The synergistic effect of EGFR tyrosine kinase inhibitor gefitinib in combination with aromatase inhibitor anastrozole in non-small cell lung cancer cell lines

Lan Shen¹, Ziming Li¹, Shengping Shen¹, Xiaomin Niu¹, Yongfeng Yu², Zonghai Li³, Meilin Liao⁴, Zhiwei Chen⁴, Shun Lu¹,⁎

¹ Shanghai Lung Cancer Center, Shanghai Chest Hospital Affiliated to Shanghai Jiao Tong University, Shanghai, 200030, China
² State Key Laboratory of Oncogenes and Related Genes, Shanghai Jiao Tong University, Shanghai, China

A R T I C L E   I N F O

Article history:
Received 6 November 2011
Received in revised form 6 August 2012
Accepted 19 August 2012

Keywords:
Anastrozole
Gefitinib
Interaction
ER
EGFR
Lung cancer

A B S T R A C T

Background: Several studies implicated that lung cancer progression was governed by the interaction between estrogen receptor (ER) and epidermal growth factor receptor (EGFR) signaling pathways. Combined targeting of EGFR and ER may have the synergistic effect in lung cancer treatment. The aim of this study was to explore the potential utility of inhibiting these two pathways with combination of anastrozole and gefitinib in non-small cell lung cancer (NSCLC) cell lines.

Materials and methods: The expression levels of ER (ER-α and ER-β) in lung cancer cell lines (A549, H460, SPC-A-1, H1299) and normal bronchus epithelial cell BEAS-2B were detected using real-time PCR and Western blot. Immunocytochemistry was used to locate ER-α and ER-β in cell line with highest ER expression levels. The cells were treated with anastrozole or gefitinib alone or in combination. The cell proliferation inhibition was detected by the CCK8 assay, cell cycle and apoptosis effects were detected by flow cytometry; the expression levels of phosphorylated-EGFR (p-EGFR), ERK, phosphorylated-ERK (p-ERK), AKT and phosphorylated-AKT (p-AKT) were detected by Western blot.

Results: Among these cell lines the expression levels of ER in A549 cells were highest. In A549 cell line, ER-α was mainly localized in the cytoplasm, whereas ER-β was mainly localized in the cytoplasm and to a lesser degree in the nucleus. The combination of two drugs increased the proliferation inhibition rates for 24 h, 48 h, 72 h to 37.66 ± 1.02%, 63.41 ± 2.02%, 70.50 ± 0.86%, respectively, which was closely associated with elevation of the G0/G1 phase fraction (P < 0.05). Apoptosis rates of A549 cells treated with anastrozole, gefitinib alone or in combination were 10.72 ± 1.12%, 17.40 ± 1.28%, 23.02 ± 2.32%, respectively (P < 0.05). The synergistic effects of the combination therapy were accompanied by reduction of p-EGFR, p-ERK and p-AKT expression compared with individual treatment.

Conclusions: The results of this study suggest that the combination of anastrozole and gefitinib compared with either drug alone can maximally inhibit cell proliferation, induce apoptosis, and affect downstream signaling pathways. Our study supports functional interaction between the ER and the EGFR pathways in lung cancer and provides a clinically exploitable strategy for non-small cell lung cancer patients.

© 2012 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Lung cancer remains the most common malignancy worldwide. The 5-year survival rate for all stages is only 15% [1]. Although systemic chemotherapy reduces the lung cancer mortality, disease progression is inevitable and dose-limiting toxicities restrict their use. Therefore, identifying new effective therapeutic treatments for lung cancer is critical.

Epidermal growth factor receptor (EGFR) belongs to the ErbB family of plasma membrane receptor tyrosine kinases, which is overexpressed in non-small cell lung cancer (NSCLC) cell lines and tissue specimens. Activation of EGFR can promote tumor cell proliferation, angiogenesis, invasion, and metastasis [2]. New molecularly targeted therapies are in clinical development to treat NSCLC through inhibiting EGFR pathways. Gefitinib (IRESSA), an EGFR tyrosine kinase inhibitor (EGFR-TKI), has been approved for its clinical benefit in the treatment of advanced NSCLC patients who have previously received platinum-based chemotherapy [3].

