Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation

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Abstract

Gefitinib (Iressa, ZD1839), a quinazoline tyrosine kinase inhibitor that targets the epidermal growth factor receptor (EGFR), is approved for patients with advanced non-small cell lung cancer (NSCLC) in several countries including Japan. However, the mechanism of drug sensitivity to gefitinib is not fully understood. In this study, we examined the molecular basis of sensitivity to gefitinib using nine human lung cancer cell lines derived from NSCLC. PC9 was the most sensitive to gefitinib of the nine NSCLC cell lines when assayed either by colony formation or MTS assays. The various cell lines expressed different levels of EGFR, HER2, HER3, and HER4, but there was no correlation between levels of EGFR and/or HER2 expression and drug sensitivity. Phosphorylation of EGFR, protein kinase B/AKT (Akt), and extracellular signal-regulated kinase (ERK) 1/2 was inhibited by much lower concentration of gefitinib in PC9 cells than in the other eight cell lines under exponential growing conditions. About 80% of cell surface EGFR in PC-9 was internalized within 10 min, whereas only about 30–50% of the cell surface EGFR was internalized in more drug-resistant cell lines in 15–60 min.

The present study is the first to demonstrate that sensitivity to growth inhibition by gefitinib in NSCLC cell lines under basal growth condition is associated with dependence on Akt and ERK1/2 activation in response to EGF signaling for survival and proliferation and also that drug sensitivity may be related to the extent of EGF-induced down-regulation of cell surface EGFR. [Mol Cancer Ther. 2004;3(4):465–472]

Introduction

Epidermal growth factor receptor (EGFR) is a prototypical member of the EGFR family that includes HER2/neu (ErB2), HER3 (ErB3), and HER4 (ErB4) (1–3). EGFR responds to a number of growth factors such as EGF/TGFα and amphiregulin. This family of receptors plays critical roles in the operation of signaling networks affecting proliferation, migration, survival, adhesion, and differentiation (3). EGFR and/or HER2 are highly expressed in many tumors of epithelial origin, including cancers of lung, breast, head and neck, and bladder (4), and patients whose tumors express high levels of EGFR and/or HER2 have a poor prognosis (5). EGFR family members exist as monomers spanning the plasma membrane, and the monomeric receptors dimerize and become functionally active after binding to the appropriate soluble extracellular ligand. Signal transduction is mediated by a large family of EGF receptors and their ligands (6). Homo- and/or heterodimerization of EGFR activates a number of intracellular signal transducing elements such as phospholipase Cγ, phosphatidylinositol-3-kinase, protein kinase B/AKT (Akt), a small G-protein (Ras), the Ras GTPase-activating protein, extracellular signal-regulated kinase (ERK) 1/2, Srf family kinases, and STATs (7). We have reported that the angiogenesis signal also operates through the EGFR/EGFR pathway (8).

Agents that target tyrosine kinase receptors may contribute to the treatment of malignancies that have relatively high levels of EGFR expression (9, 10). The tyrosine kinase inhibitor gefitinib (Iressa, ZD1839) is a synthetic anilinoquinazoline that targets EGFR (11); it has good oral bioavailability, and antitumor activity in a broad range of mouse xenograft models (9) and tumor cell lines (12). Clinically meaningful antitumor activity was observed in two phase II trials of gefitinib monotherapy in previously treated patients with advanced non-small cell lung cancer, NSCLC (IDEAL 1 and 2), and gefitinib is now approved in several countries including Japan, Australia, and the United States for the treatment of...
advanced NSCLC (13, 14). Concerning the basis of the differential sensitivity of human malignancies to the antitumor effect of gefitinib, animal experiments with xenografts of human breast cancer and other epithelial tumor cell lines have shown that tumors that overexpress HER2 are the most sensitive to gefitinib (15, 16). However, it could be argued that EGFR and/or HER2 levels (17, 18), or phosphorylation of EGFR, or other EGF/TGFα signaling mechanisms (19) control the sensitivity of cancer cells to gefitinib. Naruse et al. (20) have reported that a human leukemic cell line resistant to phorbol ester was 400-fold more sensitive to gefitinib than its parent, suggesting that gefitinib is most effective against cancer cells with non-

Materials and Methods

Materials

Gefitinib was provided by AstraZeneca (Macclesfield, United Kingdom) (8). Recombinant human EGF was purchased from PeproTech (London, United Kingdom). Anti-EGFR antibody and anti-phospho-EGFR antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies to ERK1/2, phospho-ERK1/2, Akt, and phospho-Akt were from Cell Signaling Technology (Beverly, MA). 125I-protein A was purchased from Amersham Biosciences Corp. (Piscataway, NJ).

