Implication of the Insulin-like Growth Factor-IR Pathway in the Resistance of Non–small Cell Lung Cancer Cells to Treatment with Gefitinib

Floriana Morgillo,1 Woo-Young Kim,1 Edward S. Kim,1 Fortunato Ciardiello,3 Waun Ki Hong,1 and Ho-Young Lee1,2

Abstract

Purpose: Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors have been found to be effective against lung cancer in vitro, but clinical resistance to these agents has developed as their usage has increased. In this study, we determined whether the insulin-like growth factor I (IGF-I) signaling pathway induces resistance of non–small cell lung cancer (NSCLC) cells to the EGFR tyrosine kinase inhibitor gefitinib. Experimental Design: The effects of gefitinib and cetuximab on NSCLC cells, alone or with an IGF-I receptor (IGF-IR) inhibitor, were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, the flow cytometry–based terminal nucleotidyl transferase–mediated nick end labeling assay, coimmunoprecipitation, and Western blot analysis. EGFR and IGF-IR expression in NSCLC tissues were examined by Western blot analysis. Results: Gefitinib inhibited NSCLC cell proliferation by inducing apoptosis when IGF-IR signaling was suppressed. Treatment with gefitinib, but not cetuximab, induced EGFR:IGF-IR heterodimerization and activation of IGF-IR and its downstream signaling mediators, resulting in increased survivin expression in NSCLC cell lines with high levels of IGF-IR expression. In contrast, overexpression of survivin protected cells with low IGF-IR expression from gefitinib-induced apoptosis. Most NSCLC tissues with EGFR overexpression had associated high levels of IGF-IR expression. Conclusions: IGF-IR expression may be useful as a predictive marker for gefitinib treatment of NSCLC. Suppression of IGF-IR signaling pathways may prevent or delay development of gefitinib resistance in patients with NSCLC.

Lung cancer is the leading cause of cancer-related death in both sexes in the United States and throughout the world, and its overall mortality rate has not changed significantly in decades (1). Advances in the understanding of lung tumor biology and oncogenesis have provided several molecular targets for the treatment of non–small cell lung cancer (NSCLC). Of these, inhibitors of epidermal growth factor receptor (EGFR) tyrosine kinase pathways are the most extensively studied. EGFR is overexpressed in several solid tumor types, including NSCLC (it has been found in 40–80% of cases; ref. 2). The EGFR signaling pathway activates the phosphoinositide-3-kinase/Akt and mitogen-activated protein kinase (MAPK) pathways, which mediate proliferation, differentiation, and survival in both normal and malignant epithelial cells (3, 4). Activated EGFR also promotes angiogenesis, tumor cell motility, and invasion by regulating the expression and activity of matrix metalloproteinases and by interacting with components of the integrin pathway (5). These findings indicate that EGFR-targeted agents would be effective against cancer. Small-molecular-weight EGFR tyrosine kinase inhibitors (TKI) and anti-EGFR monoclonal antibodies have been in advanced clinical development (6, 7). Monoclonal antibodies bind to the extracellular domain of EGFR. The EGFR-antibody complex is subsequently internalized, causing decreases in EGFR expression and heterodimerization in a phosphorylation status–independent manner. Treatment with cetuximab, a chimeric human-mouse anti-EGFR monoclonal immunoglobulin G1 (IgG1) antibody, has been found to block cell cycle progression by inducing G1 arrest and to inhibit human cancer xenograft growth in nude mice in vivo (8) by inhibiting tumor-induced angiogenesis (9). Treatment with cetuximab has shown therapeutic activities in patients with head and neck...
cancer or colorectal cancer (10, 11). Unlike monoclonal antibodies, TKIs do not affect internalization of the EGF receptor and are often not EGFR specific; thus, they affect the kinase activity of other ErbB family receptors. EGFR TKIs block the ATP pocket of EGFR, thereby inhibiting EGFR phosphorylation and downstream signal transduction (12).

Preclinical studies have shown that the EGFR TKI gefitinib, when used in combination with standard chemotherapeutic agents or radiotherapy, inhibits EGFR activation, causing G1 arrest and additive-to-synergistic growth inhibition (6). However, negative results from clinical trials (13, 14) have diminished the enthusiasm for gefitinib and indicate that a better understanding is needed of the mechanisms of acquired resistance to this drug. The effectiveness of gefitinib is currently being studied in a population of patients who may have a biomarker that sensitizes their tumors to gefitinib. However, mechanistic studies of gefitinib resistance have not been completed.

