Differential Constitutive Activation of the Epidermal Growth Factor Receptor in Non–Small Cell Lung Cancer Cells Bearing EGFR Gene Mutation and Amplification

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Abstract

The identification of somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) in patients with non–small cell lung cancer (NSCLC) and the association of such mutations with the clinical response to EGFR tyrosine kinase inhibitors (TKI), such as gefitinib and erlotinib, has had a substantial effect on the treatment of this disease. EGFR gene amplification has also been associated with an increased therapeutic response to EGFR-TKIs. The effects of these two types of EGFR alteration on EGFR function have remained unclear, however. We have now examined 16 NSCLC cell lines, including eight newly established lines from Japanese NSCLC patients, for the presence of EGFR mutations and amplification. Four of the six cell lines that harbor EGFR mutations were found to be positive for EGFR amplification, whereas none of the 10 cell lines negative for EGFR mutation manifested EGFR amplification, suggesting that these two types of EGFR alteration are closely associated. Endogenous EGFRs expressed in NSCLC cell lines positive for both EGFR mutation and amplification were found to be constitutively activated as a result of ligand-independent dimerization. Furthermore, the patterns of both EGFR amplification and EGFR autophosphorylation were shown to differ between cell lines harboring the two most common types of EGFR mutation (exon 19 deletion and L858R point mutation in exon 21). These results reveal distinct biochemical properties of endogenous mutant forms of EGFR expressed in NSCLC cell lines and may have implications for treatment of this condition. [Cancer Res 2007;67(5):2046–53]

Introduction

The epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein with an extracellular ligand binding domain, a transmembrane region, and a cytoplasmic tyrosine kinase domain and is encoded by a gene (EGFR) located at human chromosomal region 7p12 (1–3). The binding of ligand to EGFR induces receptor dimerization and consequent conformational changes that result in activation of the intrinsic tyrosine kinase, receptor autophosphorylation, and activation of a signaling cascade (4, 5). Aberrant signaling by EGFR plays an important role in cancer development and progression (3).

EGFR is frequently overexpressed in non–small cell lung cancer (NSCLC) and has been implicated in the pathogenesis of this disease (6, 7). Given the biological importance of EGFR signaling in cancer, several agents have been synthesized that inhibit the receptor tyrosine kinase activity. Two such inhibitors of the tyrosine kinase activity of EGFR (EGFR-TKI), gefitinib and erlotinib, both of which compete with ATP for binding to the tyrosine kinase pocket of the receptor, have been extensively studied in patients with NSCLC (8, 9). We and others have shown that a clinical response to these agents is more common in women than in men, in Japanese than in individuals from Europe or the United States, in patients with adenocarcinoma than in those with other histologic subtypes of cancer, and in patients who have never smoked than in those with a history of smoking (10–14). Mutations in the tyrosine kinase domain of EGFR have also been detected in a subset of lung cancer patients and shown to predict sensitivity to EGFR-TKIs (15–17). Indeed, the clinical characteristics of patients with known EGFR mutations are similar to those of other individuals most likely to respond to treatment with EGFR-TKIs (18–22). These mutations arise in the first four exons (exons 18–21) corresponding to the tyrosine kinase domain of EGFR, and they affect key amino acids surrounding the ATP-binding cleft (23, 24). In-frame deletions that eliminate four highly conserved amino acids (LREA) encoded by exon 19 are the most common type of EGFR mutation, with missense point mutations in exon 21 that result in a specific amino acid substitution at position 858 (L858R) being the second most common. In addition to EGFR mutations, other molecular changes may play a role in determining sensitivity to EGFR-TKIs (22, 25–28). NSCLC patients with an increased EGFR copy number, as revealed by fluorescence in situ hybridization (FISH), have thus been found to show an increased response rate to and prolonged survival after gefitinib therapy (22, 25–27).

Given that EGFR is mutated or amplified (or both) in NSCLC, it is important to determine the biological effects of such EGFR alterations on EGFR function (15, 29–32). Transient transfection of various cell types with vectors encoding wild-type or mutant versions of EGFR showed that the activation of mutant receptors by EGFR is more pronounced and sustained than is that of the wild-type receptor (15, 30). However, detailed biochemical analysis of NSCLC cell lines with endogenous EGFR mutations has been limited. We have now identified EGFR mutations in three NSCLC cell lines newly established from Japanese patients. Furthermore, we have characterized a panel of 16 NSCLC cell lines for EGFR mutations and amplification and evaluated the relation between the presence of these two types of EGFR alteration and sensitivity to gefitinib. The effects of EGFR alterations on activation status of EGFR and on downstream signaling were also evaluated.
Finally, in EGFR mutant cell lines showing constitutive EGFR activation, we assessed how the mutations activate the tyrosine kinase domain of the receptor.

