



CIP2A mediates erlotinib-induced apoptosis in non-small cell lung cancer cells without EGFR mutation



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ABSTRACT

Background: Epidermal growth factor receptor (EGFR) inhibitors show favorable clinical response in some patients with non-small cell lung cancer (NSCLC) who have no EGFR mutation, indicating alternative mechanisms for their tumoricidal effects. We previously showed erlotinib, a selective EGFR antagonist, inhibited the growth of sensitive hepatocellular carcinoma cells by inhibiting the cancerous inhibitor of protein phosphatase 2A (*CIP2A*) pathway. The aim of this study was to determine if erlotinib can also inhibit the growth of NSCLC cells by inactivating the *CIP2A*-dependent signaling pathway.

Methods: Four NSCLC cell lines (H358 H441 H460 and A549) were treated with erlotinib to determine their sensitivity to erlotinib-induced cell death and apoptosis. Expression of *CIP2A* and the downstream *AKT* were analyzed. The effects of *CIP2A* on erlotinib-induced apoptosis were confirmed by overexpression of *CIP2A* and knockdown of *CIP2A* gene expression in the sensitive cells and resistant cells, respectively. *In vivo* efficacy of erlotinib against H358 xenograft tumor was also determined in nude mice.

Results: Erlotinib induced significant cell death and apoptosis in H358 and H441 cells, as evidenced by increased caspase 3 activity and cleavage of pro-caspase 9 and PARP, but not in H460 or A549 cells. The apoptotic effect of erlotinib in the sensitive H358 cells was associated with downregulation of *CIP2A*, increase in PP2A activity and decrease in *AKT* phosphorylation. Overexpression of *CIP2A* and *AKT* protected the sensitive H358 cells from erlotinib-induced apoptosis. Knockdown of *CIP2A* gene expression by siRNA enhanced the erlotinib-induced apoptotic in the resistant H460 cells that resembled the sensitive H358 cells. Erlotinib also inhibited the growth of H358 tumors in nude mice.

Conclusions: The *CIP2A*-dependent pathway mediates the tumoricidal effects of erlotinib on NSCLC cells without EGFR mutations *in vitro* and *in vivo*. *CIP2A* may be a novel molecular target against NSCLC for future drug development.

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1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide and 80% of lung cancers are diagnosed as NSCLC [1]. Epidermal growth factor receptor (EGFR) gene mutations are identified in 10–15% of Caucasian NSCLC patients and even higher percentages in Asian patients [2]. Patients with certain EGFR mutations, such as L858R and exon 19 deletion have a higher response rate to the EGFR

targeted drugs, such as gefitinib (Iressa) and erlotinib (Tarceva) [3–6]. However, some NSCLC patients without EGFR mutations still respond to gefitinib and erlotinib [7,8], suggesting that there may be mechanism(s) other than the EGFR-pathway that mediates the tumoricidal effects of gefitinib and erlotinib. The exact mechanisms are unclear.

Cancerous inhibitor of protein phosphatase 2A (*CIP2A*) was originally identified as a cellular *PP2A* inhibitor that inhibits proteolytic degradation of *c-MYC* [9]. *CIP2A* is overexpressed in several human malignancies including HCC, gastric cancer, head and neck cancer, colon cancer, breast cancer, prostate cancer and non-small cell lung cancer [9–17]. Importantly, overexpression of *CIP2A* in NSCLC correlates with poor prognosis [14–16]. The decrease of *CIP2A* and downstream inactivation of the *AKT* pathway can inhibit proliferation and induce apoptosis in a variety of lung cancer cells [15].

In our previous study, we showed that erlotinib, a selective *EGFR* inhibitor, inhibited the growth of sensitive hepatocellular carcinoma cells by causing *CIP2A* dependent *PP2A* activation and p-*AKT* downregulation, but not in resistant cells [18]. In this study, we hypothesized that the *CIP2A*-dependent p-*AKT* pathway mediates the anti-tumor activity of erlotinib in NSCLC cells.

2. Materials and methods

2.1. Cell lines and culture

Four NSCLC cell lines were used in this study. The H358 cell line was obtained from the American Type Culture Collection (Manassas, VA) and the A549, H441, and H460 cell lines were from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The characteristics of each cell lines are listed in Table 1. The NSCLC cell lines were kept in RPMI1640 (Invitrogen, Life Technologies, Saint Aubin, France) supplemented with 10% FBS (GIBO/Life Technologies, Grand Island, NY), 100 units/mL penicillin G, and 100 µg/mL streptomycin sulfate in a 37 °C humidified incubator with 5% CO₂ in air.

2.2. Reagents and antibodies

Erlotinib (Tarceva®) was purchased from Selleck chemicals (Houston, TX). For *in vitro* studies, erlotinib at various concentrations were dissolved in DMSO and then added to the cells in serum-free RPMI1640. *PP2A* inhibitor and activator were purchased from Sigma and Merck Millipore, respectively. Antibodies for immunoblotting such as anti-*CIP2A*, *AKT* and *PARP* were purchased from Santa Cruz Biotechnology (San Diego, CA). p-*AKT* (Ser473) and caspase-9 were from Cell Signaling (Danvers, MA).