However, aberrant cancer-causing pathways incorporate multiple components. Therefore targeting a particular receptor may not be sufficient for long-term control of lung cancer due to compensatory feedback loops. Recent evidence suggests ER-α and ER-β are expressed in NSCLC cell lines and tissues [4,5]. Furthermore, the biological effect of estrogen receptor (ER) may limit the efficacy of EGFR inhibitors due to the interaction between ER and EGFR...
pathways [6]. We hypothesized that dual inhibition of ER and EGFR pathways may be one strategy to maximize the benefit of EGFR-TKI.

Aromatase, a member of the cytochrome P450 family, occurs in 60–70% of NSCLC tissues with the highly frequent co-expression of ER and high expression levels of aromatase have also been found in several NSCLC cell lines such as A549, H2122, H23 [7–9]. It can convert the androstenedione and testosterone to estrone and estradiol, respectively. The estradiol, mainly produced by aromatase, and then plays an important role in the growth of ER positive NSCLC. Therefore, higher relative levels of aromatase may predict a lower probability of survival in patients with NSCLC [10]. Anastrozole (Arimidex), a potent third-generation aromatase inhibitor, generally results in long-term superior efficacy and safety compared with tamoxifen, an antagonist of the estrogen receptor in breast cancer treatment [11]. It exerts its effects generally by interacting competitively with aromatase, blocking estrogen biosynthesis and thereby inhibiting estrogen-dependent signaling pathways and estrogen-induced growth in NSCLC [12]. The aim of this study was to explore the potential utility of targeting these two pathways by using anastrozole and gefitinib in combination in NSCLC cell lines.

2. Materials and methods

2.1. Cell culture

NSCLC cell lines A549, H460, SPC-A-1, H1299, human breast cancer cell line MCF-7, normal bronchus epithelial cell line BEAS-2B obtained from Shanghai cancer institute. The cells were grown and subcultured in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin at 37 °C in a humidified incubator with 5% CO2 and 95% air.

2.2. Drug preparation

Gefitinib and anastrozole were kind gifts from AstraZeneca. Gefitinib, 250 mg/tablet, was dissolved in DMSO to a final concentration of 25 mmol/L. Anastrozole, 1 mg/tablet, was dissolved in DMSO to a final concentration of 1 mmol/L. They were stored as aliquots at −20 °C.

2.3. Quantitative real-time PCR

A549, H460, SPC-A-1, H1299, BEAS-2B, MCF-7 cells were plated in 60-mm-diameter cell culture dishes. Total RNA was isolated from the cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. Complementary DNA (cDNA) was synthesized from total RNA with Oligo dT, Enzyme Mix and primers (Invitrogen, Carlsbad, CA). The primer sequences used were as follows:

ER-α  
forward: 5’-TGACAGATCTTGACATGGCTG-3’  
reverse: 5’-TCTCAGCTGACTTGGTGCTC-3’

ER-β  
forward: 5’-TGACAGATCTTGACATGGCTG-3’  
reverse: 5’-GCAATCCCTCTTTGAACCTGGA-3’

For amplification, cDNA was initially denatured at 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 35 s, and 72 °C for 40 s (ABI PRISM 7300 Sequence Detection System). For each PCR reaction, a cDNA standard curve was used to generate relative expression changes in ERs mRNA levels, which were normalized to β-actin gene.

2.4. Western blot analysis

In the Western blot analysis, 50,000 viable cells were plated in 100-mm-diameter dishes. For the detection of ER expression levels, the cells were collected after 48 h of incubation without any drugs. For the detection of expression levels of EGFR ERK p-EGFR, p-ERK, AKT, p-AKT, the A549 cells were treated with 20 μmol/L anastrozole or 5 μmol/L gefitinib alone or in combination for 48 h. Then cells were washed with ice-cold PBS and protein was extracted by adding 200 μL lysis buffer for 30 min on ice, collected with scraper, and microcentrifuged at 12,000×g for 15 min. Protein concentration in the supernatant was measured using the BCA-200 Protein Assay kit. For detection of protein, equal amounts of cell lysate (30 μg) were separated by size on a 10% SDS-Tricine gel (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membrane. Non-specific binding sites were blocked by incubation in 5% dry milk for 2 h at room temperature followed by incubation with primary antibodies overnight at 4 °C. The following antibodies and dilutions were used: ER-α/ER-β antibody (Abcam, USA dilution 1:200); EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA dilution 1:500); p-ERF antibody (Santa Cruz Biotechnology, Santa Cruz, CA dilution 1:200); ERK antibody (Santa Cruz Biotechnology, Santa Cruz, CA dilution 1:500); p-ERK antibody (Santa Cruz Biotechnology, Santa Cruz, CA dilution 1:500). After primary antibody incubation, the blots were washed thrice in 5%PBST (10 min each at room temperature), and horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was added at a 1:2000 dilution for all antibodies respectively. All secondary antibodies were incubated for 2 h at room temperature. Then the immunoreactive peptide was detected by SuperSignal West Pico Chemiluminescent substrate (Pierce) followed by exposure to autoradiography film.

