Influence of Gefitinib and Erlotinib on Apoptosis and c-MYC Expression in H23 Lung Cancer Cells

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Abstract. *Background: Gefitinib and erlotinib are inhibitors of epidermal growth factor receptor tyrosine kinase. The effects of these tyrosine kinase inhibitors on RAS-mutated cancer cells are unclear. Materials and Methods: Influence of gefitinib and erlotinib treatment was examined in H23 adenocarcinoma and A431 epidermoid carcinoma cells. The WST-1 assay was performed for evaluating cell growth. The phosphorylation status of extracellular-signal-regulated kinases (ERK) and AKT (protein kinase B) was examined by western blot. Flow cytometry was used for analyzing cell-cycle status and apoptosis detection. Results: In H23 cells, 20 μM erlotinib suppressed growth, while gefitinib did not suppress proliferation after 48 h of treatment. Neither gefitinib nor erlotinib affected the phosphorylation of ERK and AKT in H23 cells. Erlotinib augmented the sub-G_1 population of H23 cells, while gefitinib reduced it. Conclusion: In H23 cells, erlotinib accelerated apoptosis, while gefitinib induced G_1 arrest.*

Epidermal growth factor receptor (EGFR) was proposed as a target for cancer therapy, as cell growth depends on the activated EGFR pathway in many epithelial type of cancers. Mutation analysis of *EGFR* has revealed that the response to EGFR-tyrosine kinase inhibitor (TKI) is due to the presence of mutations, most frequently frame deletions in *exon 19* and the substitution of leucine 858 by an arginine (L858R). These mutations activate EGFR autonomously (1).

Activation of EGFR (by ligand binding or autonomous) induces the transduction of signaling pathways such as the of mitogen-activated protein kinase (MAPK) cascade *via* RAS. Although theoretically a *RAS* mutation would attenuate the therapeutic effect of EGFR inhibition, the effects of TKI on the cells have been controversial. If these TKIs have any anticancer effects even at a high dose, this might provide a clue to evaluate for new anticancer targets. These TKIs have advantages in that they have low adverse effects and simple actions on kinases, making them of use as molecular tools to search for new molecular targets.

In addition to the oncogenic changes in cancer cells, the status of *TP53* tumor suppressor gene affects target therapy. The genetic status of *RAS* and *TP53* influences cell proliferation and survival. In this study, we used two cell lines: A431 cells which express wild-type *RAS* and His-273-mutated *TP53*; and H23 cells which express *KRAS* and mutated *TP53* (2-4). Although A431 cells overexpress EGFR, EGFR this cell line is of wild-type (5, 6). In addition, *phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha* (*PIK3CA*) in these cell lines has no mutations, while H23 cells have mutant *phosphatase and tensin homolog deleted from chromosome 10* (*PTEN*) (2, 5, 7). We clarified whether TKIs have an antiproliferative effect on cells with mutated *RAS* and *TP53*.

Gefitinib and erlotinib are inhibitors of EGFR tyrosine kinase. Although their potency as TKIs differs, other differences between these two drugs remain unclear (8, 9).

Inhibition of growth factor pathways may only produce cytostatic effects if tumor cells survive in the G_0/G_1 phase (8). However, some reports have shown EGFR-TKI-induced apoptosis of cancer cells. Tracy *et al*. reported the induction of apoptosis by gefitinib in H3255 cells, which have the L858R mutation in *EGFR* (10). In addition, Gilmore *et al*. described the activation of the BCL2 antagonist of cell death (BAD) by gefitinib in insulin-like growth factor type-I receptor (IGF-R)-expressing breast cancer cells (11). In this study, we also assessed the influence on apoptosis of gefitinib and erlotinib.

In addition to the growth cascade, we focused on avian myelocytomatosis viral oncogene homolog (MYC). It is

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known that MYC is related to cell proliferation, apoptosis and transformation (12). MYC protein is stabilized by activation of RAS through the MAPK, and phosphatidylinositol-3 kinase (PI3K)/AKT (protein kinase B) pathways (13).

Here, we examined how gefitinib and erlotinib influence proliferation of lung cancer cells and phosphorylation of ERK and AKT. In addition, we gained insights into the mechanisms responsible for the distinct responses to these EGFR-TKIs.

Materials and Methods

TKIs. Gefitinib and erlotinib were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines and conditions. We obtained A431 cells from the Health Science Research Resources Bank (Osaka, Japan). H23 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) was used as culture medium and was supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and 100 μg/ml kanamycin. All cell lines were cultured at 37˚C in a humidified atmosphere with 5% CO_2.