2.3. Cell viability assay and apoptosis analysis

Four NSCLC cells were seeded in 96-well plates (3 × 10³ cells/well). To determine cell viability and proliferation, 10% WST-1 (water-soluble tetrazolium monosodium salt) (Cell Proliferation Reagent WST-1; Roche applied science, Indianapolis, IN) agent was added to the cell suspension in each well,

incubated for 1–2 h, and quantified by measuring the absorbance at 450 nm using a Biotek Synergy HT ELISA reader (Biotek, Winooski, VT) to calculate the optical density (OD) values. Apoptotic cells were measured by flow cytometry (sub-G1) and cell death was detected by Western blot. *Caspase-3* activity was measured by *caspase 3* assay kit (colorimetric) from Abcam (Paris, France).

2.4. Overexpression of *CIP2A*

CIP2A cDNA (KIAA1524) was purchased from OriGene (Rockville, MD). Briefly, following transfection, H358 cells were incubated in the presence of G418 (0.78 mg/mL) (Sigma-Aldrich, St. Louis, MO). After 8 weeks of selection, surviving colonies, i.e., those arising from stably transfected cells were selected and individually amplified. H358 cells with stable overexpression of *CIP2A* was treated with erlotinib, harvested, and processed for Western blot analysis.

2.5. *PP2A* phosphatase activity

Protein phosphatase 2A (*PP2A*) activity was measured in fresh cells as described previously [20] using *PP2A* DuoSet IC activity assay kit according to the manufacturer's description (R&D Systems, Minneapolis, MN). Briefly, an immobilized capture antibody specific for the catalytic subunit of *PP2A* that binds both active and inactive *PP2A* was used. After washing, a substrate is added that is dephosphorylated by active *PP2A* to generate free phosphate, which is detected by a sensitive dye-binding assay using malachite green and molybdic acid.

2.6. Gene knockdown using siRNA and cell transfection

Smart-pool siRNA, including control (sc-37007), *CIP2A* and *PP2A* were purchased from Santa Cruz Biotechnology (San Diego, CA). Cells were transfected with siRNA to a final concentration of 100 nM in six-well plates with the Dharma-FECT4 transfection reagent (Dharmacon, Chicago, IL). After 48 h, the medium was replaced and the cancer cells were irradiated and harvested for analysis by western blot and flow cytometry to measure apoptosis. *CIP2A* cDNA (KIAA1524) was purchased from OriGene (RC219918, Rockville, MD). Following transfection, H460 and H358 cells were incubated in the presence of 0.78 mg/mL G418 (Sigma-Aldrich, St. Louis, MO) to select the stably transfected clones. Those cells that stably expressed *CIP2A-myc* were used for treatment with erlotinib as indicated.

2.7. Quantification of *CIP2A* gene expression

Total RNA was extracted from H358 and H460 cells (approximately 5 × 10⁶) followed by erlotinib treatment using RNeasy mini kit (Qiagen, Gaithersburg, MD) then reverse transcribed by using QuantiTect Reverse Transcription Kit (Qiagen, Gaithersburg, MD). The real time quantitative PCR was performed on an Applied Roter-Gene 3000 detector (Qiagen, Gaithersburg, MD) with a specific primer set for each target gene and SYBR Green dye (Qiagen,

Table 1

Characteristic of the NSCLC cell lines used in this study with different mutation status.

Cell line	Subtype	EGFR	HER2	KRAS	BRAF	PIK3CA	TP53	PTEN
A549	Adenocarcinoma	Wild	Wild	G12S	Wild	Wild	Wild	Wild
H460	Large cell carcinoma	Wild	Wild	Q61H	Wild	E545K	Wild	Wild
H358	Adenocarcinoma	Wild	Wild	G12C	Wild	Wild	Null	Wild
H441	Adenocarcinoma	Wild	Wild	G12V	Wild	Wild	R158L	Wild

Source. This table was modified from Rikova et al. [31].

Abbreviations: EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; KRAS, Kirsten rat sarcoma viral oncogene homolog; BRAF, PIK3CA, phosphoinositide-3-kinase, catalytic, alpha polypeptide; TP53, tumor protein p53; PTEN, phosphatase and tensin homolog.

Gaithersburg, MD) for detection as described in the manufacturer's guidelines. The PCR primer sets for target genes were as follows: human *CIP2A* (Hs_KIAA1524 QuantiTect Primer Assay, NM_020890) and human *actin* (Hs_ACTB QuantiTect Primer Assay, NM_001101). An aliquot of each sample was analyzed by quantitative PCR for β -*actin* to normalize for inefficiencies in cDNA synthesis and RNA input amounts. For each sample, the average threshold (C_t) value was determined from quadruplicate assays, and the ΔC_t value was determined by subtracting the average β -*actin* C_t value from the average *CIP2A* C_t value. Three independent experiments were performed to measure the levels of *CIP2A* of H358 cells with differential time treatment.

2.8. Dual-luciferase reporter assay

To verify the transcriptional activity between the erlotinib-sensitive (H358) and erlotinib-resistance (H460) cells, the promoter activity of *CIP2A* was determined using the dual-luciferase reporter assay kit (Promega, Madison, WI). H358 and H460 cells were co-transfected in six-well plates with 2 μ g of DNA, including the luciferase reporter construct pGL4.17-*CIP2A*-promoter (firefly fluorescence reporter) and pRL-TK plasmid (renilla fluorescence reporter) as indicator for normalization of transfection efficiency, at a ratio of 9:1. Forty-eight hours post-transfection, the cells were add or not variously doses of erlotinib. After 24 h, cells lysed were collection and the luciferase activity was quantified according to the manufacturer's instructions. Cells co-transfected with pGL4.17-basic plasmids combined with the pRL-TK were used as a negative control. The promoter activity was repeated three times in parallel for statistical analysis.