Because recent studies have suggested that the insulin-like growth factor I receptor (IGF-IR) pathway is involved in NSCLC cells’ resistance to EGFR-targeting agents (15, 16), we studied whether the IGF-IR–mediated signaling pathway influences the NSCLC response to gefitinib and cetuximab. We also sought to determine the proteins mediating survival of NSCLC cells against gefitinib treatment. In the present studies, we show that treatment with gefitinib but not cetuximab stimulates the IGF-IR pathway and its downstream signaling mediators via the EGFR:IGF-IR heterodimer and induces survivin expression that protects NSCLC cells from apoptosis.

Materials and Methods

Cells and reagents. The human NSCLC cell lines H596, H226B, H226Br, H460, H1299, H358, and H322 were purchased from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Life Technologies-BRL) in a humidified atmosphere with 5% CO2. EGF was purchased from R&D Systems. Gefitinib (AstraZeneca) was prepared as a 10 mmol/L stock solution in DMSO. Cetuximab was obtained from ImClone Systems and prepared as a 20 mmol/L stock solution. AG1024, an IGF-IR TKI, was purchased from Calbiochem-Novabiochem. Adenoviruses, with and without survivin (Ad-survivin and Ad-mTOR, respectively), were amplified as described previously (17).

Cell proliferation assay. To determine the effects of gefitinib and AG1024 on NSCLC cell proliferation, we plated 3 × 10⁴ cells per well of the indicated NSCLC cell lines in 96-well plates. The next day, cells were
treated with either 0.1% DMSO as a diluent control or with different concentrations of drugs (0.1-10.0 μmol/L) in a final DMSO concentration of 0.1% in RPMI with 10% FBS or EGF (50 ng/mL). Cell medium was replaced with fresh medium containing gefitinib, AG1024, or both everyday for 3 days. At the end of the treatment period, cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The drug concentrations required to inhibit cell growth by 50% were determined by interpolation from the dose-response curves. Eight replicate wells were used for each analysis, and at least three independent experiments were done. The data from replicate wells are presented as the mean numbers of cells remaining, with 95% confidence intervals (CI). To determine the effect of the combined drug treatments, any potentiation was estimated by multiplying the percentage of cells remaining (% growth) for each agent. The classification index was calculated as described previously (18). Supra-additivity was defined as % \( A \times \% B > 1.0 \); additivity was defined as % \( A \times \% B = 0.9-1.0 \); and subadditivity was defined as % \( A \times \% B < 0.9 \). (In these equations, \( A \) and \( B \) are the effects of individual agents, and \( AB \) is the effect of the combination of the two drugs.)

**Cell cycle and apoptosis assays.** For cell cycle and apoptosis assays, the next day, the cells were treated with various concentrations of gefitinib (5 μmol/L), AG1024 (5 μmol/L), or both. Both adherent and nonadherent cells were harvested, pooled, and fixed with 1% paraformaldehyde and 70% ethanol. To determine the percentages of cells in the phases of the cell cycle (G1, S, and G2-M), we stained cells with 50 μg/mL propidium iodide and analyzed them with a flow cytometer (Epics Profile II; Beckman Coulter Inc.) equipped with a 488-nm argon laser. Apoptosis was assessed using a modified flow cytometry–based terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) assay with an APO-bromodeoxyuridine staining kit (Phoenix Flow Systems) as described previously (19). Data from at least three experiments are presented as means with 95% CIs.

**Immunoblotting and coimmunoprecipitation.** NSCLC cells were either left uninfected or infected with Ad-EV or Ad-survivin (50 infection-forming units) and then left untreated or treated with gefitinib (1-10 μmol/L), AG1024 (5 μmol/L), and cetuximab (1-10 μmol/L), alone or in combination, in growth medium that was changed daily. For growth factor stimulation, cells were cultured in serum-free medium for 1 day and then incubated with EGF (50 ng/mL). For small interfering RNA (siRNA) transfection, H460 cells in the logarithmic growth phase in six-well plates (5 × 10^4 cells per well) were transfected with 10 μL of 20 μmol/L survivin siRNA or control scrambled siRNA (Dharmacon) using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were incubated for 24 h in growth medium, and gefitinib was added. Cells were harvested after 3 days of incubation.