Cell growth and cell cycle analysis. Cell growth was determined by WST-1 assay (Boehringer Mannheim, Mannheim, Germany). Briefly, tetrazolium salt WST-1 was cleaved to form formazan dye in viable cells, and absorbance at 450 nm was measured using a microplate reader (Bio-Rad Lab., Hercules, CA, USA). Cells were plated into 96-well plates, and 24 h after seeding, different concentrations of gefitinib or erlotinib were added to the medium, and the cells were incubated for an additional 48 h. Control cells were treated with dimethyl sulfoxide (DMSO). WST-1 reagent was then added and allowed to react for 1 h.

For cell-cycle analysis, cells were cultured for 48 h with different concentrations of gefitinib or erlotinib, and cells were then washed with phosphate-buffered saline (PBS) and harvested with a trypsin-EDTA solution. Samples of 1×10^6 cells were fixed in 70% ethanol on ice for 30 min, followed by washing with PBS. Cells were then treated with RNase A (100 μg/ml) for 30 min at 37˚C, and stained for DNA with propidium iodide (10 μg/ml) for 15 min at room temperature. Fluorescence of the stained cells was measured using a flow cytometer (CyAn™ ADP; Beckman Coulter, Brea, CA, USA).

Western blot analysis. Harvested cells were lysed in a buffer containing 20 mM Tris-NaOH (pH 8), 10 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. Lysates were incubated on ice for 60 min, centrifuged at 10,000 ×*g* for 15 min with a micro-centrifuge, and the supernatant was transferred to a fresh tube. Aliquots of 20 μg protein were separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel, and were then transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies against phospho-ERK1/ERK2 (R&D Systems, Minneapolis, MN, USA), ERK1/2 (Santa Cruz Biotechnology), AKT (Phospho-Ser473) (Signalway Antibody, College Park, MD, USA), AKT (Bioworld Technology, Louis Park, MN, USA), c-MYC phosphor-Ser62 (BioAcademia, Osaka, Japan), c-MYC (Zymed Laboratories, South San Francisco, CA, USA), or α-tubulin (Santa Cruz Biotechnology), followed by incubation with the respective horseradish peroxidase-conjugated secondary antibodies. Immunoreactive proteins were visualized using the enhanced chemiluminescence detection system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Detection of apoptosis. H23 cells were exposed to gefitinib or erlotinib at a concentration of 20 μM for 48 h, followed by staining with the green fluorescent protein (GFP)-certified™ apoptosis/ necrosis detection kit (Enzo Life Sciences, Loerrach, Germany), in accordance with the manufacturer's instructions. Briefly, the kit contains an annexin V-enhanced cyanine-3 conjugate for detecting apoptosis, and 7-amino-actinomycin D (7-AAD) for detecting necrosis and late apoptosis. As a positive control for apoptosis, H23 cells were treated with 0.2 μM staurosporine (included in the kit) for 2 h, and DMSO was used as a negative control. After staining, samples were analyzed by flow cytometry (CyAnTM ADP; Beckman Coulter).

Measuring caspase-3 activity. Enzyme activity of caspase-3 was measured using ApoAlert® caspase colorimetric assay kits (Clontech Laboratories, Mountain View, CA, USA), in accordance with the manufacturer's instructions. Briefly, 2×10^6 cells were suspended in a cell lysis buffer, followed by addition of reaction buffer and Asp-Glu-Val-Asp-*p*-nitroaniline. After incubation, samples were diluted with PBS and absorbance at 405 nm was read using a spectrometer (Lambda10; PerkinElmer, Waltham, MA, USA).

Reverse transcription polymerase chain reaction. For total RNA extraction, cells were plated onto 100-mm diameter dishes. Following overnight incubation, gefitinib or erlotinib was added to the cell culture medium. Cells were harvested after 24 h of exposure, and total RNA was isolated using ISOGEN (Nippongene Co., Tokyo, Japan). Total RNA (2 μg) was reverse-transcribed with random hexamer, and the target sequence for each mRNA was separately amplified with gene-specific primers according to the manufacturer's protocol (ThermoScript RT-PCR System; Invitrogen Corp., Carlsbad, CA, USA). PCR primer sets were as follows: 5'-CTC AGA CAC CAT GGG GAA GGT GA-3' and 5'-ATG ATC TTG AGG CTG TTG TCA TA-3' for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA (14); and 5'-AAG TCC TGC GCC TCG CAA-3' AND 5'-GCT GTG GCC TCC AGC AGA -3' for *c-MYC* mRNA (15). Reaction conditions for *GAPDH* and *c-MYC* mRNA were 27 cycles of 94˚C for 30 s, 54˚C for 30 s, and 72˚C for 60 s. Amplified products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

Results

Effects of TKIs on cell proliferation. We estimated A431 and H23 cell numbers after treatment with different concentrations of gefitinib or erlotinib (Figure 1). In A431 cells, 0.5 μM gefitinib and erlotinib suppressed proliferation (Figure 1), but at concentrations higher than 1 μM, the suppressive effects were no more potent than at 0.5 μM gefitinib. Surprisingly, 20 μM erlotinib attenuated H23 cell numbers ($p<0.01$), while 20 μM gefitinib increased the cell count after 48 h of treatment ($p<0.01$) (Figure 1). The maximum concentration of 20 μM was determined from its solubility.