2.9. Xenograft tumor growth

Male NCr nude mice (5–7 weeks of age) were used. All experimental procedures using these mice were performed according to protocols approved by the Institutional Laboratory Animal Care and Use Committee of Cardinal Tien Hospital. Each mouse was inoculated subcutaneously in the dorsal flank with 1×10^7 H358 cells suspended in 0.1 mL of serum-free medium containing 50% Matrigel (BD Biosciences, Bedford, MA). When tumors reached 100–200 mm³, the mice received erlotinib (10 mg/kg) p.o. once daily. The controls received vehicle. The tumors were measured twice weekly using calipers and their volumes calculated using the following standard formula: width \times length \times height \times 0.523 [18].

2.10. Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by Tukey's subtest. The results were expressed as mean \pm standard deviation (SD). Differences were considered significant at * $P < 0.05$.

3. Results

3.1. Differential effects of erlotinib on viability and apoptosis in NSCLC cells without EGFR mutation

To investigate the antitumor effect of erlotinib on NSCLC cell lines, we first assessed the effect of treatment with erlotinib on growth inhibition in human NSCLC cell lines. As shown in Fig. 1A, erlotinib exhibited differential effects on the viability of the cells. Erlotinib caused a dose- and time-dependent reduction in cell viability in H358 and H441 cells, whereas H460 and A549 cells were more resistant. As shown in Fig. 1B, erlotinib induced significant apoptosis in H358 and H441 cells in a dose- and time-dependent manner. However, H460 and A549 cells did not show significant

apoptosis with erlotinib treatment (Fig. 1B). In the sensitive cell line, erlotinib cleaved *procaspase-9* in H358 cells, which led to the appearance of *caspase-9*, and cleaved *PARP* in both H358 and H441 cells (Fig. 1C). These were not seen in the resistant H460 and A549 cells. The activity of *caspase-3*, another apoptosis-related gene, was also measured. Erlotinib elevated *caspase-3* activity in a dose-dependent manner in H358 and H441 cells, but not in H460 or A549 cells (Fig. 1D). Erlotinib caused cell fragmentation in a dose-dependent manner in the H358 and H441 cells, but not in H460 or A549 cells (Fig. 1E). In annexin-V/propidium iodide double-staining assay, erlotinib induced significant apoptosis in H358 in a dose-dependent manner, but not in H460 (Supplementary Fig. 1). Taken together, these results suggest that erlotinib induces apoptosis and cell death in H358 and H441 cells, despite their lack of *EGFR* mutation.

Supplementary figure related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.lungcan.2014.05.024>.

3.2. Downregulation of *CIP2A* determines erlotinib-induced p-AKT inhibition and apoptosis in NSCLC cells without EGFR mutation

Next we investigated the role of *CIP2A* in erlotinib-induced apoptosis in the sensitive NSCLC cells. As shown in Fig. 2A and C, erlotinib decreased *CIP2A* protein levels and AKT phosphorylation and induced apoptosis in the erlotinib-sensitive H358 cells in dose-dependent and time-dependent manner. In contrast, erlotinib did not significantly affect levels of *CIP2A* protein or AKT phosphorylation in the resistant H460 cells.

In the sensitive H358 cells, erlotinib also increased PP2A activity, and *CIP2A* overexpression decreased PP2A activity (Fig. 2B). Erlotinib did not change PP2A activity and *CIP2A* gene knockdown by siRNA increased PP2A activity in the resistant H460 cells (Fig. 2B) and A549 cells (Supplementary Fig. 2). These data indicate that the *CIP2A* signaling pathway may play an important role in determining the sensitivity of lung cancer cells to erlotinib. In addition, erlotinib decreased *CIP2A* protein levels and induced apoptosis in H358 cells in a time-dependent analysis (Fig. 2C).

Supplementary figure related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.lungcan.2014.05.024>.

3.3. Validation of the *CIP2A*-PP2A-AKT pathway

Two approaches were used to validate the role of *CIP2A* as a mediator of erlotinib-induced apoptosis in the NSCLC cells. First, ectopic expression of *CIP2A* in the H358 cells (*CIP2A-myc* in Fig. 3A, left), which showed overexpression of *CIP2A*, partially protected the cells from apoptotic cell death induced by erlotinib (Fig. 3A). Furthermore, knockdown of gene expression of *CIP2A* increased apoptosis in the H358 cells (Fig. 3A, right) and H460 cells (Fig. 3B). These results indicate that *CIP2A* expression status is important in regulating the apoptotic effect of erlotinib in NSCLC cells.

Next, we analyzed the role of AKT, which is downstream from *CIP2A*, in mediating the effects of erlotinib. As shown in Fig. 3C, overexpression of AKT in the sensitive H358 cells partially protected cells from apoptotic death induced by erlotinib. Fig. 3D further showed that addition of okadaic acid, a known PP2A inhibitor, significantly increased AKT phosphorylation and reduced the erlotinib-induced apoptosis in the sensitive H358 cells. These results indicate that AKT activation regulates erlotinib-induced apoptosis in NSCLC cells.