We did a biochemical analysis of 14 lung tumor and 14 healthy adjacent tissue specimens from patients with NSCLC who had been treated at The University of Texas M.D. Anderson Cancer Center. This study was approved by the M.D. Anderson Cancer Center Institutional Review Board. All tissue specimens were frozen in liquid nitrogen immediately after being resected and rinsed in PBS; they were kept in a -80°C freezer until retrieved for the study. Total protein isolation and Western blot analyses were done as described previously (20).

Immunoprecipitation was done using 3 μg of protein from the total cell lysates and 3 μg of mouse monoclonal anti-EGFR antibody (Santa Cruz Biotechnology), mouse monoclonal anti–IGF-IR antibody (Novus Biologicals), or healthy preimmune serum anti-mouse antibody (sc-2025) as the negative control, followed by incubation overnight at 4°C. The immunoprecipitates were resolved by 6% SDS-PAGE and then...
analyzed by Western blot using rabbit polyclonal antibodies against human phosphorylated EGFR (pEGR; Tyr993), EGFR, and phosphorylated IGFIR (pIGF-IR and Tyr1131/Tyr1146; 1:1,000; Cell Signaling Technology); rabbit polyclonal antibodies against human IGFIR (1:500; Santa Cruz Biotechnology); rabbit polyclonal antibodies against human phosphorylated Akt (pAkt; Ser473; 1:1,000), and Akt (1:1,000) and a mouse monoclonal antibody against human anti-phosphorylated p44/42 MAPK (pp44/42 MAPK and Thr202/Tyr204; 1:500; Cell Signaling Technology); goat polyclonal antibodies against p44/42 MAPK (1:1,000; Cell Signaling Technology); and a rabbit polyclonal anti-β-actin (1:4,000, Santa Cruz Biotechnology) by 50.0% were 10 to 20 times higher than were those for H322 and H358 cells. We next determined the mechanisms responsible for the sensitivity of NSCLC cells to gefitinib. We first measured the basal levels of EGFR and pEGFR in the cell lines. As shown in Fig. 1B, all cell lines except H322 had high levels of EGFR expression. Four of these six cell lines also had high levels of pEGFR expression, indicating that no relationship exists between gefitinib response and EGFR activation. Because of the role of phosphatase and tensin homologue (PTEN) in NSCLC cells’ resistance to EGFR TKIs (21), we next determined the role of phosphatase and tensin homologue (PTEN) in

### Table 1. Synergistic indices of combination treatment with gefitinib and AG1024

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (µmol/L)</th>
<th>Treatment A</th>
<th>MGI</th>
<th>P value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Treatment B</th>
<th>MGI</th>
<th>P value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Combination treatment</th>
<th>Index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gefitinib</td>
<td>1</td>
<td>0.85</td>
<td>5.3E-05</td>
<td></td>
<td>AG1024</td>
<td>1.00</td>
<td>0.0299</td>
<td>0.73</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.65</td>
<td>1.9E-09</td>
<td></td>
<td>1.00</td>
<td>0.0299</td>
<td>0.59</td>
<td>1.3E-09</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.59</td>
<td>4.3E-10</td>
<td></td>
<td>1.00</td>
<td>0.0299</td>
<td>0.45</td>
<td>1.9E-10</td>
<td>1.022</td>
</tr>
</tbody>
</table>

<sup>a</sup>Calculated by dividing the expected growth inhibition rate by the observed growth inhibition rate. An index more than 1 indicates synergistic effect and <1 indicates less than additive effect.

<sup>b</sup>Expected growth inhibition rate of treatment A × growth inhibition rate of treatment B.

<sup>c</sup>Growth inhibition rate of combined treatment on treatments A and B.

