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Curcumin Lowers Erlotinib Resistance in Non-Small Cell Lung Carcinoma Cells With Mutated EGF Receptor

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Non-small cell lung cancer (NSCLC) patients with activating mutations in the epidermal growth factor receptor (EGFR) are responsive to erlotinib, an EGFR-tyrosine kinase inhibitor (EGFR-TKI). However, patients with secondary somatic EGFR mutations are resistant to EGFR-TKI treatment. In this study, we investigated the effect of curcumin on the tumor growth of erlotinib-resistant NSCLC cells. Cell proliferation was determined by MTT assay. Apoptosis was examined using TUNEL staining. Protein expression of genes was determined by Western blot. Tumor growth was assessed in a xenograft mouse model. Results showed that erlotinib had a stronger effect on the induction of apoptosis in erlotinib-sensitive PC-9 cells but showed a weaker effect on erlotinib-resistant H1975 and H1650 cells than cisplatin and curcumin. Furthermore, curcumin significantly increased the cytotoxicity of erlotinib to erlotinib-resistant NSCLC cells, enhanced erlotinib-induced apoptosis, downregulated the expressions of EGFR, p-EGFR, and survivin, and inhibited the NF-κB activation in erlotinib-resistant NSCLC cells. The combination of curcumin and erlotinib exhibited the same effects on apoptosis as the combination of curcumin and cisplatin in erlotinib-resistant NSCLC cells. Moreover, the combined treatment of curcumin and erlotinib significantly inhibited tumor growth of erlotinib-resistant NSCLC cells in vivo. Our results indicate that curcumin is a potential adjuvant for NSCLC patients during erlotinib treatment.

Key words: Non-small cell lung cancer (NSCLC); Curcumin; Erlotinib; Survivin; Drug resistance; NF-κB; Cancer therapy

INTRODUCTION

Lung cancer is the leading cause of cancer mortality worldwide (1,2). Around 85% of lung cancer patients are non-small cell lung cancer (NSCLC) (1,2). Overexpression of epidermal growth factor receptor (EGFR) has been shown in 40% to 80% of NSCLC biopsies and has been associated with shorter survival times in NSCLC patients (3,4). EGFR is a receptor tyrosine kinase that is aberrantly activated in several solid epithelial solid tumors especially in NSCLC (2). EGFR-driven cell signaling contributes to lung tumorigenesis and tumor progression (5,6). Therefore, targeting the inhibition of EGFR signaling has become an effective therapeutic strategy for NSCLC (7–9).

Studies have also shown that approximately 70% of NSCLC patients harbor somatic mutations in the exons of the EGFR gene that encodes the tyrosine kinase domain of the receptor. These NSCLC patients with EGFR mutations respond well to the treatment with small-molecule EGFR tyrosine kinase inhibitors (EGFR-TKIs), including erlotinib (10,11). These mutations include the deletion mutation of DE746-A750 in exon 19 and the leucine-to-arginine substitution at position 858 (L858R) in exon 21 of the *EGFR* gene (12). Nevertheless, most patients, even those who show a marked response to initial treatment, develop acquired resistance to EGFR-TKIs (13). So far, several major mechanisms involved in acquired resistance of EGFR-TKIs have been identified, including secondary mutations of EGFR (e.g., T790M in exon 20 and D761Y, in exon 19) (12) and amplification of *MET* (14). Thus, developing new agents for overcoming the resistance to NSCLC treatment would be promising.

EGFR signaling has been linked to multiple intracellular pathways that promote cell survival and inhibit apoptosis (15,16). Upon ligand-induced activation, EGFR generates phosphotyrosine sites for the activation of Ras phosphatidylinositol-3 kinase (PI3K)/Akt pathways (15). The PI3K/AKT pathway has been shown to regulate survivin expression (17). Survivin, a member of the inhibitor of apoptosis (IAP) family, inhibits caspase-dependent apoptosis (18) and is associated with drug resistance and poor prognosis (19,20). A recent study showed that

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persistent survivin expression contributed to erlotinib resistance and that inhibition of survivin reversed erlotinib resistance in EGFR-mutant NSCLC cells (21,22).

