Alexa Fluor 488 and anti-mouse IgG-Texas Red were used as secondary antibodies. Cells were analyzed using a FACSCalibur flow cytometer.

Statistical analysis

All data from MTT assay, cell count, FACS analysis, and telomerase activity were derived from at least three independent experiments. Scion Imaging software (http://www.scioncorp.com) was used for quantification of Western blots and RT-PCR products. Statistical analyses were conducted using SigmaPlot software, and values were presented as mean ± S.D. Significant differences between the groups were determined using the unpaired Student's *t*-test. A value of * $p < 0.05$ was accepted as an indication of statistical significance.

RESULTS

Gefitinib inhibits growth of MDA-MB-231 breast cancer cells, but not MCF-7 with cleavage of caspase-8 and PARP

Gefitinib is known to inhibit proliferation and induce cell death in various cancer cells. Therefore, we first evaluated the effect of gefitinib on proliferation and viability of MCF-7 and MDA-MB-231 cells by cell counting and MTT assays. As shown in Fig. 1A, gefitinib treatment significantly decreased cell proliferation and viability in MDA-MB-231 cells in a dose-dependent manner, but not MCF-7 cells. Although MDA-MB-231 cell death was slightly observed at a gefitinib concentration of 10 μM, these cells experienced marked decreases in viability (69 ± 4%) and cell number (465 ± 21) × 10^3 cells/mL following treatment with 15 μM gefitinib. Exposure to 20 μM of gefitinib further reduced cell number and viability to (332 ± 35) × 10^3 cells/mL and (29 ± 7%), respectively. Cell cycle analysis also revealed that treatment with 20 μM gefitinib resulted in a significant increase in MDA-MB-231 cells with sub-G_1 DNA content (41 ± 4%), which is indicative of apoptosis (Fig. 1B). We also investigated the status of caspase-8 following gefitinib treatment. As shown in Fig. 1C, concentrations greater than 10 μM gefitinib resulted in a significant increase in caspase-8 and PARP cleavage, which if further are indicative of apoptotic cell death. In contrast, MCF-7 cells were relatively unaffected by gefitinib treatment. These data indicate that gefitinib inhibits cell proliferation and leads to cell death in MDA-MB-231 cells, but not in MCF-7 cells.

Gefitinib inhibits EGFR phosphorylation in MDA-MB-231 cells

Previous studies have demonstrated that gefitinib is a potent and selective inhibitor of EGFR tyrosine kinase activity *in vitro* (Nicholson et al., 2001; Rho et al., 2007). In order to determine whether gefitinib has a similar effect in breast cancer, we first investigated the surface expression level of the EGFR in cultured