### Results

#### Stimulation of the IGF-IR signaling pathway in gefitinib-resistant NSCLC cell lines.

We determined the effects of gefitinib on cell proliferation in H596, H226B, H226Br, H460, H1299, H358, and H322 cell lines. We treated cells with gefitinib in the presence of 10% FBS or 50 ng/mL EGF. The MTT assay revealed that gefitinib decreased NSCLC cell proliferation in a dose-dependent manner (Fig. 1A). H322 and H358 cells were more sensitive to gefitinib than were the other cell lines [H322 in 10% FBS, 47.5% (95% CI, 41.5-53.5%; P < 0.001); H322 in EGF, 46.8% (95% CI, 40.8-52.8%; P < 0.01); H358 in 10% FBS, 52.1% (95% CI, 50.0-54.2%; P < 0.001); and H358 in EGF, 41.6% (95% CI, 37.3-45.9%; P < 0.01) after 72 h]. The gefitinib concentrations required to inhibit H596, H226B, H226Br, H460, and H1299 cell growth by 50.0% were 10 to 20 times higher than were those for H322 and H358 cells.

We next determined the mechanisms responsible for the sensitivity of NSCLC cells to gefitinib. We first measured the basal levels of EGFR and pEGFR in the cell lines. As shown in Fig. 1B, all cell lines except H322 had high levels of EGFR expression. Four of these six cell lines also had high levels of pEGFR expression, indicating that no relationship exists between gefitinib response and EGFR activation. Because of the role of phosphatase and tensin homologue (PTEN) in NSCLC cells’ resistance to EGFR TKIs (21), we next determined

![Fig. 3. Effect of gefitinib on EGFR/IGF-IR heterodimerization and activation of IGF-IR signaling pathway.](image)
The treatment of IGF-IR, Akt, and pAkt and found that most cell lines had high levels of IGF-IR expression. The levels of pIGF-IR expression were high in both resistant (H1299 and H460) and sensitive (H322 and H358) cell lines, suggesting that gefitinib treatment induces activation of the IGF-IR pathway and its downstream signaling components.

Fig. 4. Role of survivin in the resistance of NSCLC cells to gefitinib. A, immunoblotting of survivin and XIAP in H460 and H322 cells treated with the indicated concentrations of gefitinib. B, effects of treatment with 5 μmol/L gefitinib in H460 and H322 cells treated with gefitinib, H460 cells were transfected with scrambled (scr) or survivin siRNA and left untreated or treated with gefitinib for 48 h. H322 cells were infected with a control virus (Ad-EV or Ad-survivin) and incubated for 3 d in the presence of gefitinib. The protein extracts were subjected to Western blotting for evaluation of survivin and active caspase-3.

Effects of the combined blockade of the EGFR and IGF-IR pathways on the proliferation of NSCLC cells. To determine the roles of the IGF-IR signaling pathway in the development of gefitinib resistance, we evaluated the effects of gefitinib and AG1024, an IGF-IR TKI (22), on the proliferation and apoptosis of H460 cells.

Treatment with gefitinib and AG1024 efficiently blocked the gefitinib-induced increase in pIGF-IR expression in H460 cells (Fig. 2A). We observed more sensitivity to gefitinib when we simultaneously treated H460 cells with gefitinib and AG1024 than with either agent alone (Fig. 2B). In fact, the combined treatment resulted in synergistically enhanced antiproliferative effects on H460 cells [10 μmol/L gefitinib, 59% (95% CI, 55.9-62.1%; P < 0.001); 5 μmol/L AG1024, 70.1% (95% CI, 64.7-75.5%; P < 0.001); 10 μmol/L gefitinib plus 5 μmol/L AG1024, 28.5% (95% CI, 26-31%; P < 0.001; Table 1). No obvious changes in cell cycle progression were found in H460 cells that had been treated with gefitinib for 3 days; however, a decrease in the G1 phase population and an increase in the subG0/G1 phase population were found in those treated with AG1024. Combined treatment with gefitinib (5 μmol/L) and AG1024 (5 μmol/L) for the same duration led to a decrease in the S population, a further decrease in the G1 population, and an increase in the subG0/G1 population (P < 0.001 for all; Fig. 2C). Moreover, TUNEL staining of cells treated with gefitinib and AG1024 showed a marked increase in apoptosis.
administration of 5 μmol/L of both gefitinib and AG1024 as single agents induced apoptosis in 0.2% (95% CI, 0.0-2.8%; P > 0.05) and 30.1% (95% CI, 14.3-37.6%; P < 0.05) of cells, respectively, whereas combined treatment with gefitinib and AG1024 induced apoptosis in more than 90% (95% CI, 64.4-89.8%; P < 0.001). These findings suggest that the IGF-IR pathway provides an alternative proliferation or survival mechanism for NSCLC cells in which the EGFR pathway is blocked by gefitinib treatment. Thus, cotargeting the IGF-IR and EGFR pathways may be an effective therapeutic strategy for NSCLC.