**Materials and Methods**

**Cell lines.** The human NSCLC cell lines NCI-H226 (H226), NCI-H292 (H292), NCI-H460 (H460), NCI-H1299 (H1299), NCI-H1666 (H1660), and NCI-H1975 (H1975) were obtained from the American Type Culture Collection (Manassas, VA). PC-9 and A549 cells were obtained as described previously (33). Ma-1 cells were kindly provided by E. Shimizu (Tottori University, Yonago, Japan). We established seven cell lines (KT-2, KT-4, Ma-25, Ma-31, Ma-34, Ma-45, and Ma-53) from tissue or pleural effusion of Japanese patients with advanced NSCLC. These cell lines were cultured under a humidified atmosphere of 5% CO2 at 37°C.

**Growth inhibition assay.** Gefitinib was kindly provided by AstraZeneca (Macclesfield, United Kingdom) as a pure substance and was diluted in DMSO to obtain a stock solution of 20 mmol/L. For growth inhibition assays, cells (0.5 × 10^4 to 4.5 × 10^4) were plated in 96-well flat-bottomed plates and cultured for 24 h before the addition of various concentrations of gefitinib and incubation for an additional 72 h. TetraColor One (5 mmol/L tetrazolium monosodium salt and 0.2 mmol/L L-methoxy-5-methyl phenazinum methysulfate; Seikagaku, Tokyo, Japan) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo, Waltham, MA). Absorbance values were expressed as a percentage of absorbance at 490 nm with a Multiskan Spectrum instrument. Informed consent for establishment of cell lines and tumor DNA sequencing was obtained in accordance with the ethical guidelines for human genome/genetic analysis in Japan.

**Immunoblot analysis.** Cell lysates were fractionated by SDS-PAGE on a 7.5% gel, and the separated proteins were transferred to a nitrocellulose membrane. After blocking of nonspecific sites with 5% skim milk, the membrane was incubated overnight at room temperature with primary antibodies. Antibodies to phosphorylated EGFR (pY845, pY868, and pY1173), extracellular signal-regulated kinase (ERK), phosphorylated AKT, AKT, Src homology and collagen (Shc), and phosphorylated Shc were obtained from Cell Signaling Technology (Beverly, MA); antibodies to EGFR were from Zymed (South San Francisco, CA); antibodies to phosphorylated ERK (pThr202/pTyr204), AKT (pSer473), Akt (pSer473), Src (pTyr416), AKT (pSer473), and Shc (pTyr505) were obtained from Cell Signaling Technology (Beverly, MA); and antibodies to β-actin (loading control) were from Sigma. Immune complexes were detected by incubation of the membrane with a biotinylated secondary antibody and horseradish peroxidase–conjugated goat antibodies to mouse or rabbit immunoglobulin. Stripping was performed using a stripping solution containing 0.5 mol/L 2-mercaptoethanol, 1% SDS, and 20% methanol in water.
Treatment of cells with neutralizing antibodies. Cells were exposed to neutralizing antibodies (each at 12 μg/mL) for 3 h before EGF stimulation. The antibodies included those to EGF and to transforming growth factor-α (TGF-α), both from R&D Systems (Minneapolis, MN) as well as antibodies to EGFR (Upstate Biotechnology, Lake Placid, NY). Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to phosphorylated EGFR (pY1068) and to EGFR as described above.

Chemical cross-linking assay. Chemical cross-linking was done as described previously (34, 35). Cells were washed twice with ice-cold PBS and then incubated for 20 min at 4°C with 1 mmol/L bis(sulfosuccinimidyl)suberate (Pierce, Rockford, IL) in PBS. The cross-linking reaction was terminated by the addition of glycine to a final concentration of 250 mmol/L and incubation for an additional 5 min at 4°C. The cells were washed with PBS, and cell lysates were resolved by SDS-PAGE on a 4% gel and subjected to immunoblot analysis with anti-EGFR (Santa Cruz Biotechnology).

Figure 1. Detection of EGFR mutations in NSCLC cell lines. The portions of the sequencing electrophoretograms corresponding to the mutations are shown for Ma-1 (A) and KT-2 (B) cells. A, heterozygous in-frame deletion in exon 19 is revealed by the presence of double peaks. Tracings in both sense and antisense directions are shown to highlight the two breakpoints of the deletion. Wild-type (uppercase) and mutant (lowercase) nucleotide sequences. B, heterozygous point mutation (T → G) at nucleotide position 2819 in exon 21.