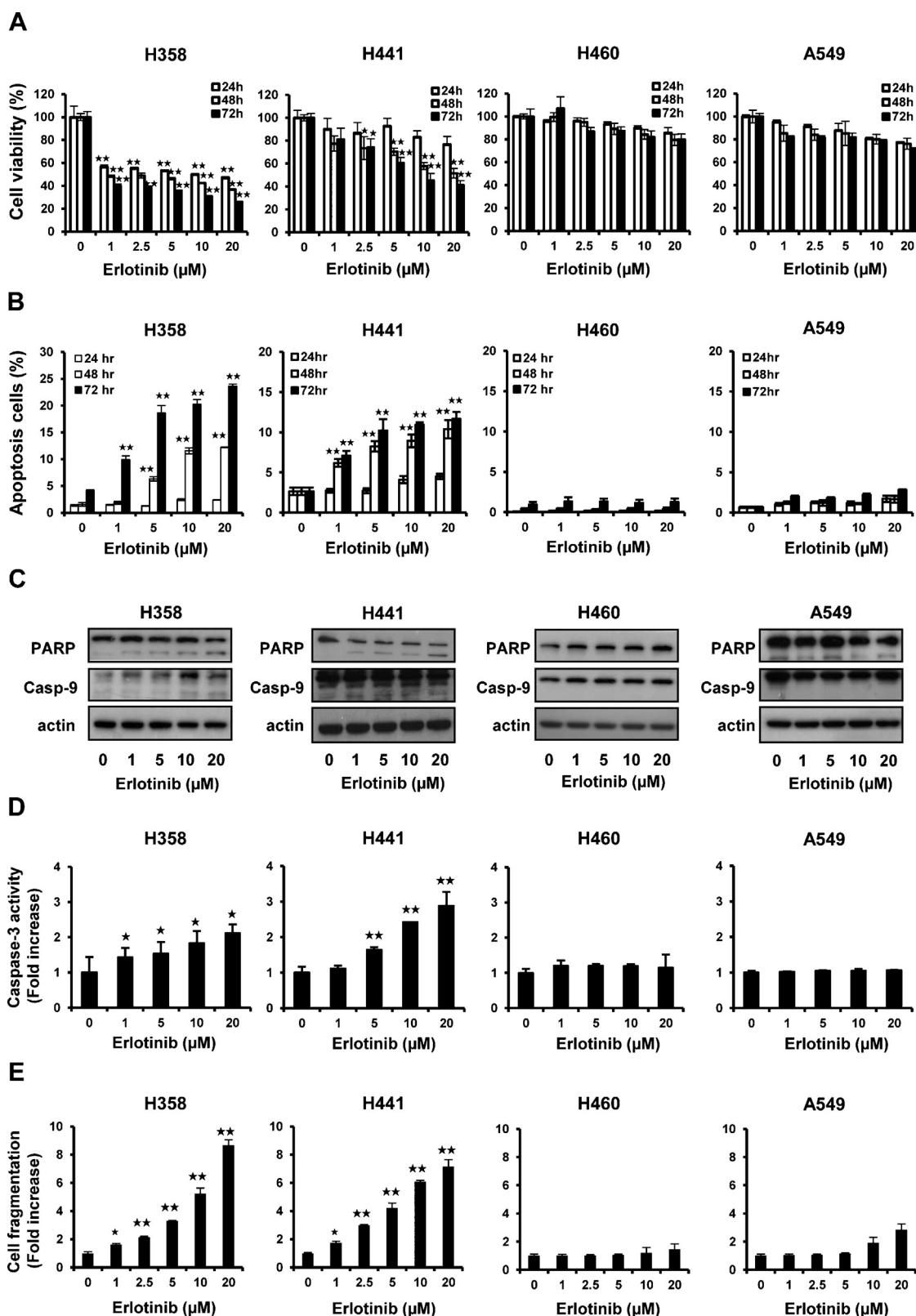


Fig. 1. Differential effects of erlotinib on cell death and apoptosis in the four human NSCLC cells. (A) Dose-dependent effects of erlotinib on cell viability in the four human NSCLC cell lines. Column, mean; bar, SD. $n=3$ for each concentration. * $p<0.05$, ** $p<0.01$, vs. no erlotinib. (B) Dose-dependent effects of erlotinib on apoptosis in the four human NSCLC cell lines. Data are mean \pm SD. $n=3$ for each concentration. * $p<0.05$, ** $p<0.01$, vs. no erlotinib. (C) Effects of erlotinib on PARP and caspase-9 in the four NSCLC cells. Cells were treated with erlotinib at the indicated concentrations for 48 h. Data are representative of three independent experiments. (D) Effects of erlotinib on caspase-3 activity. NSCLC cells were treated with erlotinib at the indicated concentrations for 48 h. Data are mean \pm SD. $n=3$ for each concentration. ** $p<0.01$, vs. no erlotinib. (E) Effects of erlotinib on cell fragmentation. NSCLC cells were treated with erlotinib at the indicated concentrations for 48 h. Data are mean \pm SD. $n=3$ for each concentration. * $p<0.05$, ** $p<0.01$, vs. no erlotinib.