We found that high-dose treatment affected the proliferation of H23 cells. There were clearly different effects on H23 proliferation between gefitinib and erlotinib.

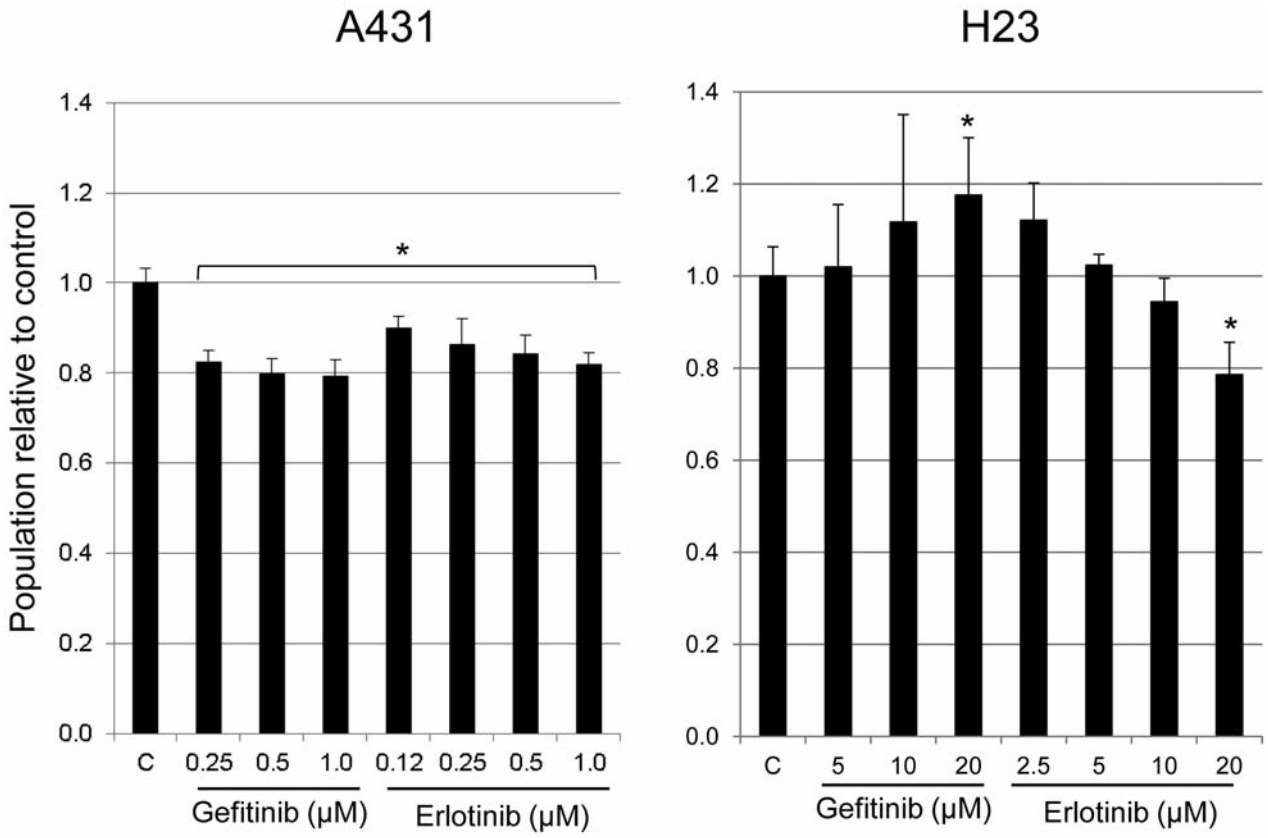


Figure 1. *WST-1 assay of three cell lines cultured with different concentrations of epidermal growth factor receptor tyrosine kinase inhibitors. At 48 h after exposure to gefitinib or erlotinib, the WST-1 assay was performed and the optical density was measured. Data are presented relative to the control (*p<0.01).*

Phosphorylation status of ERK and AKT. We examined the phosphorylation status of ERK and AKT activated *via* EGFR in cells after treatment with gefitinib or erlotinib for 6 h (Figure 2A). In A431 cells, which express wild-type RAS, gefitinib and erlotinib suppressed the phosphorylation of ERK and AKT. On the other hand, in H23 cells, which have *KRAS* and mutated *TP53*, ERK and AKT remained phosphorylated (Figure 2B).

As expected, the phosphorylation status of ERK and AKT in H23 was not affected by either gefitinib or erlotinib.