Curcumin, derived from the perennial herb *Curcuma longa Linn*, exhibits promising cancer-preventive and therapeutic properties (23,24). Curcumin inhibits cell proliferation, induces apoptosis, and inhibits invasion by modulating multiple molecular targets of tumor cells (23–25). Recent studies have shown that curcumin inhibits intrinsic EGFR tyrosine kinase activity, suppresses ligand-induced activation of EGFR (26), and downregulates *EGFR* gene expression in colon cancer cells (27). While some studies have shown that curcumin synergistically enhances the antitumor actions of chemotherapeutic drugs (28), the potential effect of curcumin combined with erlotinib for NSCLC treatment has not yet been investigated. Moreover, clinical trials have indicated that curcumin is safe even when administered at a high dose of 10 g/day (29). In the present study, we determined the effect of curcumin on EGFR-TKI-resistant NSCLC cells in vitro and in vivo and found that curcumin significantly increased the cytotoxicity of erlotinib, enhanced erlotinib-induced apoptosis, and inhibited tumor growth in EGFR-TKI-resistant NSCLC cells. Our results suggest that curcumin is a promising agent for overcoming erlotinib resistance in NSCLC treatment.

MATERIALS AND METHODS

Cell Culture and Reagents

The NSCLC H1650 cells (bearing a deletion in exon 19 of the EGFR gene, i.e., DE746-A750) and H1975 cells (EGFR L858R/T790M) (ATCC, Rockville, MD, USA) (30) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL, Life Technologies, NY). Curcumin, erlotinib, and cisplatin were purchased from Selleck Chemicals (Houston, TX, USA).

Cell Proliferation Assay

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, exponentially growing NSCLC cells (1×10^4 cells/well) were seeded into 96-well culture plates with six replicates. Twenty-four hours after seeding, cells were treated with cisplatin, curcumin, erlotinib, or the combination of curcumin and erlotinib for another 72 h. MTT (20 μl of 5 mg/ml) was added to each well and incubated for 4 h at 37°C. Formazan crystals were dissolved by adding 100 μl of dimethyl sulfoxide (DMSO). The color intensity, which is a reflection of the number of live cells, was measured at a wavelength of 570 nm. Growth inhibition is expressed as the percentage of surviving cells in drug-treated cells versus DMSO-treated control cells. The IC_{50} value is the concentration resulting in 50% cell growth inhibition by a 72-h exposure to drugs compared with control cells.

Apoptosis Assay

Apoptosis was determined by a terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) (Roche, Mannheim, Germany) assay according to the manufacturer's instructions. H1975 cells and H1650 cells (1×10^5 cells/well) were seeded in six-well plates. Cells were treated with cisplatin, curcumin, erlotinib, or the combination of curcumin and erlotinib for 48 h. Cells were fixed by 4% paraformaldehyde for 1 h and then penetrated with 0.1% Triton X-100 for 15 min. TDT enzyme and label solution were added to the fixed cells. After 1 h, the cells were observed under a fluorescence microscope (Olympus, Japan). The cells with green nuclei staining were defined as apoptotic cells. The apoptotic cells were assessed in nine randomly selected fields viewed at 200× magnification. The apoptotic index was calculated as number of apoptotic cells/total number of nucleated cells × 100%.

Western Blot

Cancer cells treated with erlotinib and curcumin were lysed with the lysis buffer. Protein concentration was measured by using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins. The SDS-polyacrylamide gels were transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated with specific primary antibodies against EGFR, phospho-EGFR (p-EGFR), survivin, cleaved caspase-3, cleaved caspase-9, cytosolic cytochrome c, phosph-p65 (p-p65), p65, and β-actin (Cell Signaling Technology, San Diego, CA, USA), and with HRP-conjugated secondary antibodies conjugated to Alexa Fluor 680 or IRdye 800 (Rockland Immunochemicals, Inc. Gilbertsville, PA, USA). The intensities of the protein bands were scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, Nebraska).