Figure 2. FISH analysis of EGFR amplification in NSCLC cell lines. The analysis was done with probes specific for EGFR (red signals) and for the centromere of chromosome 7 (green signals) in the indicated cell lines. PC-9 and Ma-1 cells manifest an EGFR/centromere copy number ratio of ≥2, whereas KT-2 and KT-4 cells manifest EGFR clusters. H1975 and H1650 cells are negative for EGFR amplification.
Results

Effect of gefitinib on the growth of NSCLC cell lines. We first examined the effect of the EGFR-TKI gefitinib on the growth of 16 NSCLC cell lines, eight of which (KT-2, KT-4, Ma-1, Ma-25, Ma-31, Ma-34, Ma-45, and Ma-53) were established from Japanese NSCLC patients for the present study. The IC_{50} values for gefitinib chemo-sensitivity ranged from 0.07 to 12.46 \mu mol/L (a 178-fold difference; Table 1).

Four cell lines (PC-9, KT-2, KT-4, and Ma-1) were relatively sensitive to gefitinib with IC_{50} values between 0.07 and 2.34 \mu mol/L, whereas the remaining 12 lines were considered resistant to gefitinib (IC_{50} > 6 \mu mol/L). No relation was apparent between sensitivity to gefitinib and histologic subtype of NSCLC for this panel of cell lines (Table 1).

**EGFR mutation and amplification in NSCLC cell lines.** We screened the 16 NSCLC cell lines for the presence of EGFR mutations in exons 18 to 21, which encode the catalytic domain of the receptor. As previously described (36–39), PC-9, H1650, and H1975 cell lines were found to harbor EGFR mutations [del(E746-A750) in PC-9 and H1650 and both L858R and T790M in H1975]. Furthermore, we detected EGFR mutations in three of the newly established cell lines (Ma-1, KT-2, and KT-4). Ma-1 cells, which were isolated from a female ex smoker with adenocarcinoma (>30 years of age), were found to harbor a small deletion within exon 19 [del(E746-A750); Fig. 1; Table 1]. Both KT-2 cells [derived from a male ex smoker with adenocarcinoma (>30 years of age)] and KT-4 cells (derived from a male nonsmoker with large cell carcinoma) harbor a point mutation (L858R) in exon 21 (Fig. 1; Table 1). Four of these six NSCLC cell lines with EGFR mutations (PC-9, Ma-1, KT-2, and KT-4) are sensitive to gefitinib (Table 1), consistent with clinical observations (15–17, 20, 22).

We next examined the 16 NSCLC cell lines for the presence of EGFR amplification by FISH analysis with a probe specific for EGFR and a control probe for the centromere of chromosome 7. Four (PC-9, Ma-1, KT-2, and KT-4) of the 16 cell lines, all of which harbor EGFR mutations, were found to be positive for EGFR amplification (Fig. 2; Table 1). PC-9 and Ma-1 cell lines, both of which harbor the same exon 19 deletion, showed an EGFR/ chromosome copy number ratio of ≥2, whereas KT-2 and KT-4, both of which harbor the L858R mutation in exon 21, showed a clustered unbalanced gain of EGFR copy number (Fig. 2). The four cell lines that manifested both EGFR mutation and amplification were sensitive to gefitinib (Table 1). The EGFR mutant cell lines H1650 and H1975 showed no evidence of EGFR amplification (Fig. 2), and both of these lines were relatively resistant to gefitinib (Table 1). None of the cell lines negative for EGFR mutations manifested EGFR amplification (Table 1), suggesting that EGFR mutation is closely associated with EGFR amplification (P < 0.05, \chi^2 test).

**EGFR expression in NSCLC cell lines.** We examined the basal abundance of EGFR in EGFR wild-type and mutant NSCLC cell lines by immunoblot analysis. The amount of EGFR in the cell lines PC-9, Ma-1, KT-2, and KT-4, all of which manifest EGFR amplification and EGFR mutation, was increased compared with that in EGFR wild-type cell lines (A549 and H1299) or EGFR mutant cell lines negative for EGFR amplification (H1975 and H1650; Fig. 3). These results, thus, reveal a close relation between increased EGFR expression and EGFR amplification in this panel of NSCLC cell lines, consistent with the results of previous analyses of NSCLC tissue specimens (6, 7).

