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

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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 hepatoacellular 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 Biotech 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 molybdc 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 × 10⁸) 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, France).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Subtype</th>
<th>EGFR</th>
<th>HER2</th>
<th>KRA5</th>
<th>BRAF</th>
<th>PIK3CA</th>
<th>TP53</th>
<th>PTEN</th>
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<tbody>
<tr>
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<td>Wild</td>
<td>Wild</td>
<td>G12S</td>
<td>Wild</td>
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<td>Wild</td>
<td>Wild</td>
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<tr>
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<td>Large cell carcinoma</td>
<td>Wild</td>
<td>Wild</td>
<td>Q61H</td>
<td>Wild</td>
<td>E545K</td>
<td>Wild</td>
<td>Wild</td>
</tr>
<tr>
<td>H435</td>
<td>Adenocarcinoma</td>
<td>Wild</td>
<td>Wild</td>
<td>G12C</td>
<td>Wild</td>
<td>Null</td>
<td>Wild</td>
<td>Wild</td>
</tr>
<tr>
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<td>Adenocarcinoma</td>
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<td>Wild</td>
<td>G12V</td>
<td>Wild</td>
<td>R158L</td>
<td>Wild</td>
<td>Wild</td>
</tr>
</tbody>
</table>

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

Abbreviations: EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; KRA5, 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_0208990) 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 (Ct) value was determined from quadruplicate assays, and the ΔCt value was determined by subtracting the average β-actin Ct value from the average CIP2A Ct 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 μg 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 added 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 × length × height × 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 ± 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 ± 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 ± 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 ± 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 ± 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 ± 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 ± SD. n = 3 for the different time intervals.
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 μM or 10 μM 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
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
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 Rabdoocotisin B can inhibit prolife-
eration 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 carcinogen-
esis 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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