Tumor Xenograft Mouse Model

Four-week-old female athymic nude mice were bred in a sterilized animal room of the Animal Experimental Centre of Shanghai Institutes for Biological Sciences (Shanghai, China). H1650 cells (1×10^6) were injected subcutaneously into the flanks of athymic nude mice. When tumor size reached around 80 mm^3 to 100 mm^3, mice were randomly assigned to treatment and control groups. Each group had five mice. Curcumin was dissolved in propylene glycol, and erlotinib was prepared in drinking water. In the treatment groups, mice received erlotinib (10 mg/kg body weight) and curcumin (1g/kg body weight) alone or their combination by oral gavage daily for 5 weeks. The control group received vehicle control. Tumor sizes

were measured by a caliper weekly. Five weeks after the treatment, animals were euthanized, and their tumors were weighed. Tumor volume (V) was calculated according to the following equation: $V\ (mm^3) = 1/2 \times a^2 b$ (a: relatively shorter diameter, b: relatively longer diameter). Animal protocols were approved by Animal Care and Facilities Committee of Zhongshan Hospital, Shanghai Medical School, Shanghai Fudan University.

Statistical Analysis

Data are expressed as means of at least three different experiments ± SD (standard deviation). The results were analyzed by Student's t test or Mann–Whitney test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Curcumin Sensitizes EGFR-Mutant NSCLC Cells to the Cytotoxicity of Erlotinib

To determine the potential effect of erlotinib on EGFR-mutant NSCLC cells, three NSCLC cell lines, PC-9 cell line ($EGFR^{19del}$), H1975 ($EGFR^{L858R+T790M}$), and H1650 ($EGFR^{DE746\text{-}A750}$) were used. MTT results showed that erlotinib dramatically inhibited cell proliferation of PC-9 cells. The IC_{50} of erlotinib for PC-9 cells is around 0.48 μM (Fig. 1A). H1975 cells and H1650 cells were markedly resistant to erlotinib, compared to PC-9 cells. The IC_{50} of erlotinib for H1975 and H1650 cells was 15.2 μM and 18.7 μM, respectively (Fig. 1A). These results demonstrated that H1650 cells and H1975 cells are erlotinib resistant and that PC-9 cells are erlotinib sensitive, consistent with previous reports (30). In contrast, we also found that the inhibition rates were not significantly different among the three NSCLC cell lines when they were treated with curcumin (Fig. 1B), suggesting that curcumin has the same inhibitory effect on different EGFR-mutant NSCLC cells. To determine whether curcumin reverses erlotinib resistance, H1975 cells and H1650 cells were treated with a combination of curcumin and erlotinib. When combined with curcumin (12.5 μM), erlotinib significantly enhanced the inhibition of cell proliferation in H1975 cells (Fig. 1C) and H1650 cells (Fig. 1D), compared to erlotinib treatment alone. These results indicate