Gefitinib induces heterodimerization of IGF-IR and EGFR in H460 cells. EGFR signaling can be modulated by several mechanisms, including heterodimerization with other members of the EGFR family, such as HER-2, and other growth factor receptors, such as IGF-IR (15, 16, 23, 24). We recently observed increased heterodimerization between EGFR and IGF-IR in NSCLC cells after treating them with erlotinib, an EGFR TKI (25). In the current study, we determined the effects of gefitinib on the interaction between EGFR and IGF-IR in H460 and H322 cells. EGFR immunoprecipitates from gefitinib-treated H460 cells showed an obvious increase in IGF-IR binding, whereas control immunoprecipitates that had been treated with preimmune serum had no immunoreactive bands (Fig. 3A). In contrast, both EGFR and control immunoprecipitates from H322 cells lacked immunoreactive bands (Fig. 3B). These results suggest that EGFR and IGF-IR interact through physical contact, and that treatment with gefitinib increases the heterodimerization of EGFR and IGF-IR in H460 cells.

Role of survivin in resistance of NSCLC cells to gefitinib. We next evaluated the signaling mediator that connects gefitinib-induced IGF-IR pathway activation and H460 cell survival. Because inhibitors of apoptosis protein (IAP) such as survivin and XIAP play a role in tumor cells’ resistance to chemotherapeutic drugs (26), we determined the effects of gefitinib on the expression of these proteins in H460 and H322 cells. As shown in Fig. 4A, the expression level of survivin but not XIAP markedly increased in H460 cells after treatment with gefitinib. Moreover, treatment with gefitinib (5 μmol/L) and AG1024 (5 μmol/L) efficiently suppressed the gefitinib-induced increase in survivin expression (Fig. 4B). We then evaluated the influence of survivin on gefitinib-induced apoptosis in NSCLC cells by knockdown of expression or overexpression of survivin. H460 cells transfected with control scrambled or survivin siRNA before treatment with 10 μmol/L gefitinib (a concentration that did not induce apoptosis) had an obvious increase in the rate of apoptosis as determined by a Western blot analysis of an increase in activated caspase-3 expression (Fig. 4C). Furthermore, among H322 cells infected with Ad-survivin, the level of gefitinib-induced apoptosis was substantially reduced (Fig. 4D). These findings suggest that NSCLC cells can escape apoptosis by inducing survivin expression. To determine whether the induction of survivin by gefitinib is related to the resistance of cell lines to the drug, we compared the expression of survivin in a subset of NSCLC cell lines that had been treated with TKI. The five nonsensitive or resistant NSCLC cell lines (H460, H1299, A596, 226B, and 226Br cells) showed an increase in survivin expression after treatment with gefitinib; no increase

Fig. 5. Effects of cetuximab on EGFR:IGF-IR interaction in NSCLC cells. A, effect of cetuximab treatment on EGFR:IGF-IR heterodimerization and activation of the IGF-IR and EGFR signaling pathways. Whole-cell extracts (3 mg) from H460 cells were left untreated or treated with cetuximab (10 and 50 μmol/L) for 3 d. They were then immunoprecipitated with anti-EGFR antibodies, and the immunoprecipitates were subjected to Western blot analysis with the indicated antibodies. Input represents cell lysates (30 μg) that were not subjected to immunoprecipitation. Control immunoprecipitation was done using mouse preimmune serum (PS) as a negative control. B, Western blot analysis of survivin, pIGF-IR, IGF-IR, pEGFR, EGFR, pAkt, and pp44/42MAPK expression in H460 cells treated with cetuximab (1, 10, and 50 μmol/L) for 3 d in the absence or presence of EGF (50 ng/mL). C, results of the MTT assay of H460 and H322 cell lines after treatment with cetuximab in RPMI 1640 containing 10% FBS for 3 d. Points, mean value of eight identical wells of a single representative experiment; bars, upper 95% CIs.
and EGFR expression.