2.5. Immunocytochemistry

A549 cells were plated on 24-well plates and allowed to attach overnight. They were fixed with 4% polyoxyethylene and 0.1% Triton-X-100 for 30 min. Then they were treated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. After non-immune serum was used to block non-specific binding for 1 h, the cells were incubated with primary monoclonal antibodies to ER-α/ER-β antibody (Abcam, USA dilution 1:50) and EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA dilution 1:50). The secondary antibodies were biotinylated IgG specific for the primary antibodies. Brown staining was considered positive. At last, they were dehydrated, cleared and mounted with neutral gums. The positive control for ER-α/ER-β was MCF-7 breast cancer cells with known immunoreactivity for the above mentioned markers. The negative control consisted of using phosphate-buffered saline instead of primary antibodies.

2.6. Cell proliferation assay

A549 cells were plated on 96-well plates and allowed to attach overnight. The cells were treated with anastrozole or gefitinib alone or in combination. After 24 h, 48 h and 72 h. CCK8 (Dojindo Laboratories, Japan) was added to each well and incubated for 2 h. Absorbance values at 490 nm were recorded using the microculture plate reader.

2.7. Cell cycle analysis

A549 cells were plated on six-well plates, allowed to attach overnight and treated with 20 μmol/L anastrozole or 5 μmol/L gefitinib alone or in combination. Cell cycle analysis was carried out after 48 h. The Tumor cell populations were stained with propidium iodide (SIGMA) and then subjected to flow cytometer (Becton, Dickinson and Company) with FACS. Then thousands events were collected from each sample. Data acquisition was carried out using Cell-Quest software and cell cycle distribution calculated using the
ModFit software (Becton, Dickinson and Company). The number of gated cells in G1, G2/M or S-phase was presented as %.

2.8. Apoptosis analysis by flow cytometry

A549 cells were plated on six-well plates, allowed to attach overnight and treated with 20 μmol/L anastrozole or 5 μmol/L gefitinib alone or in combination. The cells were harvested and collected by centrifugation. Cells were washed twice with cold PBS, then resuspended in 500 μl 1 x binding buffer and added with 5 μl Annexin V-FITC and 5 μl propidium iodide (Becton, Dickinson and Company). This was mixed well and incubated in the dark at 25 °C for 15 min, then cells were immediately analyzed by flow cytometry (Becton, Dickinson and Company) according to manufacturer’s instructions.

2.9. Statistical assay

Data were expressed as mean ± standard deviation from three independent experiments. Comparisons among groups were performed with analysis of variance. A value of P<0.05 was considered statistically significant. Statistical tests were performed using SPSS 13.0.

3. Result

3.1. The expression levels of ER-α and ER-β in NSCLC cell lines

The mRNA expression levels of ER-α and ER-β were detected by real-time PCR in H460, Spc-A1, H1299, A549 NSCLC cell lines and normal bronchial epithelial cell BEAS-2B cell line firstly. The result suggested that all lung cancer cell lines and normal pulmonary epithelial cell line expressed ER-α and ER-β mRNA, though at different levels. The ER-α mRNA was expressed at highest levels in A549 cell line and at lowest levels in BEAS-2B cell line while ER-β mRNA was expressed at highest levels in A549 cell line and at lowest levels in H460 cell line. Then protein levels of the ER (ER-α and ER-β) were detected by Western blot analysis. The A549 cell line over-expressed both ER-α and ER-β, which was consistent with the real-time PCR result (Fig. 1).