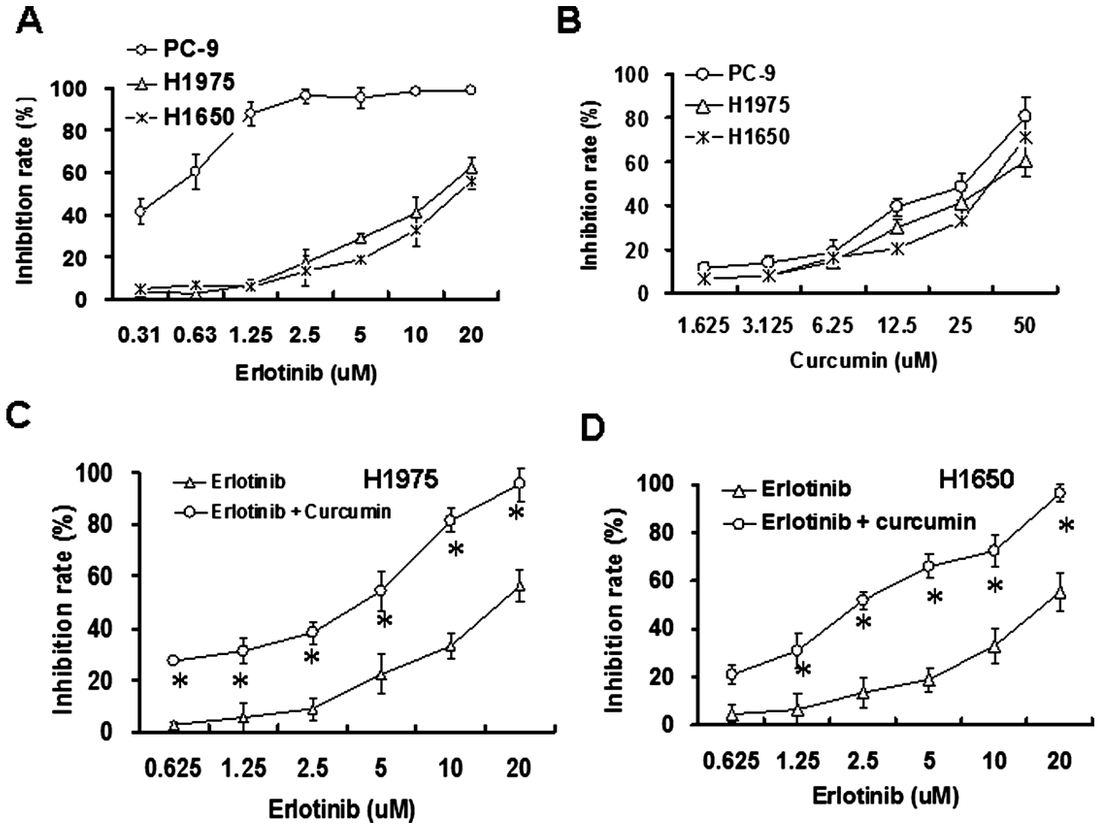

Figure 1. Curcumin sensitizes NCSLC cells to the cytotoxicity of erlotinib. (A–B) Erlotinib, curcumin, and cisplatin inhibit cell proliferation. PC-9, H1975, and H1650 cells were treated with erlotinib (A) and curcumin (B) at the indicated doses for 72 h. (C–D) Curcumin sensitizes the cytotoxicity of erlotinib to NSCLC cells. H1975 cells (C) and H1650 cells (D) were treated with erlotinib at the indicated doses alone or combined with curcumin (12.5 μM) for 48 h. Cell proliferation was determined by MTT. The data are means ± SD of three independent experiments. *$p < 0.05$, compared to erlotinib treatment alone.

that curcumin sensitizes erlotinib-resistant lung cancer cells to the cytotoxicity of erlotinib.

Curcumin Enhances Erlotinib-Induced Apoptosis of EGFR-Mutant NSCLC Cells

To assess the effect of erlotinib on apoptosis in EGFR-mutant NSCLC cells, these NSCLC cells were treated with erlotinib (1 μM) for 72 h. Erlotinib treatment markedly induced apoptosis of PC-9 cells, but only slightly induced apoptosis of H1975 cells and H1650 cells (Fig. 2A). The results suggest that H1975 cells and H1650 cells are resistant to erlotinib-induced apoptosis. To determine whether curcumin enhanced erlotinib-induced apoptosis of erlotinib-resistant cells, H1975 and H1650 cells were treated with curcumin, erlotinib, or their combination. The combined treatment significantly increased apoptosis