Cell-cycle analysis and apoptosis detection. Although both gefitinib and erlotinib had no influence on the phosphorylation of ERK and AKT in H23 cells, erlotinib was able to suppress cell growth. In order to investigate the molecular basis of this discrepancy, we analyzed cell-cycle populations (Figure 3A). The sub-G_1 population was increased when H23 cells were treated with 20 μM erlotinib, while gefitinib had little effect. As augmentation of the sub-G_1 population was considered to indicate acceleration of apoptosis, we analyzed cell death in H23 cells exposed to gefitinib or erlotinib (Figure 3B). Erlotinib increased the number of H23 cells stained with annexin-V, while gefitinib reduced it. As the S and G_2/M population did not decrease, cell-cycle arrest may not be the cause of cell growth suppression.

Next, we examined caspase-3 activation of H23 cells. In gefitinib-treated H23 cells, activity of caspase-3 was attenuated (Figure 4A), while activity was augmented by erlotinib exposure in H23 (Figure 4B). All of these results (sub-G_1 population, annexin-V staining and caspase-3 activity) indicate that apoptosis of H23 cells was increased by erlotinib but not by gefitinib.

Gefitinib suppresses expression of c-MYC. In order to elucidate the distinct effects of these EGFR-TKIs, we focused on c-MYC, which may be involved in the proliferation and apoptosis of cells. We examined the expression of c-MYC in H23 cells treated with gefitinib and erlotinib. Gefitinib attenuated the expression of c-MYC protein but erlotinib did not (Figure 5A). On RT-PCR, gefitinib was found to suppress the expression of *c-MYC* mRNA, while erlotinib did not (Figure 5B).

MYC proteins have multiple phosphorylation sites, and phosphorylation of Ser62 by MAPK/ERK increases MYC stability (16). However, neither gefitinib nor erlotinib affected

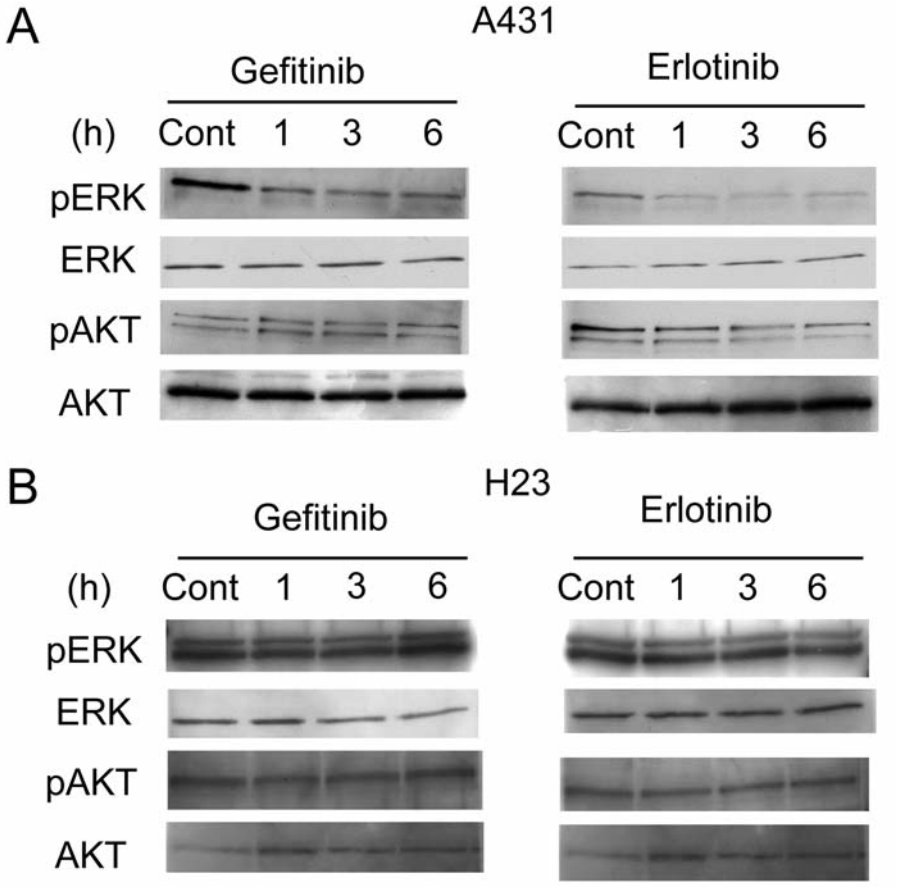


Figure 2. *Extracellular-signal-regulated kinases (ERK) and AKT (protein kinase B) in A431 (A) and H23 (B) cells treated with epidermal growth factor receptor tyrosine kinase inhibitors for 6 h. After 24 h of pre-culture, EGFR-TKIs were added to the culture medium. Concentrations of gefitinib and erlotinib were 0.5 μM for A431 cells, and 20 μM for H23 cells (pERK: phospho-ERK, pAKT: phospho-AKT).*

the phosphorylation of ERK in H23 cells, as shown in Figure 2B. These results suggested that decrease of c-MYC was mainly due to transcription attenuation.