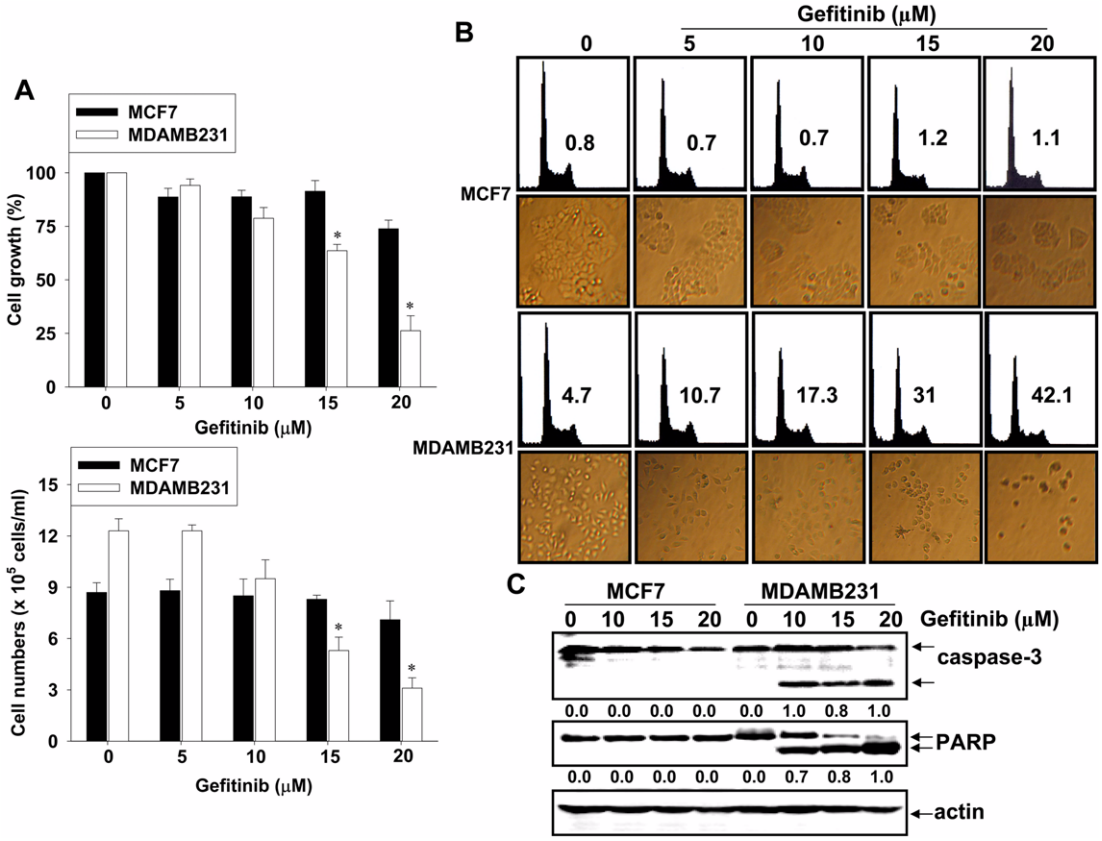


Fig. 1. Gefitinib triggers growth inhibition and apoptosis in MDA-MB-231 cells. Cells were seeded at 4×10^4 cells/mL and treated with the indicated concentrations of gefitinib for 72 h. (**A**) Cell number and viability were determined by MTT assay (*upper panel*) and hemocytometer counts of trypan blue-excluding cells (*lower panel*), respectively. (**B**) Flow cytometric analysis of MCF-7 and MDA-MB-231 cells treated with gefitinib for 72 h was examined. Cells were harvested at the indicated times, stained with PI and 10,000 events were analyzed for each sample (*upper panel*). DNA content is represented on the *x*-axis; the number of cells counted is represented on the *y*-axis. Data are representative of three independent experiments. The morphology of cells treated with or without gefitinib for 72 h was examined under light microscopy (× 400) (*lower panel*). (**C**) Cells, treated with gefitinib (0, 10, 15 and 20 μM) for 72 h, were lysed for protein extraction. Samples were subjected to 10% SDS-PAGE and Western blotting for detection of caspase-8 and PARP as indicated. Representative results from 3 independent experiments are shown. β-Actin was used as a loading control. Data are expressed as mean ± S.D. of three independent experiments. The significance was determined by Student's *t*-test (*$p < 0.05$ *vs.* untreated control).

MCF-7 and MDA-MB-231 cells. Immunofluorescence analysis demonstrated that MCF-7 cells do not express significant levels of EGFR (green color) and that MDA-MB-231 cells express very high levels of EGFR when grown in 10% serum-containing media (Fig. 2A). Surface EGFR expression was also confirmed using flow cytometry. As shown in Fig. 2B, MCF-7 cells did not express significant levels of surface EGFR (6.1 ± 2.4%) and MDA-MB-231 cells expressed high levels of surface EGFR (42.2 ± 5.1%).