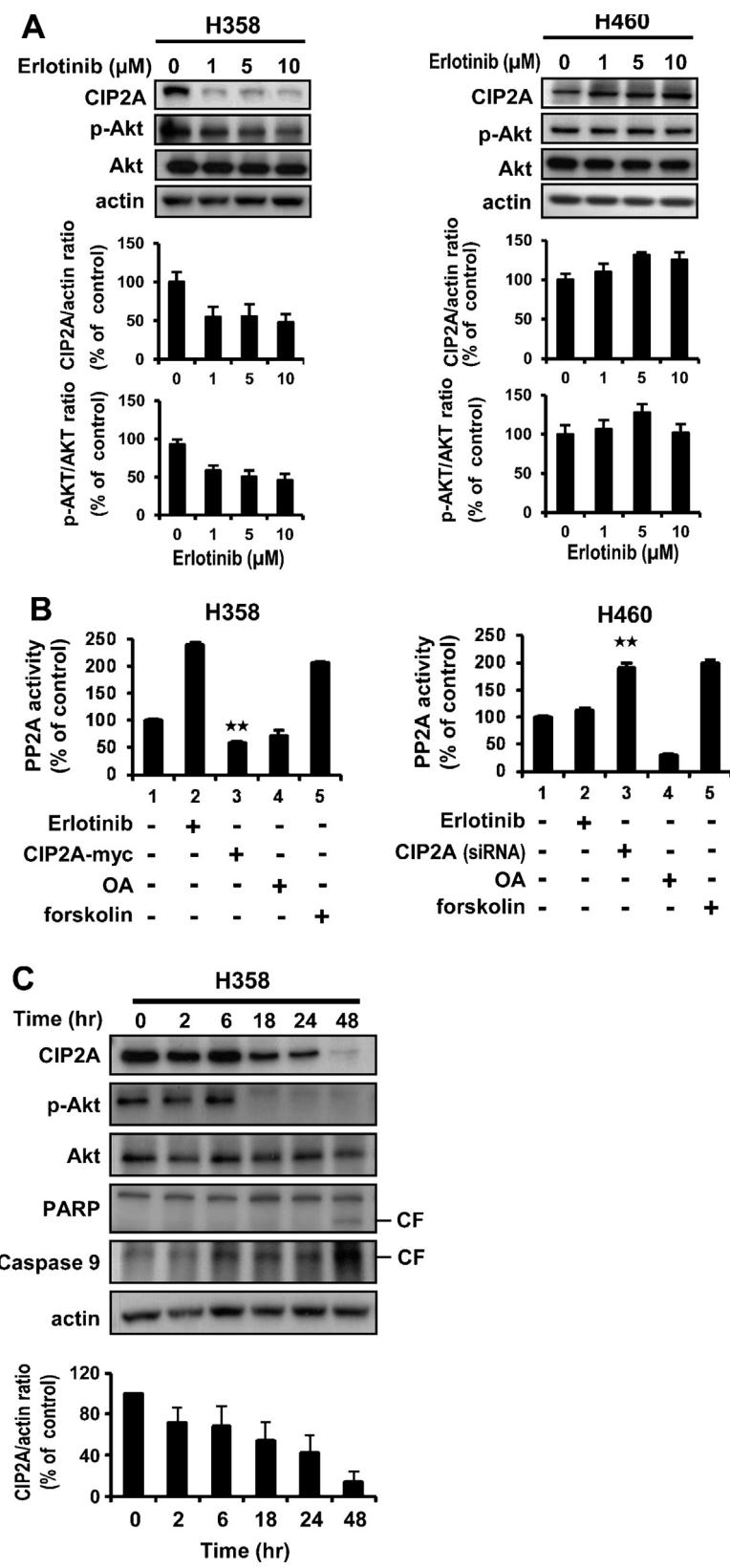


Fig. 2. Downregulation of *CIP2A* determines the effects of erlotinib on p-AKT and apoptosis in NSCLC cells through activation of PP2A. (A) Dose-dependent effects of erlotinib on *CIP2A*, p-AKT and AKT. NSCLC cells were exposed to erlotinib at the indicated concentrations for 48 h. Immunoblots were scanned by a UVP BioSpectrum AC image system and quantitated using VisionWork LS software to determine the ratio of the level of *CIP2A* to actin or p-AKT to AKT. Data are mean \pm SD. $n = 3$ for each experiment. (B) Effects of erlotinib on PP2A activity. Transfection with *CIP2A-myc* in H358 cells and *CIP2A* siRNA in H460 cells were also performed. OA and forskolin treatment serves as negative and positive controls for PP2A activity. Data are mean \pm SD. $n = 3$ for each experiment. ** $p < 0.01$, vs. no treatment. (C) Time-dependent effects of erlotinib on *CIP2A*, p-AKT and apoptosis-related proteins in the sensitive H358 cells. H358 cells were exposed to 10 μM erlotinib for up to 48 h. CF, cleaved form (activated form). Data are mean \pm SD. $n = 3$ for the different time intervals.