Although the suppression of c-MYC by gefitinib would be thought to induce cell-cycle arrest in H23 cells, cell-cycle analysis of H23 cells treated with EGFR-TKI after 48 h of gefitinib exposure (Figure 3A) did not show any signs of obvious influence. We again analyzed the cell cycle after longer exposure (72 h). Gefitinib increased the G_0/G_1 population of H23 cells, and attenuated the S-phase population (Figure 6A). Proliferation of H23 cells might continue at this point, as the G_2/M population remained present. However, G_1-arrest had antiproliferative effects upon further treatment with gefitinib (Figure 6B).

Discussion

In this study, gefitinib and erlotinib apparently had opposite effects on cell proliferation in H23 cells. Erlotinib attenuates cell growth by promoting apoptosis. On the other hand, the mechanisms responsible for increased numbers of H23 cells after gefitinib exposure may be the result of apoptosis inhibition rather than increase of proliferation. However, gefitinib also seems to have antiproliferative effects on H23 cells, as it induced G_1 arrest. Although the concentration of gefitinib (20 μM) used in this study is not suitable for clinical use, more potent drugs may be able to suppress c-MYC in the clinical setting.

Although the potency of EGFR inhibition differed, the antiproliferative effects were similar in A431 cells (8, 9). In H23 cells, antiproliferative effects were induced by higher concentrations of gefitinib and erlotinib in comparison with A431 cells. This difference may be related to the mutation status of *RAS* and *TP53*.

We examined the influence of gefitinib and erlotinib on the phosphorylation status of ERK and AKT, and found that the effects were almost identical. In H23 cells, which have *K-RAS* and mutant *TP53*, treatment with gefitinib and erlotinib did not affect phosphorylation of ERK and AKT. With regard to sensitivity to EGFR-TKIs in cells having