Gefitinib is known to affect the phosphorylation of EGFR. Therefore, we next investigated whether gefitinib could inhibit EGFR phosphorylation in these cells. MCF-7 cells expressed low levels of p-EGFR and total EGFR regardless of the gefitinib treatment. However the levels of phosphorylated EGFR in MDA-MB-231 cells dramatically decreased and total EGFR was slightly downregulated in a time-dependent manner following treatment with gefitinib (Fig. 2C). These data indicated that both expression and phosphorylation of EGFR may be required to render cells sensitive to gefitinib.

Gefitinib represses telomerase activity in MDA-MB-231 cells through transcriptional downregulation of hTERT, but not of TEP-1

Telomerase functions to maintain the length of telomeres and prevent the induction of apoptosis. Aberrant activation of telomerase can immortalize cells and is nearly a ubiquitous feature of malignant cells (Sebastian et al., 2005). Our results indicate that treatment with gefitinib induces apoptosis and

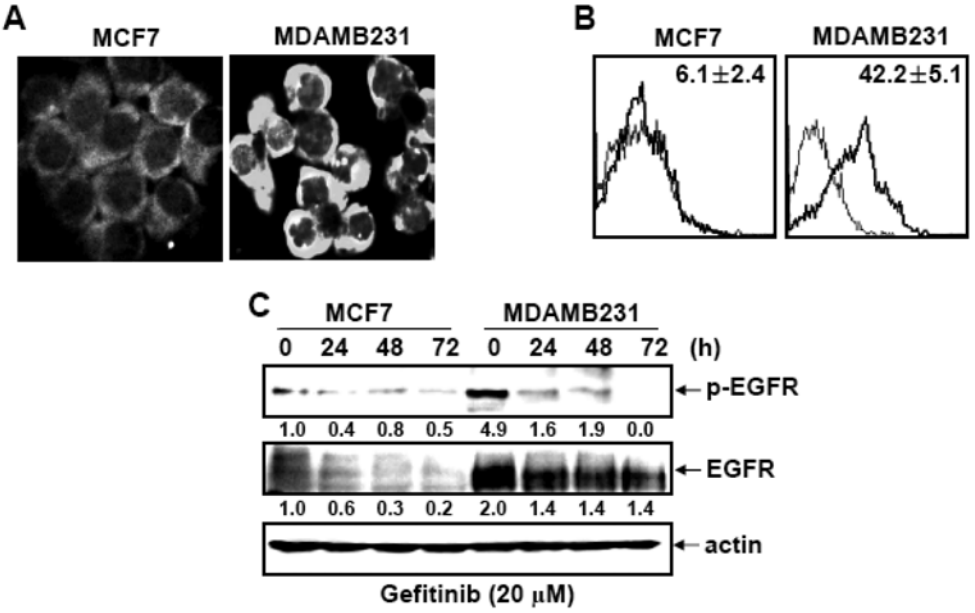


Fig. 2. Surface expression of EGFR is different in human breast cancer cell lines. (**A**) Untreated cells were stained with DAPI, and anti-EGFR antibody and anti-mouse IgG conjugated with Alexa 488 antibody. Green and blue indicate EGFR expression and nuclear staining, respectively. (**B**) Thick peaks from flow cytometry analysis represent the amount of EGFR on the surface of MCF-7 and MDA-MB-231 cells. Outlined peak represents isotype control (normal mouse IgG_1). Counts indicate number of events. (**C**) Cells, treated with gefitinib (20 μM) for 72 h, were lysed for protein extraction. Samples (30 μg protein/lane) were subjected to 10% SDS-PAGE and Western blotting for detection of specific proteins as indicated. Representative results from 3 independent experiments are shown. β-Actin was used as a loading control. Data are expressed as mean ± S.D. of three independent experiments. The significance was determined by Student's *t*-test (*$p < 0.05$ *vs.* untreated control).

inhibits EGFR phosphorylation in MDA-MB-231 cells, however telomerase activity in human breast cancer cells remains unknown. In order to elucidate a potential association, we cultured cells in the absence or presence of gefitinib, and measured telomerase activity using a TRAP-ELISA kit. As shown in Fig. 3A, gefitinib (20 μM) significantly decreased telomerase activity in MDA-MB-231 cells (19 ± 7%), but not in MCF-7 cells (77 ± 11%). Additionally, we assessed that gefitinib time-dependently induces suppression of telomerase activity in MDA-MB-231 cells. As shown in Fig. 3B, time-dependent reduction of telomerase activity in MDA-MB-231 cells was observed after gefitinib treatment.