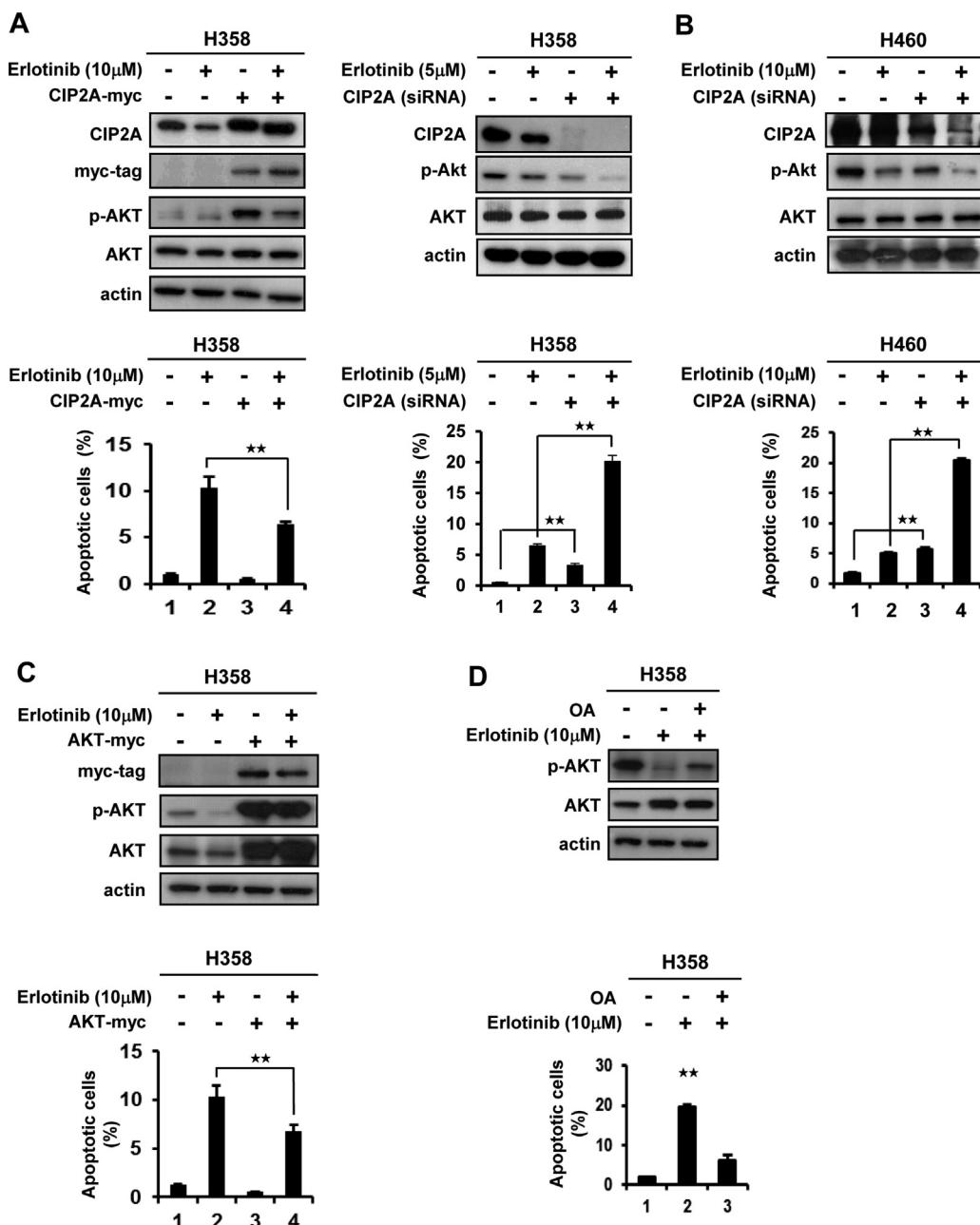


Fig. 3. Validation of CIP2A-PP2A-AKT pathway. (A) Ectopic expression of CIP2A (CIP2A-myc) increased p-AKT and attenuated the effects of erlotinib on apoptosis in H358 cells (left figure). H358 cells overexpressing CIP2A were treated with 10 μ M erlotinib for 48 h. Knockdown of CIP2A expression by siRNA increased the sensitivity to erlotinib-induced apoptosis in H358 cells. Cells were transfected with either control or CIP2A siRNA for 48 h then exposed to 5 μ M erlotinib for 24 h. (right figure). (B) Knockdown of CIP2A expression by siRNA increased the sensitivity to erlotinib-induced apoptosis in H460 cells. Cells were transfected with either control or CIP2A siRNA for 48 h then exposed to 10 μ M erlotinib for 48 h. (C) Ectopic expression of AKT (AKT-myc) attenuated the effects of erlotinib on apoptosis in H358 cells. H358 cells overexpressing AKT were treated with 10 μ M erlotinib for 48 h. (D) Okadaic acid (OA), a PP2A inhibitor, increased pAKT and inhibited the effects of erlotinib on apoptosis in H358 cells. Data are mean \pm SD. n = 3 for each condition. **p < 0.01, vs. no treatment.

3.4. Erlotinib downregulates transcription of CIP2A in NSCLC cells without EGFR mutation

To examine the mechanisms by which erlotinib inhibited CIP2A expression, we investigated whether erlotinib affected CIP2A protein degradation. After protein translation was blocked by cycloheximide, the rate of CIP2A degradation did not change significantly with or without erlotinib treatment in either H358 or H460 cells (Fig. 4A). We next investigated whether erlotinib affected CIP2A gene transcription. Our data showed that the mRNA levels of CIP2A decreased in a time- and dose-dependent manner in H358

cells but not in H460 cells (Fig. 4B). To further explore the inhibition of CIP2A transcription by erlotinib, H358 and CH460 cells were transfected a luciferase reporter construct for CIP2A promoter. Erlotinib significantly down-regulated the activity of CIP2A promoter in a dose-dependent manner in H358 cells (Fig. 4C). However, erlotinib did not alter the luciferase activity H460 cells. Erlotinib may inhibit CIP2A expression in the sensitive H358 cells by affecting the transcriptional factor Elk-1 DNA binding [19] so we treated H358 and H460 cells with 2 μ M or 10 μ M erlotinib for 24 h and processed for the ChIP assays. The expression of Elk-1 was reduced in a dose-dependent manner in the sensitive H358 cells, but not