3.2. The locations of ER-α/ER-β and EGFR in A549 cell line which has the highest expression levels of ER

The locations of ER-α/ER-β and EGFR in A549 cell line were detected by immunocytochemistry. The results showed representative immunocytochemical staining of ER-α was mainly localized in the cytoplasm of A549 cell line, whereas staining of ER-β was mainly localized in the cytoplasm and to a lesser degree in the nucleoli (Fig. 2). In addition, EGFR was mainly localized in the cytoplasm and cytoplast of A549 cell line (Fig. 2).

3.3. Effects of anastrozole, gefitinib or in combination on A549 cellular proliferation

The A549 cells were treated with anastrozole or gefitinib alone or in combination. The cell proliferation inhibition was detected by CCK8 assay. A549 cells were treated with 2.5, 5, 10, 20 or 40 μmol/L anastrozole respectively for 24, 48 and 72 h, and the resulting IC50 values were 117.94 μmol/L, 56.06 μmol/L, and 33.67 μmol/L, respectively. Then A549 cells were treated with 1.25, 2.5, 5, 10, 20 μmol/L gefitinib respectively for 24, 48 and 72 h, and the resulting IC50 values for gefitinib were 74.75 μmol/L, 10.67 μmol/L, and 1.40 μmol/L, respectively. As shown in Table 1, the single agent of anastrozole or gefitinib inhibited the proliferation of A549 cells in a time- and dose-dependent manner.
Based on the IC50 of two drugs, A549 cells were treated with 20 μmol/L anastrozole or 5 μmol/L gefitinib alone or in combination. Fig. 3 showed that the proliferation inhibition ratios of A549 cells treated with anastrozole or gefitinib alone for 24, 48, 72 h were 23.32 ± 0.97%, 31.81 ± 1.30%, 35.88 ± 0.78% and 21.32 ± 1.57%, 34.44 ± 0.47%, 39.56 ± 0.45%, respectively. The combination of two drugs increased the proliferation inhibition rates for 24, 48, 72 h to 37.66 ± 1.02%, 63.41 ± 2.02%, 70.50 ± 0.86%, respectively (P < 0.05).

3.4. Effects of anastrozole or gefitinib alone or in combination on cell-cycle analysis

The percentage of G0/G1, S-, G2/M phase in A549 cells untreated were 51.99 ± 1.12%, 36.82 ± 2.56%, 11.12 ± 0.88%, respectively. The percentage of G0/G1, S, G2/M phase in A549 cells treated with anastrozole or gefitinib for 48 h were 57.07 ± 1.04%, 33.05 ± 0.45%, 9.57 ± 0.42% and 59.11 ± 0.27%, 31.47 ± 1.93%, 9.09 ± 1.97%, respectively. The percentage of G0/G1, S, G2/M phase in A549 cells treated with both drugs were 64.99 ± 0.36%, 27.67 ± 1.57%, 7.47 ± 1.17%. As shown in Fig. 4, the combined application of both drugs strongly enhanced the percentage of G0/G1 phase cells and distinctly diminished the percentage of S- and G2/M-phase cells (P < 0.05).

3.5. Effects of anastrozole or gefitinib alone or in combination on cell apoptosis

Apoptosis rates of A549 cells treated with anastrozole, gefitinib alone or in combination were 10.72 ± 1.12%, 17.40 ± 1.28%, 23.02 ± 2.32%, respectively (P < 0.05). As shown in Fig. 4, the combined application of both drugs significantly induces more cell apoptosis.

3.6. Gefitinib or anastrozole alone or in combination modify expression levels of EGFR signaling related proteins

In an effort to understand the signaling from ER through EGFR, the expression levels of EGFR, p-EGFR ERK, p-ERK, AKT and p-AKT were evaluated in A549 cells. As shown in Fig. 5, Separate incubation of A549 cells with anastrozole or gefitinib alone or in combination did not evoke significant effects on expression levels of EGFR, ERK and AKT. The levels of p-EGFR were lowered by the single drug regimen while levels of p-EGFR were lowest in A549 cells with combined drug use. The levels of p-ERK were not lowered by the single drug regimen while diminished in the presence of both drugs. However the levels of p-AKT were not lowered by anastrozole but lowered by gefitinib, while the levels of p-AKT were lowest in A549 cells with combined drug use.