Cell Culture

Cell lines H522, H322, H358 (American Type Culture Collection, Manassas, VA), QG56 and PC9 (Kyushu Cancer Center, Fukuoka, Japan), and LK2 (Japanese Collection of Research Bioreources, Tokyo, Japan) were cultured in RPMI supplemented with 10% fetal bovine serum (FBS). A549 (Japanese Collection of Research Bioreources) was cultured in MEM supplemented with 10% fetal bovine serum (FBS). EBC-1 (Japanese Collection of Research Bioreources) was cultured in MEM supplemented with 10% FBS and NEAA. EGFR-Down-Regulation

EGFR Driven Signaling Limits Sensitivity to Gefitinib

Cell Viability Assay

CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega Corp., Madison, WI) was used to evaluate cytotoxicity in LK2 and its stable transfectants. One hundred microliter aliquots of exponentially growing cell suspension (3–5 × 10^5 cells) were seeded into 96-well plates, and 24 h later, various concentrations of gefitinib were added. After incubation for 72 h at 37°C, 100 µl of CellTiter-Glo reagent were added and luminescence measured with a multilabel counter (Wallac, Tokyo, Japan). Each experiment was performed in three replicate wells for each drug concentration.

MTS Assay

A CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega) was used to evaluate cytotoxicity. One hundred microliter samples of an exponentially growing cell suspension (5–8 × 10^3 cells) were seeded into a 96-well microtiter plate, and various concentrations of gefitinib were added. After incubation for 72 h at 37°C, 20 µl of CellTiter 96® AQueous One Solution were added to each well and the plates were incubated for a further 4 h at 37°C. Absorbance was measured at 490 nm with a 96-well plate reader. Each experiment was performed in three replicate wells for each drug concentration. The IC_{50} value is defined as the concentration needed for a 50% reduction in absorbance calculated from the survival curves.

Western Blot Analysis

To examine EGF-stimulated phosphorylation of proteins, confluent tumor cells were cultured in serum-free medium for 24 h. The cells were pretreated with gefitinib at concentrations up to 5 μM for 3 h before exposure to 20 ng/ml EGF for 15 min at 37°C. To examine phosphorylation under basal conditions, subconfluent tumor cells cultured in medium supplemented with 10% FBS were incubated with various concentrations of gefitinib for 3 h at 37°C. The cells were then rinsed with ice-cold PBS and lysed in Triton X-100 buffer. The cell lysates were subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA). After transfer, the blots were incubated with blocking solution and probed with various antibodies followed by washing. Proteins were visualized with HRP-conjugated secondary antibodies followed by enhanced chemiluminescence (ECL, Amersham).

Colony Formation Assay

Cell survival was determined by plating 3–9 × 10^3 cells in 35-mm dishes. After 24 h, various concentrations of gefitinib were added, followed by incubation for 7–10 days at 37°C. Gefitinib was solubilized in DMSO and controls for all experiments were carried out by adding equivalent volumes of DMSO. Colonies were counted after Giemsa staining, as described previously (21). IC_{50} values and SDs were obtained from the best fit of the data to a sigmoidal curve using GraphPad software.

Materials and Methods

EGFR-Driven Signaling Limits Sensitivity to Gefitinib

Cell viability was determined by plating 3–9 × 10^3 cells in 35-mm dishes. After 24 h, various concentrations of gefitinib were added, followed by incubation for 7–10 days at 37°C. Gefitinib was solubilized in DMSO and controls

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lysed with 1 N NaOH to determine the fraction of radioactivity (22). The linear regression coefficient of the dependence of this ratio on time represents the specific rate constant for down-regulation ($K_e$).