Expression of hTERT and TEP-1 are known to tightly regulate telomerase activity in various cancer cells (Feng et al., 1995; Harrington et al., 1997; Holt et al., 1997). In order to determine whether the effect of gefitinib on telomerase activity involves hTERT or TEP-1, we performed RT-PCR to investigate expression of these subunits. As indicated in Fig. 3C, treatment with 20 μM gefitinib reduced hTERT mRNA levels in MDA-MB-231 cells, but not in MCF-7 cells. The expression of TEP-1 was unaffected by gefitinib treatment in either cell line. These data indicated that gefitinib-induced apoptosis may be partially due to inhibition of telomerase activity through downregulation of hTERT at the transcriptional level.

Gefitinib suppresses c-myc expression in MDA-MB-231 cells

The hTERT promoter site (-181 bp) has two c-myc binding regions and c-myc has previously been shown to directly regulate telomerase activity (Wu et al., 1995; Wang et al., 1999). Therefore, we were interested in determining whether gefitinib alters c-myc expression in breast cancer cells. As shown in Fig. 4A and 4B, RT-PCR and Western blotting analysis determined that treatment of MDA-MB-231 cells with 20 μM gefitinib significantly decreased c-myc mRNA and protein levels, but not MFC-7 cells. DNA binding activity of c-myc was also downregulated by gefitinib in only MDA-MB-231 cells (Fig. 4C). These results show that gefitinib repressed c-myc expression, and this could be a potential mechanism by which telomerase activity is decreased in MDA-MB-231 cells.

Gefitinib deactivates Akt and prevents phosphorylation of hTERT, thereby inhibiting translocation into the nucleus of MDA-MB-231 cells