4. Discussion

Several studies have provided evidence of an important role for estrogen in the genesis and progression of lung cancer [13]. In addition, among NSCLC cells expressing either ER-α or ER-β, treatment with estradiol can stimulate cell proliferation and promote tumor progression [14–16]. The growth of NSCLC is potentially regulated by ER through two different mechanisms: genomic and non-genomic [17,18]. The genomic mechanism involves estrogen ligand binding to the nuclear ER. The ER then dimerizes and interacts with specific estrogen response elements, which leads to transcriptional regulation of target genes. The non-genomic mechanism involves estrogen ligand binding to the extranuclear ER. The ER can interact directly with membrane tyrosine kinase receptors such as EGFR, which leads to altered function and regulation of gene expression. This suggests that estrogen may stimulate cell proliferation and survival through bidirectional signaling between ER and EGFR in cells expressing both two receptors.

In our study, we investigated that ER-α and ER-β were indeed expressed in A549, H460, SPC-A1 and H1299 lung cancer cell lines by real-time PCR and Western blot. However, the expression levels of ER in A549 cells were highest among these cell lines, which might show biological responses to estrogen more significantly. In an effort to confirm that whether genomic or non-genomic ER signaling was activated in lung cancer cells, the ER location of A549 cell line was detected. We found that ER-α was in the cytoplasm of lung cancer cells, whereas ER-β was in both cytoplasm and nucleus of lung cancer cells. This result was consistent with the studies of Kawai [19]. So the biological effects of estrogen may be mediated through convergence of genomic and non-genomic actions in A549 cell line.

Furthermore, we also found that EGFR was expressed in the cytomembrane and cytoplasm of A549 cell line. Previous studies [20,21] have identified that wild-type EGFR is expressed in A549 cell line which required concentrations >1 μmol/L of gefitinib for 50% inhibition (IC50). So targeting EGFR in combination with anti-estrogen therapy in A549 cells may especially enhance the antitumor efficacy of gefitinib because it potentially provides a...
second mechanism for inhibiting the EGFR by reducing its transactivation. So we have targeted both ER and EGFR pathways in A549 cells with anastrozole and gefitinib. We found that anastrozole additively increases the antiproliferative effect of gefitinib in A549 cells tested. Combined targeting of ER and EGFR pathways may have the synergistic effect. This combined benefit was particularly evident in suppressing tumor cell growth, which was also closely associated with elevation of the G0/G1 phase fraction. In addition, combined therapy can also achieve greater apoptotic effects than using either agent alone.

The possible explanations for these additive effects of antitumor activity may be mediated by the interaction of these two drugs. In an effort to explore more detailed mechanisms, changes in the expression levels of EGFR signaling protein were also investigated. The Western blot analysis revealed strong changes in a panel of EGFR signaling related proteins in A549 cells. Compared with gefitinib used alone, simultaneous application of anastrozole and gefitinib profoundly diminished the expression levels of p-EGFR and p-AKT, whereas the expression levels of p-ERK were additively downregulated. These findings suggest that cooperative interaction between ER and EGFR signaling pathways results in the downregulation of p-EGFR, p-AKT, p-ERK expression levels, which may cause the retardation of G1–S transition and the induction of apoptosis.

Recent data [22–25] have also revealed that ER, especially membrane-associated ER forms appear to elicit rapid signaling in cooperation with various kinase cascades, including the EGFR and its downstream effectors, such as mitogen-activated protein kinase (MAPK) and PI3K/AKT-kinase. This occurs through a linked path that involves activation of G proteins, Src kinase, and matrix metalloproteinases (MMPs). Increased MMP function leads to the liberation of HB-EGF, which then binds and activates the EGFR pathway. On the basis of functional interactions between ER and EGFR pathways, combination drug therapy in lung cancer models has been reported. As Stabile reported [26], treatment

**Fig. 2.** The locations of ER-α and ER-β in A549 cell line were detected by immunocytochemical (S-P ×400). Appropriate positive control (MCF-7 breast cancer cell) and negative control were done in parallel to confirm specificity of the immunoassays. (A) A549 ER-α (+), (B) MCF-7 ER-α (+) (positive control), (C) A549 ER-α (−) (negative control), (D) A549 ER-β (+), (E) MCF-7 ER-β (+) (positive control), and (F) A549 ER-β (−) (negative control). The locations of EGFR in A549 cell line were detected by immunocytochemical (S-P ×400). (G) A549 EGFR (+), (H) A549 EGFR (−) (negative control). The figure shows one representative from three separate experiments.
Inhibitory ratios of A549 cells treated with anastrozole or gefitinib alone or in combination were detected by cck8 assay. Tumor cells were treated with 20 μmol/L anastrozole or 5 μmol/L gefitinib alone or in combination. Controls remained untreated. Cells were counted after 24 h, 48 h and 72 h using the cck8 assay. The inhibitory ratios were calculated as (mean absorbancecontrol – mean absorbancetreatment)/mean absorbancecontrol. * indicates difference to single drug treatment (P < 0.05).