**Results**

**Sensitivity to Gefitinib in NSCLC and Epidermoid Carcinoma Cell Lines**

We first compared the effect of gefitinib on a panel of nine NSCLC cell lines, and two epidermoid carcinoma cell lines as controls, by both colony formation and MTS assay. Eight of the nine NSCLC cell lines and the two epidermoid cancer cell lines showed considerable resistance to gefitinib. Dose-response curves to gefitinib for 10 of the 11 human cancer cell lines assessed by the colony formation assay are presented in Fig. 1, and IC$_{50}$ values for the 11 cell lines are given in Table 1. The seven NSCLC lines, A549, H522, H322, H358, H157, QG56, and LK2 showed 100- to 200-fold greater resistance to gefitinib than PC9 cells; the latter had an IC$_{50}$ of 0.06 μM. The drug sensitivity of all 11 human cancer cell lines was also examined by MTS assay, and the IC$_{50}$ values are also presented in Table 1. By the MTS assay, the IC$_{50}$ of PC9 was 4 μM, and A549, H522, H322, H358, EBC-1, H157, QG56, and LK2 had 5- to 10-fold greater resistance to gefitinib (Table 1). In the colony formation assay, one of the epidermoid carcinoma cell lines, KB3-1, showed 25-fold higher resistance to gefitinib than the other cell line, A431, which had an IC$_{50}$ of 0.4 μM (Fig. 1 and Table 1). When assayed by the MTS assay, the KB3-1 cells showed only a 2-fold higher resistance to gefitinib than the A431 cells (Table 1). Although the relative resistance of the other NSCLC and epidermoid cell lines compared with PC9 and A431 cells was much less when assayed by MTS than by colony formation, both assays concurred in indicating that the PC9 and A431 cells were more sensitive to gefitinib than the other cell lines examined in this study.

**Expression of EGFR and Its Family of Receptor Proteins, HER2, HER3, and HER4**

We examined expression of EGFR, HER2, HER3, and HER4 in all the cell lines used in this study by Western blot analysis. Expression of EGFR and its family members, HER2, HER3, and HER4, in the NSCLC cell lines varied considerably (Fig. 2). The level of expression of the receptors in each of the NSCLC and epidermoid cancer cell line is given in Fig. 2 relative to expression levels in the drug-sensitive lines PC9 and A431, respectively. One of the

**Table 1.** Cell lines employed in this study, sensitivities to gefitinib, and EGF-induced stimulation of EGFR, Akt, and ERK1/2

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Origin</th>
<th>IC$_{50}$ (μM)$^a$</th>
<th>Colony Formation</th>
<th>MTS</th>
<th>Fold Stimulation by EGF$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>EGFR</td>
</tr>
<tr>
<td>PC9</td>
<td>Human NSCLC (Adenocarcinoma)</td>
<td>0.06 (1.0)</td>
<td>4 (1.0)</td>
<td>1.6</td>
<td>4.0</td>
</tr>
<tr>
<td>A549</td>
<td>Human NSCLC (Adenocarcinoma)</td>
<td>13 (217)</td>
<td>21 (5.3)</td>
<td>3.5</td>
<td>11.1</td>
</tr>
<tr>
<td>H522</td>
<td>Human NSCLC (Adenocarcinoma)</td>
<td>13 (217)</td>
<td>20 (5.0)</td>
<td>2.4</td>
<td>1.9</td>
</tr>
<tr>
<td>H322</td>
<td>Human NSCLC (Adenocarcinoma)</td>
<td>6.8 (113)</td>
<td>27 (6.8)</td>
<td>6.6</td>
<td>2.8</td>
</tr>
<tr>
<td>H358</td>
<td>Human NSCLC (Adenocarcinoma)</td>
<td>2.0 (33)</td>
<td>12 (3.0)</td>
<td>3.2</td>
<td>2.1</td>
</tr>
<tr>
<td>EBC-1</td>
<td>Human NSCLC (Squamous cell carcinoma)</td>
<td>ND$^c$</td>
<td>21 (5.3)</td>
<td>1.3</td>
<td>1.1</td>
</tr>
<tr>
<td>H157</td>
<td>Human NSCLC (Squamous cell carcinoma)</td>
<td>12 (200)</td>
<td>30 (7.5)</td>
<td>125</td>
<td>4.1</td>
</tr>
<tr>
<td>QG56</td>
<td>Human NSCLC (Squamous cell carcinoma)</td>
<td>7.8 (130)</td>
<td>42 (10.5)</td>
<td>1.5</td>
<td>3.8</td>
</tr>
<tr>
<td>LK2</td>
<td>Human NSCLC (Squamous cell carcinoma)</td>
<td>8.0 (133)</td>
<td>20 (5.0)</td>
<td>nd$^d$</td>
<td>3.3</td>
</tr>
<tr>
<td>A431</td>
<td>Human epidermoid carcinoma</td>
<td>0.4 (1.0)</td>
<td>10 (1.0)</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td>KB3-1</td>
<td>Human epidermoid carcinoma</td>
<td>10 (25)</td>
<td>15 (1.5)</td>
<td>31.3</td>
<td>4.2</td>
</tr>
</tbody>
</table>

$^a$Drug sensitivity of nine human non-small lung cancer cell lines and two epidermoid cancer cell lines to gefitinib was assayed by both colony formation and MTS. IC$_{50}$ value for each cell line is presented from two independent assays, and relative activity is presented in parentheses when normalized by IC$_{50}$ for PC9 cells.