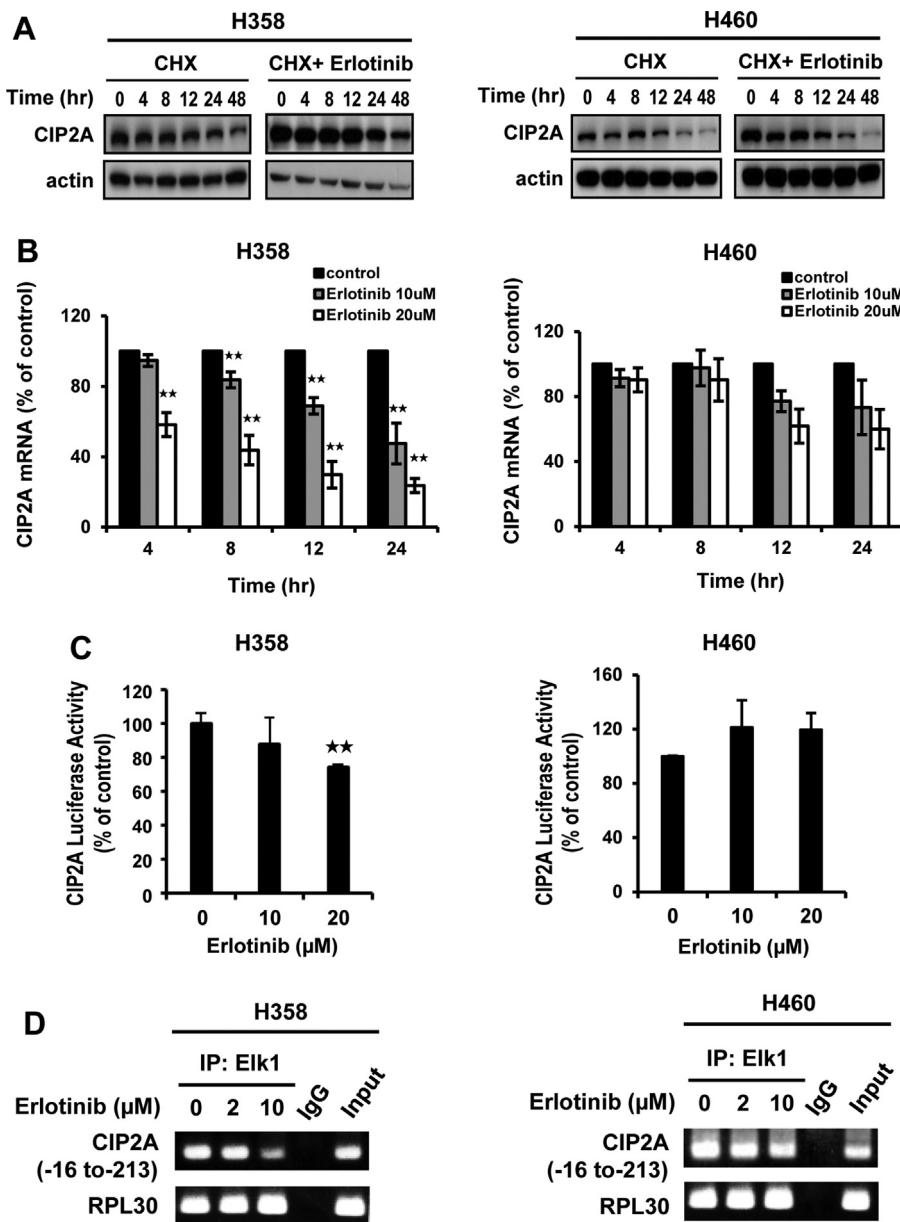


Fig. 4. Erlotinib-induced downregulation of CIP2A in NSCLC cells. (A) H358 or H460 cells were treated with 100 μg/mL cycloheximide (CHX) in the presence or absence of erlotinib for the indicated length of time. (B) H358 and H460 cells treated with erlotinib at 10 μM or 20 μM for the designated incubation time. Erlotinib inhibits CIP2A mRNA in a dose- and time-dependent manner, especially in H358 cells. Data are mean ± SD. n = 3 for each time point. **p < 0.01, vs. no treatment control. (C) Effects of erlotinib on CIP2A promoter activity. H358 and H460 cells were transfected by CIP2A reporter and Renilla vectors for 24 h then treated with 10 or 20 μM erlotinib for an additional 24 h. Cell lysates were prepared for analysis of luciferase activity. Erlotinib decreased CIP2A luciferase activity in H358 cells, but not in H460 cells. Data are mean ± SD. n = 3 for each condition. **p < 0.01, vs. no erlotinib. (D) Chromatin immunoprecipitation assays of the CIP2A promoter. H358 and H460 cells were treated with 2 or 10 μM erlotinib for 24 h and processed for the ChIP assays. Soluble chromatin was immunoprecipitated with Elk-1 or IgG (negative control) antibodies. Immunoprecipitates were subjected to PCR with primer pair specific to CIP2A promoter (−16 to −213 bp) and RPL30 (internal control). The gel shown is representative of three independent experiments.

in the resistant H460 cells. These data suggest that erlotinib may inhibit CIP2A expression in the sensitive H358 cells by affecting the transcriptional regulation

3.5. Evaluation of the therapeutic effect of erlotinib on H358-bearing mice

To determine whether or not the *in vitro* effects of erlotinib on H358 cells could be reproduced *in vivo*, mice were implanted with H358 xenograft. No apparent differences in body weight or toxicity were found in any mice (Fig. 5A). Treatment with erlotinib significantly inhibited H358 xenograft tumor growth and tumor sizes were nearly one-fifth of those of control mice (Fig. 5B).