of human xenograft mouse models of NSCLC by the combinatorial therapy of fulvestrant and gefitinib reduced tumor volume as much as 60%, in comparison with 49% or 32% reduction by using gefitinib or fulvestrant alone, respectively. Moreover, combination therapy also increased more apoptosis in cancer cells, decreased phospho-p44/p42 MAPK expression. Shen H [27] demonstrated that co-treatment with gefitinib and tamoxifen decreased the proliferation and increased the apoptosis of A549 and H1650 adenocarcinoma cell lines, when compared with either drug alone.

Márquez-Garbán et al. [28] found that faslodex and erlotinib tandem elicited enhanced blockade of the growth of NSCLC xenografts in vivo, and antitumor activity exceeded that of either agent given alone. So dual targeting of ER and EGFR signaling can maximally inhibit cell proliferation, induce apoptosis, and affect downstream signaling pathways.

It is well known that super-responses to EGFR-TKI in clinic have been ascribed to some types of gene mutations in the tyrosine kinase domain of EGFR. Therefore it is also much meaningful to try

Cell cycle analyses of A549 cells were carried out after 48 h by flow cytometric analysis. Tumor cells were treated with 20 μmol/L anastrozole or 5 μmol/L gefitinib alone or in combination (Ana/Gef). Controls remained untreated. The cell population at each specific checkpoint is expressed as percentage of the total cells analyzed. One representative experiment of three is shown. The definition of quadrant in the read-out of flow cytometry: the upper left quadrant (An−/PI+) is counted as the necrotic cells. The upper right quadrant (An+/PI+) is counted as late apoptotic cells. The lower left quadrant (An−/PI−) is counted as the live cells. The lower right quadrant (An+/PI−) is counted as early apoptotic cells.
this combination regimen in other cancer cells harboring EGFR activating mutations. As Stabile et al. [26] reported, combined targeting of the ER and the EGFR also shows enhanced antitumor effects in an EGFR mutation lung cancer cell line. However, combination therapy may be especially beneficial for patients whose tumors lack EGFR mutation because it potentially provides a second mechanism for inhibiting the EGFR by reducing its transactivation by ER. So we focused on the combined antitumor effect of the A549 cell line in our study. Clinically, testing of dual EGFR/ER inhibition has yielded promising results to date. A pilot study [29] using the combination of gefitinib and fulvestrant in postmenopausal women with advanced NSCLC has been completed. The combination therapy was determined to be well-tolerated and suggested antitumor efficacy of the tandem treatment. Of special note, a phase II clinical trial of therapy with the EGFR inhibitor erlotinib together with fulvestrant in patients with advanced NSCLC is now underway [30]. However to our knowledge, this is the first report suggesting the effects of aromatase inhibitor anastrozole in combination with EGFR-TKI gefitinib on human lung adenocarcinoma cells. Therefore, it is remarkable that combination of these two drugs may be an attractive regimen to enhance the antitumor efficacy in the treatment of NSCLC patients.

Conflict of interest statement

All authors declare no conflict of interest.

Acknowledgments

We thank AstraZeneca Pharmaceuticals for generously providing Iressa and anastrozole for use in these studies. Support for this work was provided by Wu Jie Ping-AstraZeneca Medical Special Foundation.

References


**Fig. 5.** Western blot analysis of EGFR signaling proteins was listed in Section 2. Tumor cells were treated with 20 μmol/L anastrozole or 5 μmol/L gefitinib alone or in combination (Ana/Gef). Controls remained untreated. Drugs were applied for 48 h. Cell lysates were subjected to SDS–PAGE and blotted on the membrane incubated with the respective monoclonal antibodies. GAPDH served as the internal control. The figure shows one representative from three separate experiments.