$^b$The fold stimulation by EGF of EGFR, Akt, and ERK1/2 is presented for each cell line when normalized by untreated control in the absence of EGF (see Fig. 4).

$^c$ND, not determined because of poor colony-forming ability of the cell line.

$^d$nd, not detected because of poor phosphorylation.
NSCLC cells, LK2 that expressed almost no detectable EGFR and HER2, was relatively resistant to gefitinib. On the other hand, QG56 cells that expressed more EGFR than PC9 cells were much less sensitive than the latter to gefitinib (Table 1). Expression of the four family members, EGFR, HER2, HER3, and HER4, varied among the NSCLC cell lines. Of two epidermoid cancer cell lines, gefitinib-resistant KB3-1 cells had only 10% of the EGFR level of A431 cells. In contrast, five of the NSCLC cell lines (H358, A549, H522, EBC1, QG56) had more HER2, while four NSCLC cell lines had lower levels of HER3 than PC9. KB3-1 cells had 10–20% of the EGFR, HER2, HER3, and HER4 of A431 cells (Fig. 2). Thus, expression of the four EGFR family members is not correlated with the cytotoxicity of gefitinib. We next examined if increasing the expression of EGFR in LK2 cells, which expressed very low levels, if any, of EGFR (see Fig. 2) would render them sensitive to gefitinib. Two EGFR transfectants (LK2/EGFR2 and LK2/EGFR5) were isolated by introducing cDNA of human EGFR into the LK2 cells. These EGFR transfectants had much higher EGFR levels than their parent, or mock transformed LK2 cells (Fig. 3A). However, their sensitivity to gefitinib was similar to that of the parent strain when assayed by the cell viability assay (Fig. 3B).

Effect of Gefitinib on Phosphorylation of EGFR, Akt, and ERK1/2

EGF/TGFα causes phosphorylation of EGFR by its tyrosine kinase activity, and leads to activation of a number of downstream cytoplasmic signaling molecules (6). We first examined the effect of gefitinib on phosphorylation of EGFR, Akt, and ERK1/2 in response to EGF (EGF-induced phosphorylation) in the cell lines used in this study.

Figure 4 shows the inhibitory effect of gefitinib on EGF-induced autophosphorylation of EGFR, and phosphorylation of Akt and ERK1/2 in three of the NSCLC cell lines. EGF stimulated EGFR autophosphorylation and activation of Akt and ERK1/2 in PC9 (A), A549 (B), and QG56 (C) cells, and activation was blocked to different extents by gefitinib. Table 2 presents IC50 (μM) values for EGF-induced autophosphorylation of EGFR, and for phosphorylation of Akt and ERK1/2. IC50 gefitinib doses for EGF-induced autophosphorylation of EGFR were similar in all the NSCLC cell lines including PC9 although the drug-resistant lines EBC-1 and H157 had 2.7- and 0.2-fold higher IC50 (μM) values. EGF-induced Akt phosphorylation in four of the NSCLC cell lines was 10-fold or more resistant to gefitinib than in PC9 cells while the other cell lines showed similar IC50 values to PC9. ERK1/2 phosphorylation was highly
resistant to gefitinib in most of the NSCLC cell lines, while the remaining three were only weakly resistant (1.1- to 3.1-fold). The two epidermoid carcinoma cell lines, A431 and KB3-1, showed similar IC_{50} values for gefitinib with respect to EGFR, Akt, and ERK1/2 phosphorylation (Table 2).