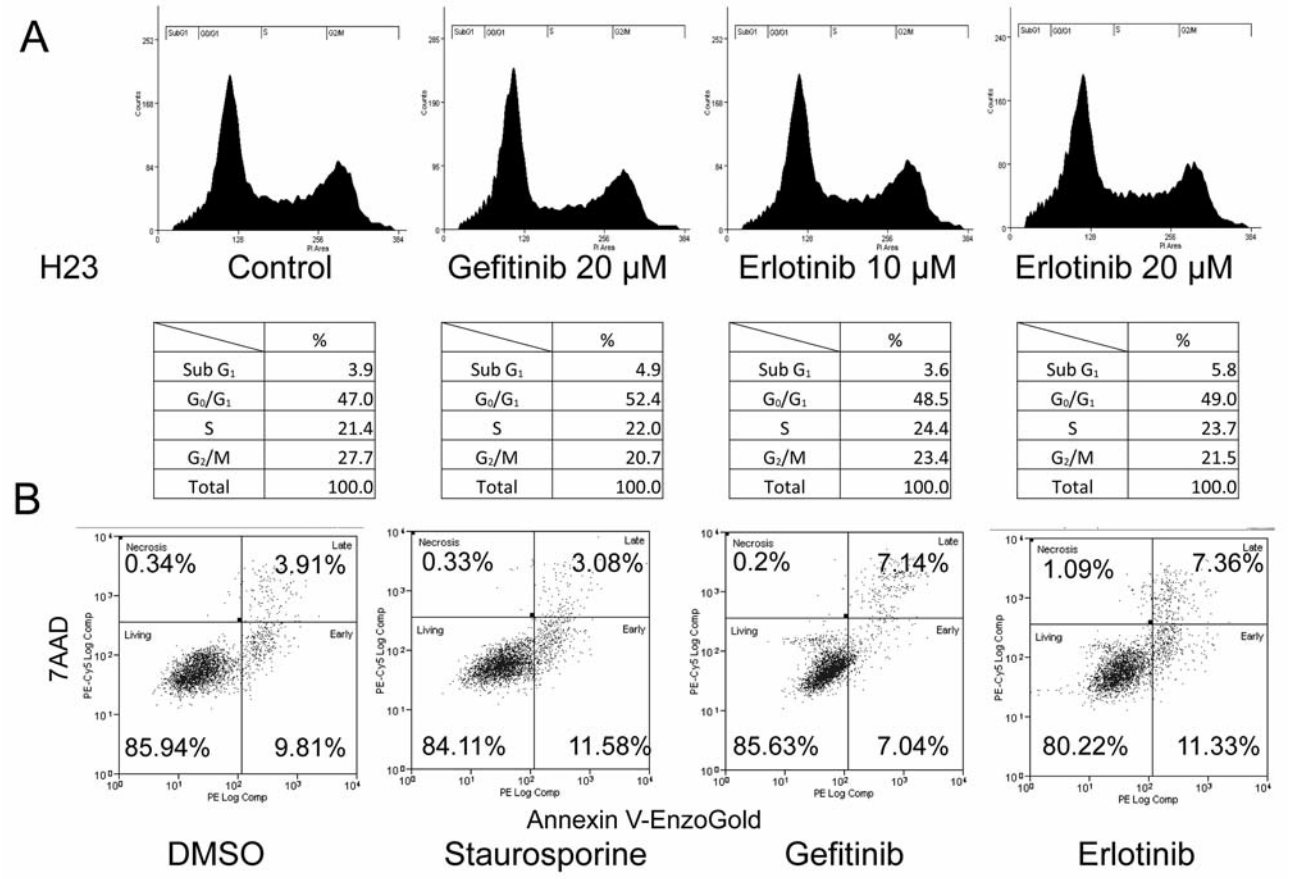


Figure 3. *A: Cell-cycle analysis of H23 cells treated with epidermal growth factor receptor tyrosine kinase inhibitors. Cells were exposed to the indicated concentrations of gefitinib or erlotinib for 48 h. Collected cells were stained with propidium iodide and analyzed. B: Detection of apoptosis. H23 cells were exposed to staurosporine (0.2 μM, 2 h), gefitinib (20 μM, 48 h) or erlotinib (20 μM, 48 h). After incubation, collected cells were stained with 7-amino-actinomycin D (7-AAD) and annexin V-EnzoGold. A flow cytometer was used to analyze the fluorescence of samples. Low 7-AAD/low EnzoGold indicates living cells, high 7-AAD/low EnzoGold indicates necrotic cells, low 7-AAD/high EnzoGold indicates early apoptotic cells and high 7-AAD/high EnzoGold indicates late apoptotic cells.*

activated RAS, the conclusions of previous reports have been inconsistent (17-19). Although higher concentrations of EGFR-TKIs are necessary to suppress cell growth in cells that express K-RAS, our data suggest that EGFR-TKI might have an effect on RAS mutant-positive cells through tyrosine kinase and non-tyrosine kinase targets.

In H23 cells, erlotinib increased apoptosis after less than 48 h of treatment, while gefitinib attenuated apoptosis and induced cell-cycle arrest within 72 h of exposure. Our results suggest that suppression of c-MYC is one of the mechanisms of apoptosis attenuation induced by gefitinib (16). These anti-apoptosis effects of gefitinib in H23 cells were surprising, as apoptosis was induced to some extent in H23 cells, even under normal culture conditions.

We previously reported the influence of gefitinib on p27 expression in these cell lines (20). In A431 cells, expression of p27 was augmented by gefitinib after 48 h and G_1 arrest was observed. In H23 cells, p27 was not affected by gefitinib. As an attenuation of the S- and G_2/M-phase populations was observed after 72 h of treatment, we believe that longer term exposure to gefitinib induces antiproliferative effects in H23 cells. These effects appear similar, but probably operate *via* different mechanisms. G_1 arrest in A431 cells by gefitinib treatment is related to cyclin-dependent kinase inhibitors; however, in H23 cells, suppression of c-MYC attenuates the S and G_2/M populations.

Although we herein report an increased sub-G1 population, augmentation of annexin-V-positive cells, and increase of caspase-3 activity, the mechanisms of apoptosis increase by erlotinib in H23 are not apparent. As H23 cells express Insulin-like growth factor-I receptor, activation of BAD is one of the potential mechanism, but as erlotinib did not affect the phosphorylation of ERK and AKT in H23 cells, these pathways may keep BAD inactivated (11, 21).