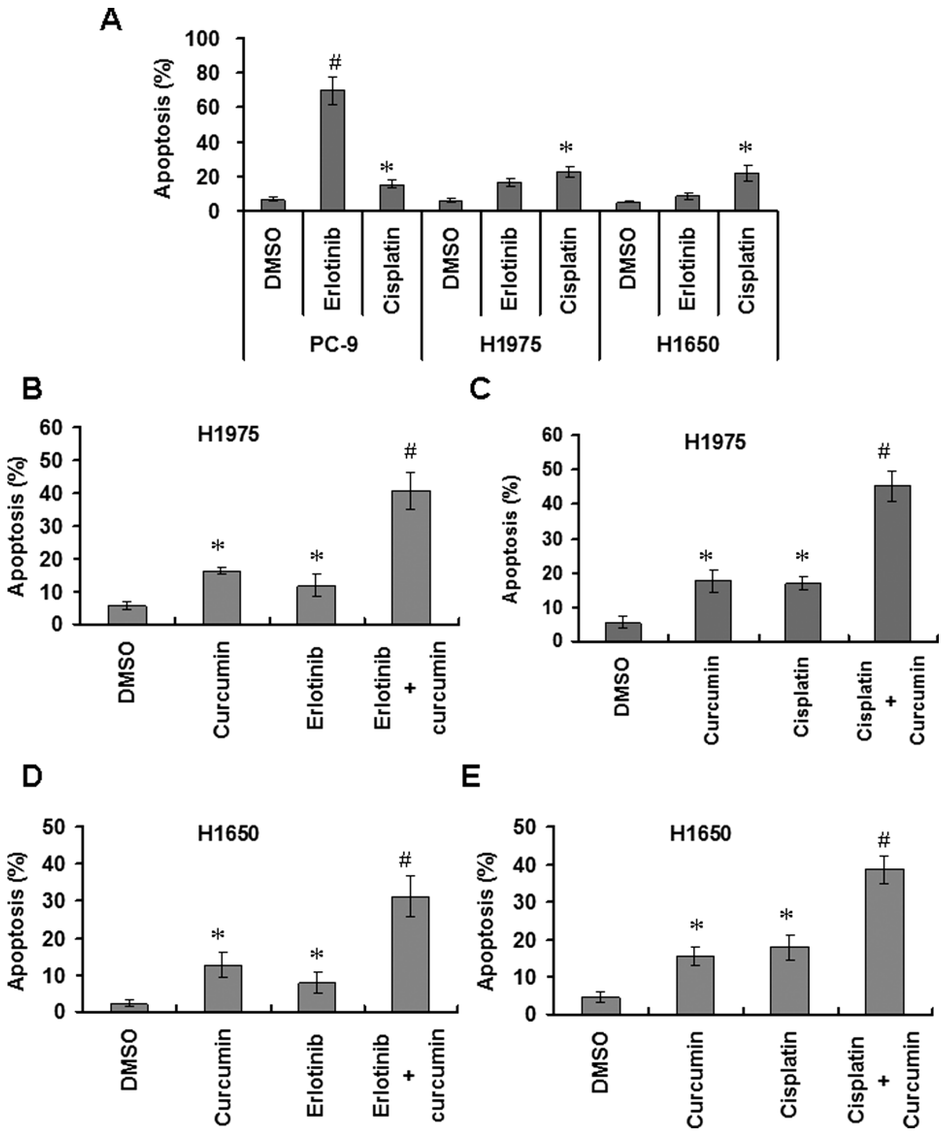

Figure 2. Curcumin enhances erlotinib and cisplatin-induced apoptosis in NSCLC cells. (A) Erlotinib- and cisplatin-induced apoptosis in NSCLC cells. PC-9, H1975, and H1650 cells were treated with erlotinib (1 μM) for 72 h. Apoptosis was determined by TUNEL. #$p<0.01$, compared to H1975 cells and H1650 cells; *$p<0.01$, compared to erlotinib treatment. (B–E) Curcumin enhances erlotinib and cisplatin-induced apoptosis in erlotinib-resistant NSCLC cells. H1975 cells (B) and H1650 cells (D) were treated with curcumin (12.5 μM), erlotinib (1 μM), or a combination of curcumin (12.5 μM) and erlotinib (1 μM) for 48 h. H1975 cells (C) and H1650 cells (E) were treated with curcumin (12.5 μM), cisplatin (1 μM) (B), or a combination of curcumin (12.5 μM) and cisplatin (1 μM) for 48 h. Apoptosis was determined by TUNEL. The data presented are means ± SD of three independent experiments. *$p<0.01$, compared to DMSO treatment; #$p<0.01$, compared to curcumin, erlotinib, or cisplatin treatment alone.

in both H1975 cells (Fig. 2B) and H1650 cells (Fig. 2C) compared to erlotinib or curcumin treatment alone. We also determined the effect of cisplatin, a widely used chemotherapeutic drug, on erlotinib-sensitive and erlotinib-resistant NSCLC cells. We found that cisplatin showed weaker induction of apoptosis in PC-9 cells but exhibited a stronger effect on erlotinib-resistant H1975 and H1650 cells than erlotinib did (Fig. 2A). However, the combination of curcumin and erlotinib showed the same effect on apoptosis of erlotinib-resistant cells as the combination of curcumin and cisplatin (Fig. 2B–E). The results indicate that curcumin overcomes erlotinib resistance in NSCLC cells.