marked with a circle had increased IGF-IR

used as a loading control. The samples

against EGFR in NSCLC cells.

resistance to EGFR TKIs, but not to the monoclonal antibody

sionareatleastpartlyresponsiblefortheinductionofacquired

EGFR, activation of IGF-IR, and induction of survivin expres-

findings suggest that heterodimerization between IGF-IR and

proliferation assay showed that H460 cells were more resistant

imab decreased EGF-stimulated pEGFR expression in H460

EGFR and IGF-IR (Fig. 5A). Moreover, treatment with cetux-

We evaluated the effects of cetuximab on the interaction

between IGF-IR and EGFR. EGFR precipitates from cetuximab-

specimens from patients with NSCLC.

Expression of EGFR and IGF-IR in human lung tissue. Our
data suggested that IGF-IR expression has an important role in
determining NSCLC cells’ sensitivity to EGFR TKIs. Therefore,
we determined the levels of EGFR and IGF-IR expression in
NSCLC tissue. Nine of the fourteen tumor specimens had
higher levels of EGFR expression than did normal tissue
specimens from the same patients. Eight of those specimens
also had higher levels of IGF-IR expression than those did in
normal tissue (Fig. 6).

Discussion

We have previously shown that erlotinib, an EGFR TKI,
induces heterodimerization of EGFR/IGF-IR, resulting in the
activation of IGF-IR and induction of Akt/mTOR-mediated
synthesis of survivin protein, which protects NSCLC cells from
the drug-induced apoptosis (25). In the present study, we show
that the activation of the IGF-IR via heterodimerization of
EGFR/IGF-IR and consequent induction of survivin expression
also mediate gefitinib resistance in NSCLC cells, confirming
and extending our previous findings (25). Our studies also
show that IGF-IR pathway did not affect antiproliferative
activities of cetuximab, a monoclonal antibody blocking EGFR.

Overall, these findings suggest that heterodimerization
between IGF-IR and EGFR, activation of IGF-IR, and induction
of survivin expression contribute to the acquired resistance to
EGFR TKIs, but not to the monoclonal antibody against EGFR in
NSCLC cells.

In clinical trials, the EGFR TKIs, such as erlotinib and
gerfinib, were found to be effective in the treatment of patients
with NSCLC (27, 28). These agents were approved for NSCLC
treatment after resulting in response rates of 10% and survival
advantages in patients previously treated with chemotherapy
(29). However, large-scale phase III clinical trials in advanced
NSCLC have had contrasting results (13, 14, 29, 30). In the
current study, we found that gefitinib treatment did not inhibit
NSCLC cell proliferation at doses sufficient to suppress EGFR
activation, suggesting that the development of resistance to
EGFR TKIs is caused by the activation of alternative cell survival
signaling mechanisms.

To develop better anticancer therapeutic strategies using
gerfinib, we sought to identify the pathways whereby NSCLC
cell trigger alternative survival signaling. PTEN expression and
EGFR and Kirsten ras (KRAS) somatic mutation have been
involved in the cellular response to EGFR-targeted therapy
(31–33). Bianco et al. (34) found that the loss of PTEN
expression in the MDA-468 breast cancer cell line contributed
to gefitinib resistance. These findings suggest that PTEN- 
deficient cell lines maintain their Akt activity and survive when
EGFR is inactivated. However, deletion of PTEN does not
frequently occur in NSCLC cells (31, 35). In fact, all NSCLC cell
lines used in our study expressed PTEN, suggesting that
gerfinib resistance in NSCLC cells is not caused by a PTEN
deficiency. Similarly, the NSCLC cell lines without somatic
mutations of EGFR responded to gefitinib, which is consistent
with previously reported findings (36, 37). Moreover, in our
study, gefitinib showed antiproliferative effects on H358 cells
that have somatic mutation in KRAS (38). Together, these
findings indicate that expression of PTEN and mutational status
of EGFR and KRAS are not entirely responsible for NSCLC cells’
resistance to gefitinib.