Figure 5 presents the effects of gefitinib on phosphorylation of EGFR, Akt, and ERK1/2 in three cell lines under basal growth condition in the presence of 10% serum. EGFR autophosphorylation and activation of Akt and ERK1/2 in PC9 (A), A549 (B), and QG56 (C) cells were inhibited to different extents. In PC9 cells, EGFR autophosphorylation as well as Akt and ERK1/2 activation were almost completely blocked by 0.1 μM gefitinib (Fig. 5A). By contrast, in the other two cell lines, A549 and QG56, there was only slight if any inhibition of the activation of Akt and ERK1/2 despite the fact that EGFR phosphorylation was completely inhibited at 0.5–5 μM (Fig. 5, B and C). Under basal growth condition, all three processes, EGFR autophosphorylation and activation of Akt and ERK1/2 were the most susceptible to inhibition by gefitinib in PC9 cells. The IC_{50} for EGFR autophosphorylation was only 0.07 μM in PC9 while the IC_{50} values in the other cell lines were about 4-fold or more higher. KB3-1 cells also showed a 3-fold higher IC_{50} for EGFR autophosphorylation than the A431 cells. Gefitinib inhibited Akt activation under basal growth condition with an IC_{50} of 0.08 μM in PC9, whereas the IC_{50} values for QG56 and the other seven cell lines were 4- to 125-fold higher. The dose of gefitinib to inhibit ERK1/2 activation in PC9 cells under basal growth condition was almost 200-fold lower than that required to inhibit the other seven NSCLC cell lines (Table 2). The IC_{50} value for ERK1/2 activation in H358 cells was about 3-fold higher than in PC9 cells, while the IC_{50} values for both Akt and ERK1/2 phosphorylation in KB3-1 were much higher than in the drug-sensitive A431 cells (Table 2).

![Figure 4](image-url). Dose-dependent inhibition of EGF-induced EGFR autophosphorylation, Akt and ERK1/2 phosphorylation in three human cancer cells. Serum-starved cancer cells were treated for 3 h with the indicated concentrations of gefitinib, followed by the addition of EGF (20 ng/ml) for 15 min. Protein extracts were resolved by 7.5% SDS-PAGE and probed with either antibody. Immunoreactive proteins were visualized by enhanced chemiluminescence. A, PC9 cells; B, A549 cells; and C, QG56 cells.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>EGF-Induced (IC_{50}, μM)</th>
<th>Basal Condition (IC_{50}, μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-EGFR</td>
<td>P-Akt</td>
</tr>
<tr>
<td>PC9</td>
<td>0.30 (1.0)</td>
<td>0.05 (1.0)</td>
</tr>
<tr>
<td>A549</td>
<td>0.22 (0.7)</td>
<td>0.06 (1.2)</td>
</tr>
<tr>
<td>H522</td>
<td>0.30 (1.0)</td>
<td>0.50 (10)</td>
</tr>
<tr>
<td>H322</td>
<td>0.43 (1.4)</td>
<td>0.13 (2.0)</td>
</tr>
<tr>
<td>H358</td>
<td>0.40 (1.3)</td>
<td>0.13 (2.0)</td>
</tr>
<tr>
<td>EBC-1</td>
<td>0.80 (2.7)</td>
<td>5&lt; (100&lt;)</td>
</tr>
<tr>
<td>H157</td>
<td>0.06 (0.2)</td>
<td>0.07 (1.4)</td>
</tr>
<tr>
<td>QC56</td>
<td>0.20 (0.7)</td>
<td>0.90 (18)</td>
</tr>
<tr>
<td>LK2</td>
<td>nd b</td>
<td>0.50 (10)</td>
</tr>
<tr>
<td>A431</td>
<td>0.04 (1.0)</td>
<td>0.10 (1.0)</td>
</tr>
<tr>
<td>KB3-1</td>
<td>0.04 (1.0)</td>
<td>0.12 (1.2)</td>
</tr>
</tbody>
</table>

aIC_{50} values were obtained from 50% inhibitory doses of gefitinib on phosphorylation of EGFR, Akt, and ERK1/2 under EGF-stimulated or basal (10% serum) culture condition as presented in Fig. 2. The relative activity for IC_{50} of each cell line is presented in parentheses when normalized by the IC_{50} value in PC9 and A431, respectively.

bnd, not detected because of poor phosphorylation.
EGF-Induced EGFR Down-Regulation and Expression of Cbl Protein

Cell surface EGFR is internalized during EGF/TGFα-driven receptor recycling in exponentially growing cells. We compared the rates of EGF-induced internalization of EGFR in the cell lines expressing relatively high amounts of EGFR (Fig. 6). After exposure to EGF, EGFR was rapidly down-regulated from the cell surface, and we found that 80% and 60% of the cell surface EGFR was internalized within 15 min in PC9 and A431 cells, respectively. In contrast, there was only 30–50% loss of cell surface EGFR 15–40 min after EGF stimulation in EBC-1, A549, and QG56 cells. Sixty percent to 80% of the EGFR molecules thus appeared to be rapidly internalized from the cell surface in gefitinib-sensitive cells, whereas much fewer EGFR molecules were internalized in the resistant cells and internalization was slower.