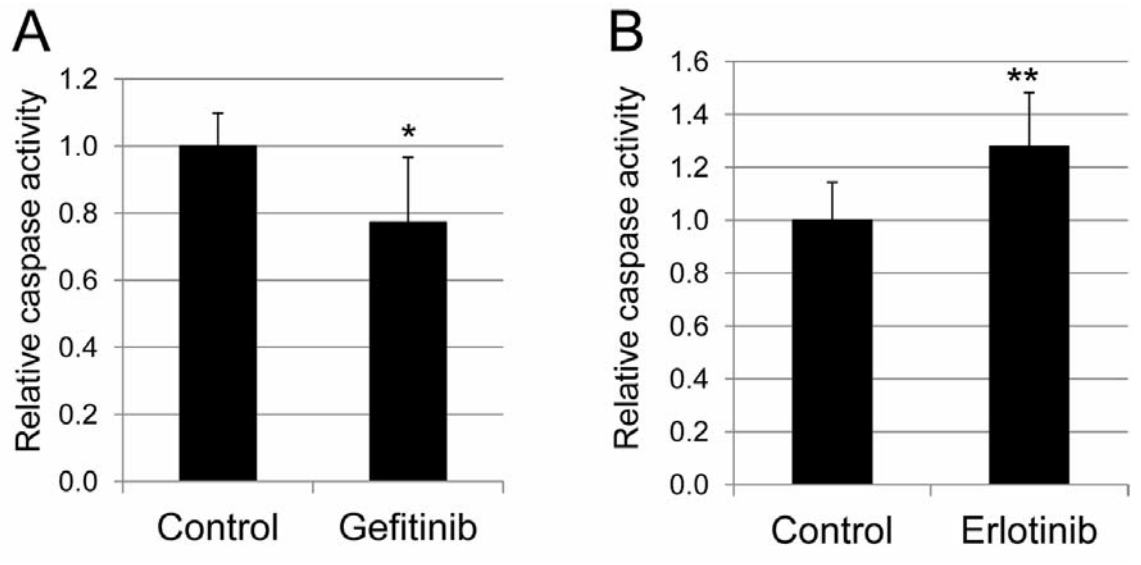


Figure 4. *A: Suppressive effects of gefitinib on caspase-3 activity in H23 cells. Cells were treated with 20 μM gefitinib for 48 h and harvested. Samples were reacted with substrates, and after incubation, absorbance at 405 nm was measured in order to determine the concentration of p-nitroaniline. Data show the ratio of activity in gefitinib-treated cells to those with DMSO. B: After treatment with 20 μM erlotinib, H23 cells were collected and the activity of caspase-3 was measured as describe above (*p=0.027, **p=0.018).*

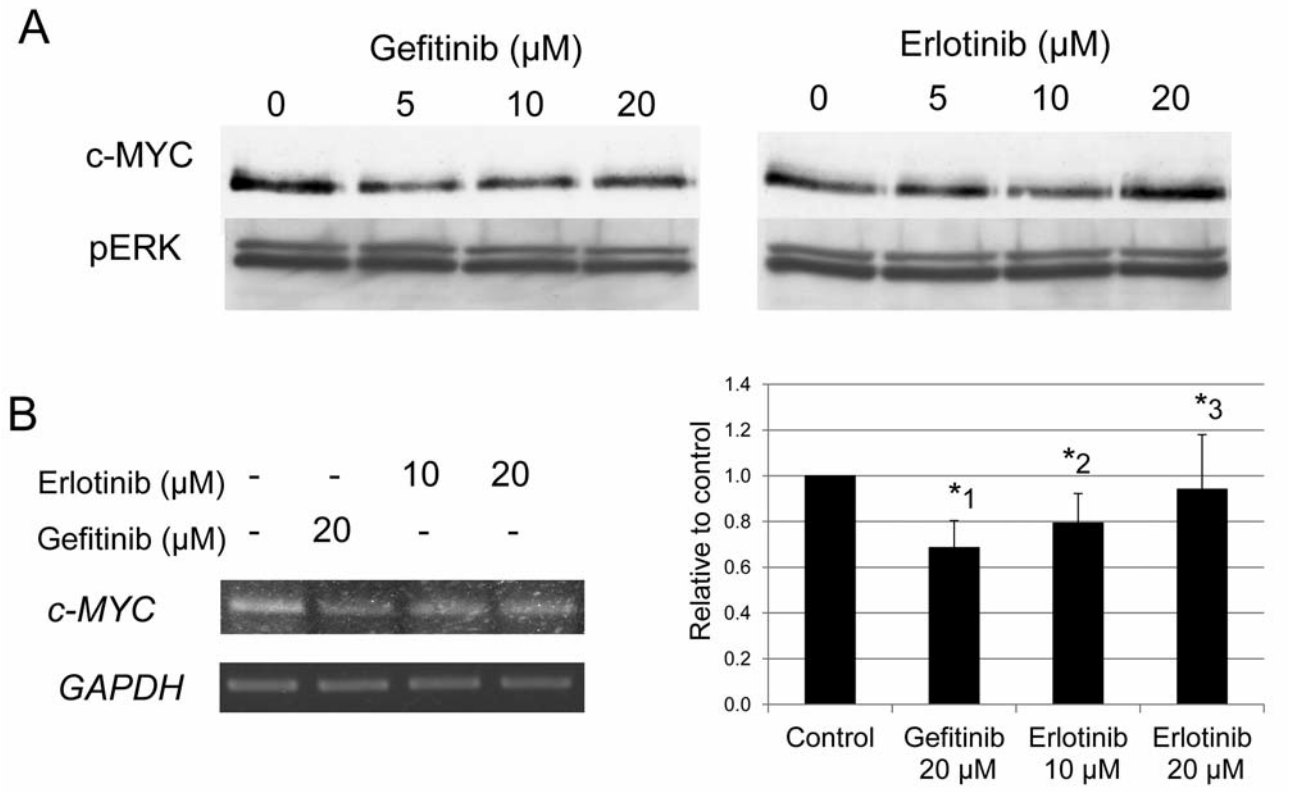
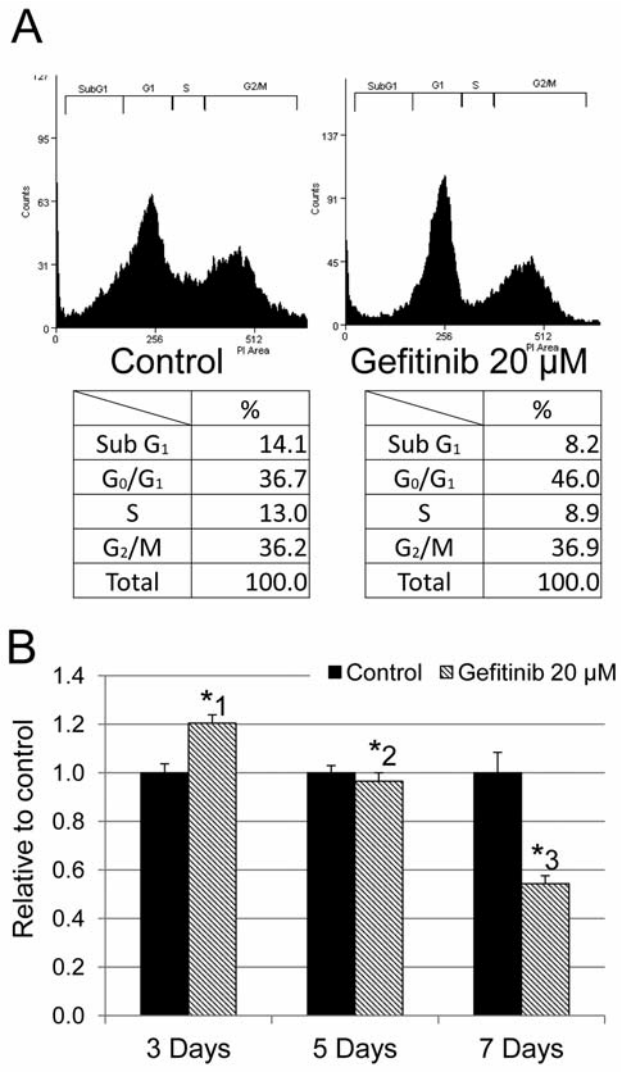