4. Discussion

Although NSCLC patients with EGFR-mutation respond well to EGFR inhibitors, such as erlotinib, some NSCLC patients without EGFR mutations also show favorable response. In the phase 3 Sequential Tarceva in Unresectable NSCLC (SATURN) trial, erlotinib, used as second-line NSCLC maintenance therapy after first-line chemotherapy without selection for EGFR status, showed better progression free survival than placebo in patients without EGFR mutation (hazard ratios 0.78, 95% CI 0.63–0.96; P = 0.0185) [7]. In the Tarceva In Treatment of Advanced NSCLC (TITAN) study, which explored the efficacy and tolerability of second-line erlotinib versus chemotherapy in patients with refractory NSCLC, there was

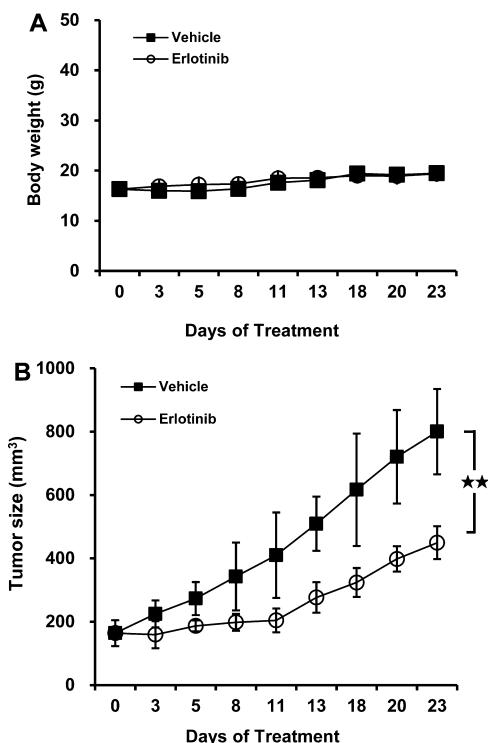


Fig. 5. *In vivo* effect of erlotinib on H358 xenograft in mice. (A) Effects on body weight of mice. Mice were treated with vehicle, or oral erlotinib at 10 mg/kg daily for 3 weeks ($n = 10$ each). (B) Effects of erlotinib on tumor size. Erlotinib inhibited the growth of H358 tumors by >80%. Data are mean \pm SD ($n = 10$ each); *** $p < 0.001$.

no significant differences in efficacy between erlotinib and docetaxel or pemetrexed in patients without *EGFR* mutation (hazard ratios 0.85, 95% CI 0.59–1.22; $P = 0.37$) [8]. These two studies offer strong clinical evidence that erlotinib has an anti-tumor effect even in NSCLC patients without *EGFR* mutation, indicating the presence of alternative mechanisms. Our study showed that the *CIP2A*-dependent signaling pathway may be one such mechanism.

Our study revealed that erlotinib induced cell death and apoptosis in the NSCLC H358 and H441 cells, but not in the H460 and A549 cells. The differential sensitivity to erlotinib was related to the status of *CIP2A* since erlotinib inhibited mRNA and protein expression of *CIP2A* in the NSCLC H358 and H441 cells but not in the NSCLC (H322 and H460). In addition, overexpression of *CIP2A* in the sensitive H358 cells partially protected the cells from apoptotic cell death induced by erlotinib. Knockdown of *CIP2A* gene expression in the resistant H460 cells increased apoptotic cell death induced by erlotinib. The *CIP2A* effects were mediated by downstream activation of *PP2A* and inhibition of *AKT* activation. Erlotinib treatment increased *PP2A* activity and decreased *AKT* phosphorylation in the sensitive H358 cells but not in the resistant H460 cells. Overexpression of *CIP2A* in the erlotinib sensitive H358 cells increased phosphorylated *AKT* and inhibited apoptosis. Knockdown of *CIP2A* gene expression in the erlotinib resistant H460 cells decreased the phosphorylated *AKT* and increased erlotinib-induced apoptosis, thus changing the phenotype of the resistant H460 cells to resemble the sensitive H358 cells. Our results, however, did not exclude the possibility that other pathways, such as *KRAS* (Kirsten rat sarcoma viral oncogene homolog) and *TP53* (tumor protein *p53*), may also be involved in determining the differential sensitivity to erlotinib since the protective effects by altering the *CIP2A* status was incomplete. This needs to be investigated in future research.

Our findings have several important clinical implications: First, we showed that overexpression of *CIP2A* inhibited erlotinib-induced cell death in the sensitive H358 cells. These results explain

the poor prognosis in patients whose lung tumors overexpresses *CIP2A* [14–16]. Therefore, *CIP2A* status in the tumor may serve as a useful marker for predicting the response to erlotinib. Besides being used as a prognosis biomarker, we identified *CIP2A* as a major molecular determinant of the therapeutic effect of erlotinib in NSCLC patients without *EGFR* mutation. This was supported by our *in vivo* data that showed erlotinib reduced H358 xenograft tumors in mice and that altering the expression of *CIP2A* could change the resistant H460 cells into a phenotype resembling the sensitive H358 cells. These results provided further support that *CIP2A* may be used as a novel anti-cancer target [9,15,18,20–28]. Liang Ma et al. demonstrated that Rabdoocetin B can inhibit proliferation and induce apoptosis in a variety of lung cancer cells by down-regulation of *CIP2A* and inactivation of *AKT* pathway [15]. Importantly, the *AKT* pathway plays a major role in carcinogenesis and drug resistance in NSCLC [29]. Several studies have also shown that tumors with the activation of *AKT* signaling become more aggressive and are associated with poor prognosis in patients with NSCLC [30].

In conclusion, erlotinib induced apoptotic cell death in NSCLC cells without *EGFR* mutation through a novel mechanism that was *CIP2A*-dependent. The expression status of *CIP2A* could be a molecular determinant of the sensitivity of NSCLC cells to erlotinib. Development of novel therapies targeting *CIP2A* would increase our therapeutic options for patients with NSCLC.

Conflict of interest statement

All authors declared that no competing interests exist.

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