Curcumin Downregulates Expressions of EGFR, p-EGFR, and Survivin in EGFR-Mutant NSCLC Cells

To investigate the underlying mechanisms by which curcumin enhances erlotinib-induced apoptosis in EGFR-mutant NSCLC cells, we determined the effects of curcumin on EGFR and apoptosis-related proteins. Western blot showed that erlotinib decreased p-EGFR level but did not affect EGFR expression (Fig. 3A). However, curcumin downregulated p-EGFR expression (Fig. 3A). The combined treatment of curcumin and erlotinib exhibited stronger inhibition of EGFR and p-EGFR expressions than curcumin or erlotinib treatment alone did (Fig. 3A).

Survivin has been shown to be linked to the inhibition of apoptosis in erlotinib-resistant NSCLC cells (21). We also found that erlotinib did not affect expression of survivin and failed to induce the cleavage of caspase-3 and caspase-9 and release of cytochrome c in H1650 cells (Fig. 3B). In contrast, curcumin markedly decreased survivin expression, induced cleavage of caspase-3 and caspase-9, and stimulated release of cytochrome c in H1650 cells (Fig. 3B). Moreover, the combined treatment of curcumin and erlotinib increased the cleavage of caspase-3 and caspase-9 and the release of cytochrome c to a greater degree than curcumin or erlotinib treatment alone (Fig. 3B). These results suggest that the inhibition of survivin expression contributes to the curcumin-induced decrease in erlotinib resistance in EGFR-mutant NSCLC cells.

NF-κB activation promotes erlotinib resistance in NSCLC cells (31–33). Curcumin inhibits NF-κB activation (34). Thus, we investigated the effect of curcumin on NF-κB activation in erlotinib-resistant EGFR-mutant lung cancer models. We found that curcumin markedly inhibited p-p65 expression, while erlotinib did not affect p-p65 expression in H1650 cells. The results suggest that inhibition of NF-κB activation may be one of the mechanisms by which curcumin overcomes erlotinib resistance.

Curcumin Potentiates the Antitumor Effect of Erlotinib in NSCLC In Vivo

To investigate whether curcumin enhances the antitumor action of erlotinib in vivo, we determined the effects

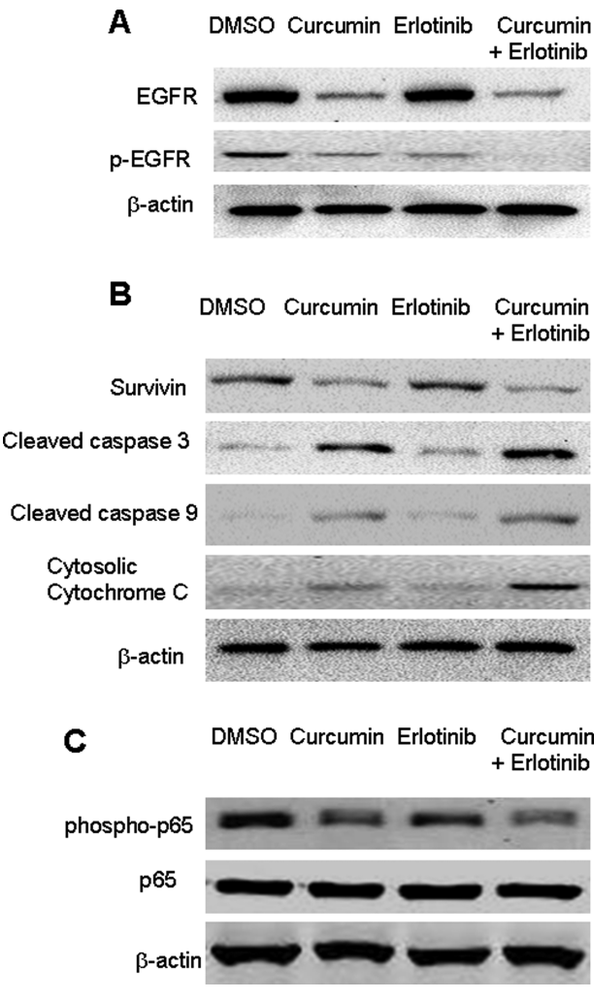

Figure 3. Curcumin regulates expressions of EGFR and apoptosis-related proteins. H1650 cells were treated with curcumin (12.5 μM), erlotinib (1 μM), or their combination for 48 h. Protein expressions of EGFR and p-EGFR (A), apoptosis-related proteins (B), and p-p65 (C) were determined by Western blot.