Our in vitro data suggest that the cross-talk between the IGF-IR
and EGFR signaling pathways is involved in the development of
gefitinib resistance in NSCLC cells. First, we found that the levels
of IGF-IR and pIGF-IR expression in NSCLC cells were inversely
associated with the antiproliferative effects of gefitinib. Second,
gefitinib induced phosphorylation of IGF-IR and its downstream
mediators in NSCLC cell lines with high levels of IGF-IR
expression. Third, gefitinib induced heterodimerization between
EGFR and EGFR and survivor expression in the high IGF-IR–
expressing H460 cells but not in the low IGF-IR–expressing
H322 cells. Finally, gefitinib exhibited apoptotic activity in H460
cells, in which IGF-IR activation was blocked. These NSCLC cell
lines have shown a similar response to the erlotinib treatment
(25). EGFR and IGF-IR have similar extracellular domain
structures (39); therefore, it is plausible that EGFR TKIs induce
a direct interaction between EGFR and IGF-IR, leading to the
activation of IGF-IR pathways and induction of survivin
expression and, thus, maintain NSCLC cell proliferation. Recent
reports showing a direct interaction between EGFR and IGF-IR
(40, 41) also support our hypothesis.
The induced expression of survivin found in our study seemed to protect H460 cell from apoptosis in the presence of gefitinib: survivin overexpression protected H322 cells from gefitinib-induced apoptosis. In addition, gefitinib induced apoptosis in H460 cells, in which transfection with survival-specific siRNA silenced survivin expression. Survivin is a member of the IAP family and is associated with both cancer progression and drug resistance (42, 43). Therefore, the resistance and sensitivity of NSCLC cells to gefitinib may be determined, at least in part, by their ability to activate the EGFR-IR-mediated pathway and induce survivin expression. Most strikingly, we found no evidence of EGFR and IGF-IR heterodimerization, IGF-IR activation, and induction of survivin expression in H460 cells after cetuximab treatment. These findings suggest that activation of the EGFR-IR pathway and the subsequent expression of survivin are at least partly responsible for the sensitivity of NSCLC cells to EGFR TKIs but not to anti-EGFR monoclonal antibodies.

In summary, we showed that the cross-talk between IGF-IR and EGFR has a specific role in inducing gefitinib resistance in NSCLC cells. Gefitinib induced heterodimerization of EGFR and IGF-IR; stimulated IGF-IR and downstream pathways, including phosphoinositide-3-kinase/Akt/mTOR and p44/42 MAPK; and increased survivin expression in NSCLC cell lines. Overexpression of the EGFR family and its ligands have been found in 30% to 80% of NSCLC (30, 44, 45). However, we found no interaction between EGFR and IGF-IR in NSCLC cells with low levels of IGF-IR expression, suggesting that the basal level of IGF-IR expression is important for initiating the formation of the EGFR-IGF-IR complex. Nine of the fourteen (64%) tumor specimens in our study exhibited higher levels of EGFR expression, and eight of those had related higher IGF-IR expression compared with paired normal tissue. The number of IGF-IRs may determine the response to IGF-1: activated IGF-IRs in sufficient numbers change the mode of IGF’s effect from nonmitogenic to mitogenic, resulting in cell cycle progression, translation, and DNA synthesis (46). Overexpression of IGF-IR has been associated with survival of NSCLC patients treated with gefitinib (47). Therefore, our newly identified mechanism of EGFR TKI resistance could provide new therapeutic avenues for NSCLC. Treatment with the anti-EGFR monoclonal antibody cetuximab may be effective once resistance to EGFR TKIs has been established, and the use of combination regimens with EGFR TKIs and IGF-IR inhibitors may be effective at treating NSCLC in patients with high levels of EGFR and IGF-IR expression. However, the results of an in vitro study with a limited number of cell lines are not sufficient to generalize the roles of the IGF-IR signaling pathway and survivin expression in gefitinib resistance in NSCLC cells. Further clinical trials are needed to determine whether combined treatment with EGFR TKIs and IGF-IR pathway inhibitors enhances objective responses and survival in patients with NSCLC.

References

Treatment with Gefitinib in Small Cell Lung Cancer Cells to−

Implication of the Insulin-like Growth Factor-IR Pathway in the Resistance of Non−small Cell Lung Cancer Cells to Treatment with Gefitinib

Floriana Morgillo, Woo-Young Kim, Edward S. Kim, et al.