Cbl is a key protein limiting the initial step of EGFR endocytosis (6), and the EGFR signaling complex is degraded in a coordinate manner with Cbl after interacting with EGF (23). We examined expression of Cbl protein in the nine NSCLC cell lines and two epidermoid carcinoma cell lines (Fig. 7). Almost all the cell lines differed in their expression of Cbl with EBC-1 and QG56 cells expressing considerably less Cbl than the other cell lines. The relative expression levels of Cbl protein in both the NSCLC and epidermoid cancer cell lines are presented in Fig. 7. Cbl expression appears not to be correlated with either the rate of EGF-induced down-regulation (see Fig. 6) or sensitivity to gefitinib (Table 1).

Discussion

Of the nine NSCLC cell lines, PC9, derived from an adenocarcinoma, was the most sensitive to the EGFR-targeting agent, gefitinib, and of the two epidermoid carcinoma cell lines, A431 was more sensitive to gefitinib than KB3-1. The sensitivity of PC9 was similar to that of A431. All these cell lines except LK2 expressed some, although variable, levels of EGFR, HER2, HER3, and HER4. EGFR expression in PC9 was similar to that of some of resistant NSCLC cell

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**Figure 6.** Time kinetics for EGF-induced down-regulation of EGFR. All cell lines (PC9, EBC-1, A549, QG56, A431) were further incubated with 500 ng/ml EGF at 37°C for the indicated periods. Then cells were incubated for 1 h at 4°C with a monoclonal antibody against human EGFR receptor which specifically recognizes extracellular domain of EGFR and for another 1 h with 125I-protein A. Relative amount of 125I-protein A bound to the EGFR antibody is plotted and cell surface EGFR was determined by 125I-protein A (see Materials and Methods; Ref. 22). Points, average of triplicate dishes; bars, SD.

**Figure 7.** Expression of Cbl and EGFR protein in nine NSCLC cell lines and two epidermoid carcinoma cell lines. Protein levels of Cbl, EGFR, and β-actin determined by Western blot analysis using 100 μg protein of cell lysate of each cell line from NSCLC and EC lines are presented when PC9 (NSCLC) and A431 (EC) are respectively normalized as 100%.
Under basal growth conditions in the presence of 10% serum, inhibition of EGFR autophosphorylation in seven of the NSCLC cell lines required 4-fold (or higher) levels of gefitinib than PC9 (Table 2). To our surprise, activation of both Akt and ERK1/2 in almost all the NSCLC lines was found to be much less susceptible to the inhibitory effect of gefitinib under basal growth condition than in the drug-sensitive PC9 cells (Table 2). The activation of EGFR, Akt, and ERK1/2 in A431 cells was also highly sensitive to inhibition in comparison with KB3-1. Taken together, these findings indicate that under basal growth condition, high sensitivity to gefitinib in NSCLC and epidermoid cancer lines is closely correlated with dependence on Akt and ERK1/2 activation in response to EGFR signaling for survival and proliferation (see Fig. 8). Consistent with this notion, Barnes et al. (24) have reported that gefitinib inhibited EGFR and mitogen-activated protein kinase (MAPK) activation efficiently in exponentially growing cutaneous carcinoma cells.

We also observed an apparent difference in EGF-induced down-regulation of cell surface EGFR between drug-sensitive PC9 or A431 cells and drug-resistant EBC-1, A549, QG56, and KB3-1 cells (see Fig. 6). Almost all cell surface EGFR molecules were rapidly internalized in PC9 or A431 cells when only 30–50% of the cell surface EGFR molecules were slowly internalized in gefitinib-resistant cell lines. EGFR molecules in the sensitive cell lines seem to be highly susceptible to EGF stimulation with the result that they transduce the signals more effectively than the drug-resistant cells. We also examined expression of Cbl protein (Fig. 7). Cbl is a key protein affecting receptor internalization in general. However, there was no correlation between level of Cbl protein and EGF-induced down-regulation of EGFR.

In conclusion, one NSCLC cell line, PC9, of the nine strains examined was especially sensitive to the effect of gefitinib. This sensitivity appears to follow from the fact that this cell line is much more dependent than the others on the EGFR receptor/ERK1/2 and Akt pathway for its survival and proliferation (see Fig. 8). The sensitivity of the EGF pathway could be useful for predicting the likely effectiveness of gefitinib in NSCLC patients. Further analysis of other signaling processes in addition to EGFR phosphorylation are called for using clinical specimens.

References
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