of curcumin and erlotinib on tumor growth in a xenograft mouse model of H1650 cells. The results showed that curcumin or erlotinib treatment significantly inhibited tumor growth (Fig. 4A) and reduced tumor weight (Fig. 4B) compared to the control treatment. More importantly, the combined treatment with curcumin and erlotinib exhibited stronger inhibition of tumor growth and reduction of tumor weight than curcumin or erlotinib treatment alone (Fig. 4A, B). These results suggest that curcumin enhances the response of EGFR-mutant NSCLC to erlotinib and potentiates the antitumor effect of erlotinib in vivo.

DISCUSSION

In this study, we found that curcumin significantly increased erlotinib inhibition of cell proliferation in

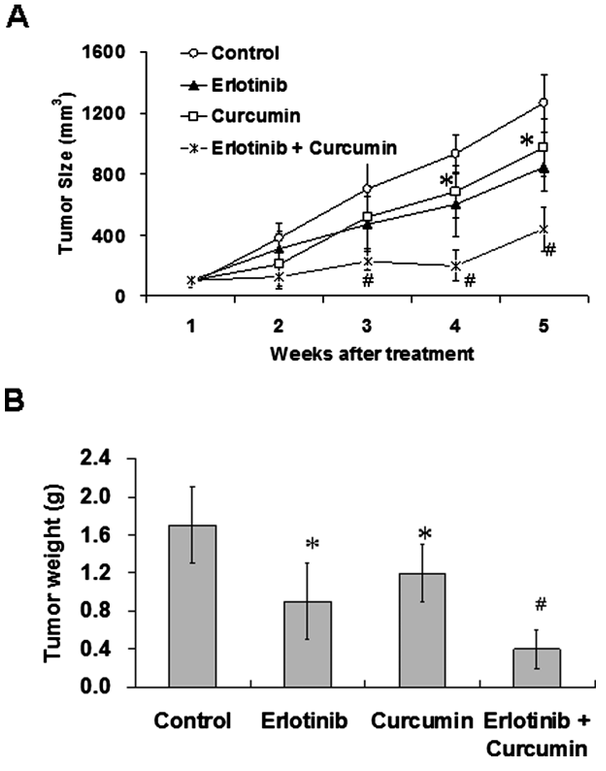

Figure 4. Curcumin enhances the antitumor effect of erlotinib in vivo. H1650 cells were injected subcutaneously into the flanks of athymic nude mice. The tumor-bearing mice received curcumin, erlotinib, or their combination treatment for 5 weeks. (A) Tumor volume was measured weekly. The tumor volume presented is means ± SD of five mice. (B) Tumor weight was weighed at the last day of the experiment. Tumor weights presented are means ± SD of five mice. $*p < 0.01$, compared to DMSO treatment; $\#p < 0.01$, compared to curcumin or erlotinib treatment alone.

EGFR-mutant NSCLC cells. The combination of curcumin and erlotinib markedly downregulated the expressions of EGFR, p-EGFR, and survivin, inhibited the activation of NF-κB, induced apoptosis, and inhibited tumor growth of EGFR-mutant NSCLC cells in vivo. Our results demonstrate that curcumin could overcome erlotinib resistance and enhance the antitumor action of erlotinib in EGFR-mutant NSCLC cells.