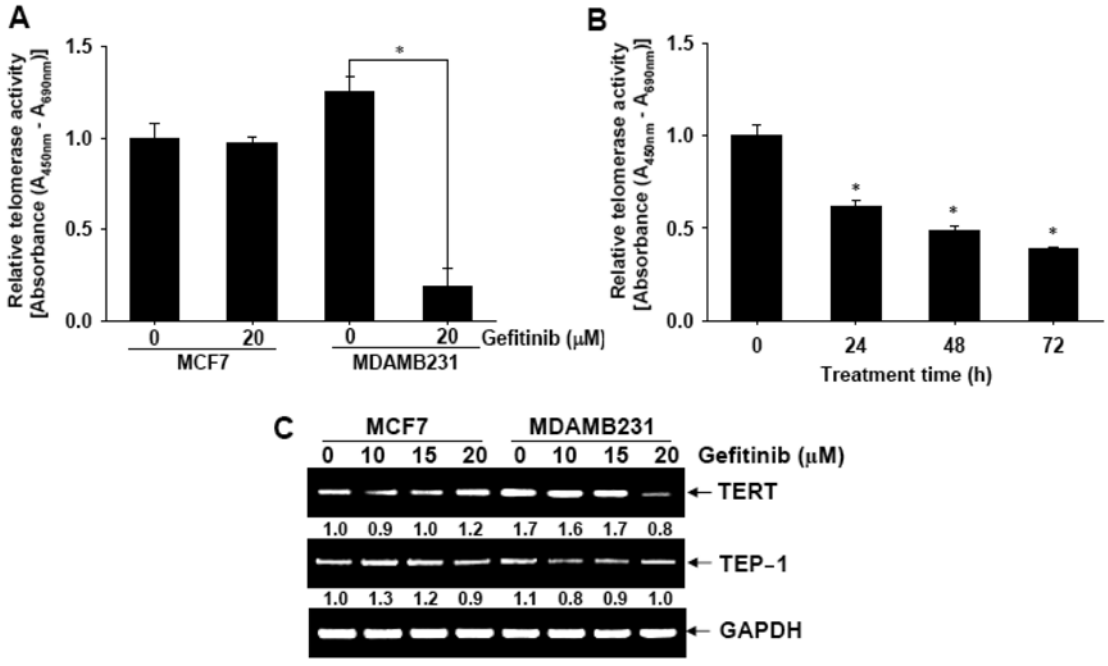


Fig. 3. Gefitinib treatment induces downregulation of hTERT and inhibition of telomerase activity in MDA-MB-231. (**A**) Cells were incubated with the indicated concentrations of gefitinib for 72 h. After 72 h incubation with gefitinib, telomerase activity of breast cancer cells was measured using a TRAP-ELISA kit as described in materials and methods. (**B**) MDA-MB-231 cells were treated with 20 μM gefitinib and telomerase activity was measured in the indicated times. (**C**) Total RNA from MDA-MB-231 cells was isolated using a Trizol reagent. One μg of RNA were reverse-transcribed. The resulting cDNA was subjected to PCR with hTERT and TEP-1 primers, and visualized by EtBr staining. GAPDH was used as an internal control. Fold activation (density of PCR band) is expressed relative to the control value obtained with untreated cells.