Figure 5. *A: H23 cells were exposed to gefitinib or erlotinib for 48 h and harvested. For examination of expression of avian myelocytomatosis viral oncogene homolog (c-MYC), immunoblotting was performed. B: Reverse transcription polymerase chain reaction for c-MYC and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). After 48 h of treatment with gefitinib or erlotinib, cells were harvested (*1: p=0.03, *2: p=0.07, *3: p=0.38).*



	%
Sub G_1	14.1
G_0/G_1	36.7
S	13.0
G_2/M	36.2
Total	100.0

	%
Sub G_1	8.2
G_0/G_1	46.0
S	8.9
G_2/M	36.9
Total	100.0

Figure 6. *A: Cell-cycle analysis of H23 cells subjected to longer-term gefitinib treatment. Cells were exposed to 20 μM gefitinib for 72 h. Collected cells were stained with propidium iodide and analyzed. B: WST-1 assay of H23 cells treated with gefitinib for 3, 5 and 7 days. Data are relative to that of the control (*1 p<0.01, *2 p=0.02, *3 p<0.01).*

Proteins in the *MYC* family affect cell growth, proliferation, differentiation and apoptosis. The *MYC* promoter is controlled by multiple signal transduction cascades, including the RAS/MAPK, transforming growth factor-β and nuclear factor-kappa B (NF-κB) pathways (22). Gefitinib attenuated the expression of *c-MYC* mRNA in H23 cells. Although multiple signaling pathways control the function of MYC by post-translational modifications, we were able to determine that *c-MYC* mRNA suppression by gefitinib is a potential mechanism of c-MYC suppression in H23 cells. Further studies are necessary to elucidate the pathway of *c-MYC* mRNA suppression by gefitinib; however, suppression of *c-MYC* mRNA is a potential therapeutic target in cancer therapy.

Our data suggest that gefitinib induced apoptosis inhibition and cell-cycle arrest simultaneously *via* c-MYC suppression in H23 cells. The antiapoptotic effects of gefitinib may therefore be able to offset the cytotoxic effects of drugs, if used concomitantly.

We herein present the different effects of gefitinib and erlotinib on H23 cells. Erlotinib increased apoptosis, whereas gefitinib suppressed c-MYC expression, followed by apoptosis inhibition and G_1 arrest. These findings indicate that these TKIs might have anticancer effects with unknown mechanisms, including anticancer target molecules other than EGFR. It is necessary for further experiments to elucidate the molecular mechanisms of the effects of TKIs.

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