**EGFR phosphorylation in NSCLC cell lines.** We examined tyrosine phosphorylation of endogenous EGFRs in NSCLC cell lines by immunoblot analysis with phosphorylation site–specific antibodies. In cells (A549) that express only wild-type EGFR, phosphorylation of the receptor at Y845, Y1068, or Y1173 was undetectable in the absence of EGF but was markedly induced on
exposure of the cells to this growth factor (Fig. 4). Similar results were obtained with H1650 cells, which are positive for the deletion in exon 19 of EGFR but negative for EGFR amplification. In contrast, PC-9 and Ma-1 cells, which are positive for both the exon 19 deletion and EGFR amplification, manifested an increased basal level of EGFR phosphorylation at Y1068, indicative of constitutive activation of the EGFR tyrosine kinase. Exposure of PC-9 or Ma-1 cells to EGF induced EGFR phosphorylation at Y845 and Y1173, showing that the mutant receptors remain sensitive to ligand stimulation. Furthermore, the cell lines (H1975, KT-2, and KT-4) with the L858R point mutation manifested an increased basal level of EGFR phosphorylation at Y845 and Y1173, showing that the mutant receptors remain sensitive to ligand stimulation. Furthermore, the cell lines (H1975, KT-2, and KT-4) with the L858R point mutation manifested an increased basal level of EGFR phosphorylation at Y845 and Y1173, showing that the mutant receptors remain sensitive to ligand stimulation. Furthermore, the cell lines (H1975, KT-2, and KT-4) with the L858R point mutation manifested an increased basal level of EGFR phosphorylation at Y845 and Y1173, showing that the mutant receptors remain sensitive to ligand stimulation.

Table 1

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Figure 4. Phosphorylation of EGFR and downstream signaling molecules in NSCLC cell lines. Serum-deprived cells were incubated for 15 min in the absence or presence of EGF (100 ng/mL), after which cell lysates (40 μg of protein) were subjected to immunoblot analysis with antibodies to phosphorylated forms of EGFR (pEGFR), ERK (pERK), or AKT (pAKT) as well as antibodies to all forms of the corresponding proteins, as indicated. Representative of three independent experiments.

Phosphorylation of signaling molecules downstream of EGFR in NSCLC cell lines. Given that constitutive activation of EGFR was detected in NSCLC cell lines with endogenous EGFR mutations, we examined whether signaling molecules that act downstream of the receptor are also constitutively activated in these cell lines. We first examined the basal levels of phosphorylation of AKT and ERK, both of which mediate the oncogenic effects of EGFR. Immunoblot analysis with antibodies to phosphorylated forms of AKT or ERK revealed that these molecules are indeed constitutively activated in the EGFR mutant lines (PC-9, Ma-1, H1975, KT-2, and KT-4) that manifest constitutive activation of EGFR, although the extent of phosphorylation varied (Fig. 4). The increased levels of AKT and ERK phosphorylation in these mutant cell lines are consistent with the increased level of EGFR phosphorylation on Y1068, which serves as the docking site for phosphatidylinositol 3-kinase and growth factor receptor binding protein 2, molecules that mediate the activation of AKT and the Ras-ERK pathway, respectively (2, 40). We next examined whether the differences in the pattern of constitutive tyrosine phosphorylation of EGFR apparent between NSCLC cell lines harboring the exon 19 deletion and those with the L858R mutation in exon 21 are associated with distinct alterations in downstream signaling pathways. Given that Y1173, a major docking site of EGFR for the adapter protein Shc (2, 40, 41), is constitutively phosphorylated in cells with the L858R mutation but not in those with the exon 19 deletion, we compared Shc phosphorylation between cell lines with these two types of EGFR mutation. Ligand-independent tyrosine phosphorylation of the 52- and 46-kDa isoforms of Shc was apparent in cell lines with either type of EGFR mutation (Fig. 5). However, cell lines (KT-2 and KT-4) that harbor the L858R mutation exhibited a markedly greater basal level of phosphorylation of the 66-kDa isoform of Shc than did those (PC-9 and Ma-1) that harbor the exon 19 deletion or those (A549) that harbor only wild-type EGFR. These data suggest that the constitutively active mutant forms of EGFR induce selective activation of downstream effectors as a result of differential patterns of receptor autophosphorylation.
Ligand-independent dimerization and activation of EGFR mutants. Evidence suggests that EGFR ligands, including EGF and TGF-α, secreted by tumor cells themselves might be responsible for activation of mutant receptors in an autocrine loop (29, 42). To investigate whether EGFR is constitutively activated as a result of such an autocrine mechanism in EGFR mutant NSCLC cell lines, we treated the cells with a combination of three neutralizing antibodies (anti-EGF, anti-TGF-α, and anti-EGFR) for 3 h and then examined the effect of EGF on EGFR phosphorylation. The ligand-dependent activation of EGFR in A549 cells (which express only wild-type EGFR) was blocked by such antibody treatment (Fig. 6A). In contrast, treatment of the EGFR mutant cell lines PC-9 or KT-4 with the neutralizing antibodies failed to inhibit the constitutive phosphorylation of EGFR on Y1068. These observations suggest that the constitutive phosphorylation of the mutant receptors is not attributable to autocrine stimulation, although we are not able to exclude a possible role for other EGFR ligands.