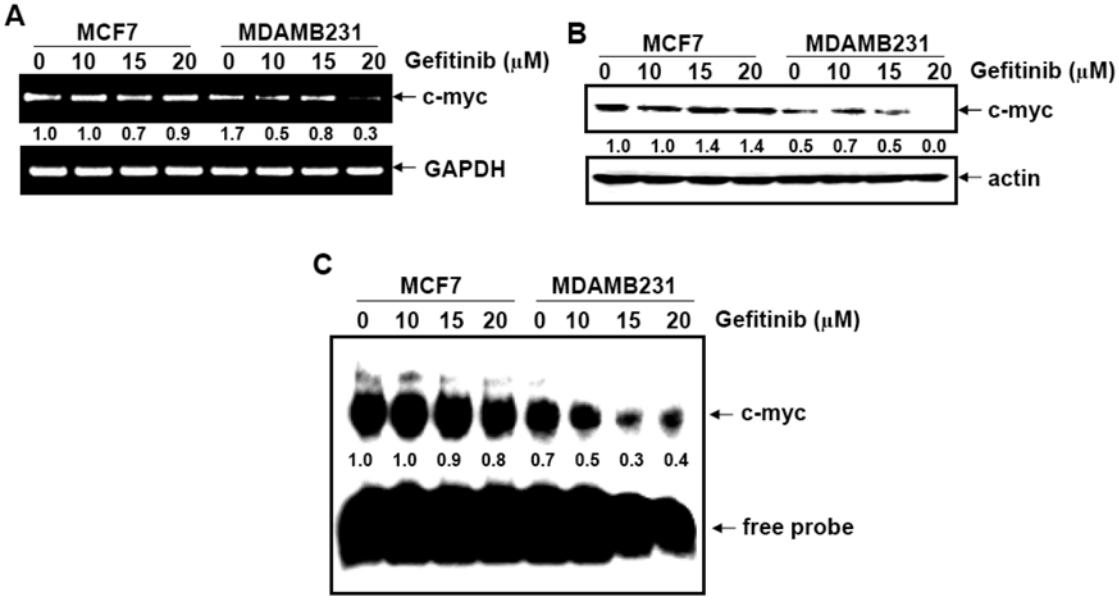
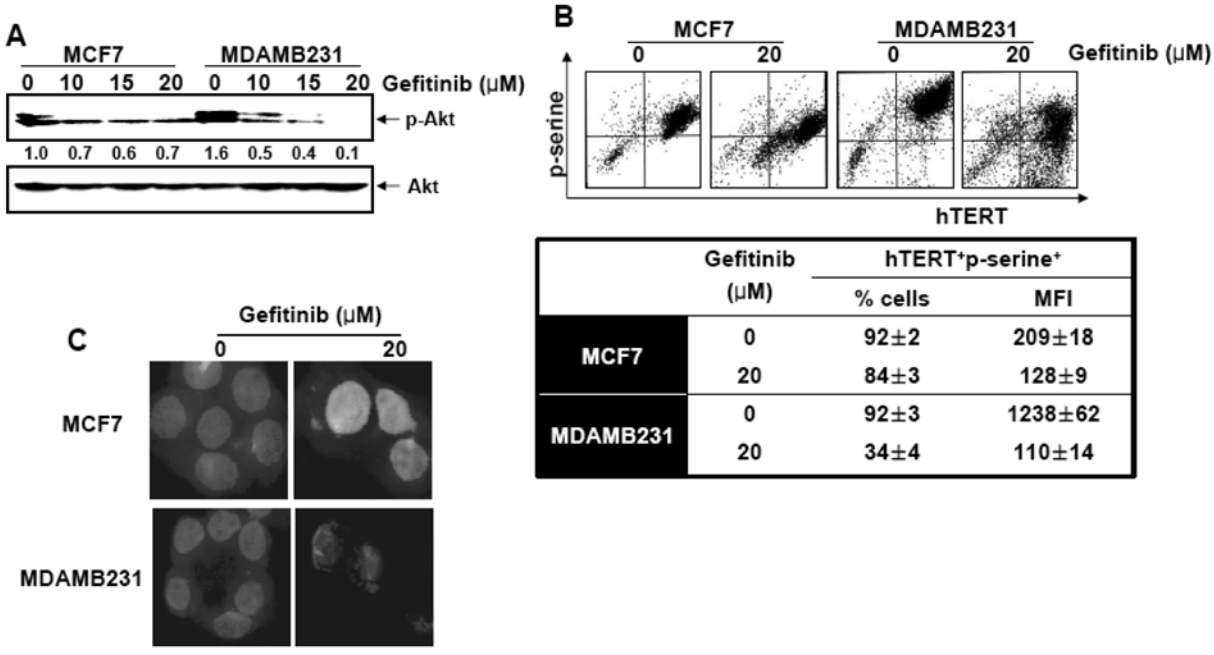


Fig. 4. Gefitinib treatment down-regulates c-myc expression and DNA-binding activity in MDA-MB-231 cells. (**A**) Cells were incubated with the indicated concentrations of gefitinib for 72 h. RNA was subjected to RT-PCR with c-myc specific primers and visualized by EtBr staining. GAPDH was used as an internal control. Fold activation (density of PCR band) is expressed relative to the control value obtained with untreated cells. (**B**) Breast cancer cells were harvested at the indicated times after incubation with gefitinib, protein was loaded onto a 10% gel, subjected to SDS-PAGE, and transferred to a nitrocellulose membrane. Protein levels were analyzed by Western blotting using anti-c-myc antibodies and normalized to β-actin. (**C**) After 72 h incubation with gefitinib, c-myc DNA binding activity was analyzed by LightShift™ chemiluminescent EMSA kit.



	Gefitinib (μM)	hTERT⁺p-serine⁺	
		% cells	MFI
MCF7	0	92±2	209±18
	20	84±3	128±9
MDAMB231	0	92±3	1238±62
	20	34±4	110±14

Fig. 5. Gefitinib inhibits phosphorylation of Akt and thereby prevents translocation to the nucleus in breast cancer cells. (**A**) Cells were incubated with gefitinib (0, 10, 15 and 20 μM) for 72 h. Cells were harvested after incubation with gefitinib and protein was loaded onto a 10% gel, subjected to SDS-PAGE, and transferred to a nitrocellulose membrane. Protein levels were analyzed by Western blotting using anti-p-Akt Ser^{473} and anti-Akt antibodies, and anti-Akt antibodies. Gefitinib (20 μM) treated cells were stained with anti-hTERT and anti-p-serine antibody. Cells were then incubated with anti-rabbit IgG conjugated with Alexa 488 antibody and anti-mouse IgG conjugated with Texas Red antibody. Analysis was done by flow cytometry (**B**) and fluorescence microscope (**C**).