Clinical studies have demonstrated that EGFR-targeted treatment may provide advantages over traditional chemotherapy for the advanced NSCLC (7,11). Erlotinib has been demonstrated to be an effective drug for NSCLC patients with EGFR mutations (35,36). However, most patients eventually develop resistance to currently available EGFR-TKIs, including erlotinib. Effective therapies for erlotinib-resistant patients remain to be developed. Curcumin has long been known as a potential preventive and therapeutic agent for human diseases (23). Curcumin has been demonstrated to inhibit multiple signaling molecules of tumor cells, such as protein kinases and apoptosis-related proteins (24), to modulate immune function (23, 37), to reverse drug resistance (38), and to exhibit strong antitumor effects (24). In this study, we found that curcumin could sensitize EGFR-mutant NSCLC cells to the cytotoxicity of erlotinib and enhance erlotinib-induced apoptosis of H1650 cells and H1975 cells. A previous study has shown that a novel EGFR inhibitor promotes apoptosis of H1975 cells, but not that of H1650 cells (30). We found that curcumin potentiates erlotinib-induced apoptosis in both cells. Furthermore, the combined treatment of curcumin and erlotinib significantly inhibited tumor growth of H1650 cells in vivo. These results demonstrate for the first time that curcumin enhances the antitumor action of erlotinib in EGFR-mutant and erlotinib-resistant NSCLC cells and implicate that curcumin may be a potential agent for overcoming erlotinib resistance in treating NSCLC patients. We also found that erlotinib had a stronger induction of apoptosis in erlotinib-sensitive PC-9 cells than cisplatin, while erlotinib showed a weaker effect on erlotinib-resistant H1975 cells and H1650 cells than cisplatin. The combination of curcumin and erlotinib showed the same effect on apoptosis of erlotinib-resistant cells as the combination of curcumin and cisplatin (Fig. 2D). The results indicate that curcumin overcomes erlotinib resistance in EGFR-mutant NSCLC cells.

EGFR signaling has been recognized as important regulators of cell proliferation and apoptosis (15). EGFR overexpression is associated with lung cancer development and progression. The EGF binds to EGFR and activates EGFR signaling (7). Inhibition of EGFR expression, therefore, decreases the activation of EGFR signaling (3). Our results showed that erlotinib could inhibit the level of p-EGFR, but not affect the expression of EGFR. Previous studies have shown that curcumin suppresses the intrinsic EGFR kinase activity and blocks ligand-induced EGFR activation in cancer cells but does not affect EGFR expression (21,22). In contrast, we found that curcumin not only decreased p-EGFR level, but also inhibited endogenous EGFR expression in erlotinib-resistant NSCLC cells. The results suggest that curcumin overcomes erlotinib resistance through downregulating endogenous EGFR level, which results in the inhibition of intrinsic EGFR activity.

Overexpression of survivin has been shown to be linked to drug resistance (39). Previous studies have shown that EGFR signaling activation of the PI3K-AKT pathway upregulates survivin expression (40); persistent survivin expression is associated with erlotinib resistance in EGFR mutation-positive NSCLC cells. Downregulation of survivin contributes to EGFR-TKI-induced apoptosis in EGFR mutation-positive NSCLC cells (21). Curcumin has been shown to inhibit survivin expression in various types of cancer cells (24,41). Our results showed that erlotinib did not downregulate survivin expression and failed to induce apoptosis in H1650 cells, consistent with

a previous report (21). Curcumin significantly decreased survivin expression and induced apoptosis in H1650 cells. Furthermore, the combination of curcumin and erlotinib markedly induced apoptosis, released cytosolic cytochrome c, and inhibited survivin expression. Consequently, the combination of curcumin and erlotinib resulted in the increased cleavage of caspase-3 and caspase-9 and release of cytorchrome c. Our results are similar to a previous report that the downregulation of survivin by YM155 (a specific inhibitor of survivin) increased erlotinib-induced apoptosis in H1650 cells (21). The results demonstrate that the downregulation of survivin is one mechanism for the curcumin-enhanced erlotinib antitumor effect.

EGFR signaling has been shown to activate the NF-κB pathway that promotes EGFR-TKI resistance (31,32). Genetic or pharmacologic inhibition of NF-κB by siRNA or NF-κB-specific inhibitors enhanced erlotinib-induced apoptosis in erlotinib-resistant EGFR-mutant lung cancer cells (33). Curcumin has been demonstrated to inhibit NF-κB activation in cancer cells (34). We found that curcumin markedly decreased the level of p-p65 in H1650 cells. The result suggests that inhibition of NF-κB activation may be another mechanism by which curcumin overcomes erlotinib resistance.

In summary, our results demonstrated that curcumin could overcome erlotinib resistance and significantly enhance erlotinib-inhibited cell proliferation and erlotinib-induced apoptosis in erlotinib-resistant NSCLC cells. The downregulation of expressions of EGFR, p-EGFR, and survivin and inhibition of NF-κB activation are the major mechanisms by which curcumin overcomes erlotinib resistance in NSCLC cells. Our findings suggest that curcumin might be a potential adjuvant for NSCLC patients during erlotinib treatment.

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