Evidence has shown that hTERT is an authentic substrate for Akt kinase. Furthermore, human telomerase activity is enhanced phosphorylation of hTERT by Akt kinase (Kang et al., 1999). Therefore, we investigated whether the decreased telomerase activity after gefitinib treatment was due to the inactivation of Akt. As shown in Fig. 5A, total Akt levels did not change following treatment with various doses of gefitinib in either cell line. However, the levels of Akt phosphorylation at Ser^{473} were significantly decreased in a dose-dependent manner in MDA-MB-231 cells, but not in MCF-7 cells. MDA-MB-231 cells had higher basal p-Akt levels and a more sensitive deactivation ratio than MCF-7 cells. These results suggest that gefitinib deactivates Akt kinase by preventing phosphorylation of Ser^{473} residues.

Because activated Akt phosphorylates hTERT, we next examined whether phosphorylation of hTERT is inhibited by gefitinib treatment. As shown in Fig. 5B, flow cytometry analysis shows that 20 μM of gefitinib significantly decreased the phosphorylation of hTERT from 92 ± 3% to 34 ± 4% in MDA-MB-231 cells, however the percentage of $hTERT^+$/p-$serine^+$ cells was also slightly reduced from 92 ± 2% to 84 ± 3% in MCF-7 cells.

Because phosphorylation of hTERT is also necessary for its nuclear translocation, we subsequently examined the localization of hTERT in MCF-7 and MDA-MB-231 cells with or without gefitinib treatment. As shown in Fig. 5C, hTERT protein is normally localized in the nucleus and cytoplasm. Treatments with 20 μM of gefitinib decreased overall hTERT staining (red) intensity and eliminated nuclear staining in MDA-MB-231 cells, but not in MCF-7 cells. Collectively these results suggest that gefitinib decreases phosphorylation of hTERT and inhibits its translocation into the nucleus through blockage of Akt phosphorylation.

DISCUSSION

In this study, we observed that gefitinib decreases telomerase activity and downregulation of hTERT mRNA exclusively in MDA-MB-231 cells, but not in MCF-7 cells. These results are in agreement with a recent study that demonstrated that hTERT mRNA and telomerase activity were decreased after gefitinib treatment in human non-small lung cancer A549 cells and squamous vulvar cancer A431 cells (Suenaga et al., 2006). Gefitinib (20 μM) also caused significant cytotoxic effects and reduced proliferation of MDA-

MB-231 cells, however treatment with this drug had little effect on MCF-7 cells. Therefore, inhibition of telomerase activity by gefitinib can be partially interpreted as an important regulator that leads to apoptosis.

The hTERT promoter contains two c-myc, five Sp1, one Ets, and two Inr binding sites (Cong et al., 1999). Especially, c-myc has also been shown to bind to the E-boxes at the promoter of hTERT and activates the hTERT gene promoter leading to telomerase activation (Li et al., 2002; Greenberg et al., 1999). Because telomerase maintenance has been proposed as an essential prerequisite to human cancer development, c-myc inhibitors are thought to be good prospective drugs for treating cancer (Claassen and Hann, 1999; Sedivy, 1998). In order to confirm the involvement of c-myc in gefitinib-induced reduction of telomerase activity in breast cancer cells, we measured c-myc levels and DNA binding activity at 72 h after gefitinib treatment. In this study, we observed that, similar to hTERT, c-myc mRNA and protein levels were decreased. Furthermore, c-myc DNA binding activity was significantly repressed by gefitinib treatment in MDA-MB-231 cells. These results are in agreement with some previous reports where c-myc mRNA and/or protein levels were decreased by anti-telomerase agents in mouse melanoma and in human colon cancer and prostate cancer cells (Yan et al., 1999; Ouchi et al., 2005). Cancer cells are likely to use multiple signals to affect hTERT transcription and telomerase activity. Therefore, further studies are necessary to confirm the roles of other transcription factors in MDA-MB-231 cells.

Recently, the regulation of telomerase and hTERT has received much attention. It is clear that telomerase activity may be regulated via multiple mechanisms involving hTERT, including direct phosphorylation of hTERT by Akt (Kang et al., 1999), alternative splicing of hTERT mRNA, or by transcriptional regulation of hTERT (Ulaner et al., 1998). Phosphorylation of hTERT by Akt can affect nuclear localization from a presumably nonfunctional cytoplasmic location to a physiologically relevant under compartment (Liu et al., 2001). Our results suggest that gefitinib acts at the posttranslational level to downregulate phosphorylation of hTERT through the Akt pathway. Although Akt is essential for hTERT activation, posttranscriptional activation of the enzymatic activity of hTERT by phosphorylation is also regulated through the kinases PKC and ERK1/2 (Liu, 1999; Li et al., 1998). Moreover, Li et al have reported that protein phosphatase 2A (PP2A) markedly inhibited telomerase activity in human breast cancer cells (Li et al., 1997). Therefore, we cannot rule out that PKC, ERK1/2, and PP2A also exert a direct effect on hTERT phosphorylation and translocation into the nucleus.

The present study reports that treatment with 20 μM gefitinib is very effective at inhibiting the expression of telomerase-related proteins in MDA-MB-231 cells, but not in MCF-7 cells. The inhibition of telomerase by gefitinib might provide a plausible explanation for the anti-cancer effects in MDA-MB-231 cells, however treatment with 20 μM gefitinib for 72 h did not reduce cell viability or the expression of telomerase-related proteins in MCF-7 cells. Gefitinib is a selective EGFR tyrosine kinase inhibitor that is able to induce reversible inhibition or a delay in the growth of cancer cell lines and human tumor xenografts expressing high levels of EGFR (Moasser et al., 2001; Moulder et al., 2001). Additionally, gefitinib has been also reported to possess antiproliferative effects independent of EGFR expression in human breast cancer cell lines (Nicholson et al., 2001). Therefore, EGFR expression levels are not always an accurate predictor of gefitinib sensitivity. Our data demonstrate that treatment with 20 μM gefitinib was effective at inducing apoptosis through telomerase inactivation in MDA-MB-231 cells, but not in MCF-7 cells. These data partially demonstrated that phosphorylation, but not expression of EGFR, was involved in mediating apoptosis in MDA-MB-231 cells treated with gefitinib. Previous reports have also clearly showed that HER2 and androgen receptors are expressed in breast carcinomas and that these proteins are targets for gefitinib (Gilmore et al., 2002). However, our study only investigated *in vitro* expression and phosphorylation of EGFR. Thus, further studies are required in order to determine whether this drug regulates the expression and/or activation of other receptors involved in regulating telomerase activity in MDA-MB-231 cells.

In summary, the results of the present study demonstrate that the apoptotic effects of gefitinib is accompanied by significant downregulation of telomerase activity and that these effects can be greatly induced by transcriptional and posttranslational mechanisms of hTERT in MDA-MB-231 cells, but not in MCF-7 cells. Based on our results, gefitinib could partially regulate telomerase activity through phosphorylation and expression of EGFR in MDA-MB-231 cells. Although gefitinib may regulate telomerase activity through repression of transcriptional and posttranslational modification of hTERT, further efforts to explore the other regulatory molecules and targets of gefitinib are needed.

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