Ligand-induced EGFR dimerization is responsible for activation of the receptor tyrosine kinase (4, 5). To determine whether mutant receptors are constitutively dimerized, we treated EGFR wild-type or mutant cell lines with a cross-linking agent before immunoblot analysis with antibodies to EGFR. Whereas ligand-induced dimerization of wild-type EGFR was observed in A549 cells, receptor dimerization in PC-9 and KT-4 cells, which express mutant receptors, was apparent in the absence of ligand and was not increased substantially by exposure of the cells to EGF (Fig. 6B). These data indicate that ligand-independent receptor dimerization is responsible for the constitutive activation of the mutant forms of EGFR.

Discussion

The discovery of somatic mutations in the tyrosine kinase domain of EGFR and of their association with a high response rate to EGFR-TKIs has had a substantial effect on the treatment of advanced NSCLC (15–17, 20, 22). Asian patients with NSCLC seem to have a higher prevalence of these mutations, ranging from 20% to 40% (18, 20, 21, 43–45). We have now identified EGFR mutations in three of eight newly established cell lines from Japanese patients with advanced NSCLC. Characterization of these eight new cell lines and eight previously established NSCLC lines revealed that, consistent with previous observations (29, 31, 36), those cell lines that harbor EGFR mutations are more likely to be sensitive to gefitinib than are those without such mutations. Not all EGFR mutant cell lines (e.g., H1650 and H1975) are sensitive to this EGFR-TKI, however, suggesting the existence of additional determinants of gefitinib sensitivity. In addition to the L858R mutation in exon 21 of EGFR, H1975 cells contain the T790M mutation in exon 20, which has been shown to confer resistance to EGFR-TKIs (38, 39). H1650 cells, which do not harbor mutations in EGFR other than the exon 19 deletion, manifest loss of the tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (37), which may result in resistance to EGFR-TKIs. EGFR amplification in NSCLC cells has also been shown to correlate with a better response to gefitinib (22, 25–27). Given that little is known of the relation between EGFR mutation and amplification in NSCLC, we examined the 16 NSCLC cell lines used in this study for EGFR amplification by FISH. Four of the six cell lines with EGFR mutations were found to be positive for gene amplification, whereas none of the 10 mutation-negative cell lines manifested EGFR amplification. This finding thus suggests that EGFR mutation and amplification are linked. Cappuzzo et al. showed that 6 of 9 (67%) NSCLC patients with EGFR amplification also had EGFR mutations (25). Furthermore, Takano et al. sequenced EGFR and determined the EGFR copy number by real-time PCR analysis for the tumors of 66 NSCLC patients (22); all of the patients with a high EGFR copy number (≥6.0 per cell) also had EGFR mutations. Moreover, PCR analysis revealed selective amplification of the mutant EGFR alleles in the patients with a high EGFR copy number. Our sequencing electrophoretograms for the EGFR mutant cell lines positive for EGFR amplification also revealed that the mutant signals were dominant, and the wild-type sequence was barely detectable (Fig. 1), indicative of selective amplification of the mutant alleles. We used the recently proposed definition of EGFR amplification as determined by FISH (25, 27) and found that the pattern of gene amplification seemed to be dependent on the

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type of EGFR mutation; gene clusters were observed in cells with the L858R mutation in exon 21, whereas an EGFR/chromosome copy number ratio of ≥2 was detected in those with the small deletion [del(E746-A750)] in exon 19. Together, these data support the notion that EGFR mutation and amplification may be co-selected for during the growth of NSCLC cells. The four cell lines (PC-9, Ma-1, KT-2, and KT-4) positive for both EGFR and amplification were sensitive to gefitinib, suggesting that EGFR amplification may increase sensitivity to gefitinib in EGFR mutant cells.

Previous biochemical studies of cells transiently transfected with vectors for wild-type or mutant forms of EGFR suggested that EGFR mutations increase EGF-dependent receptor activation (15, 30). Infection of NIH 3T3 cells with a retrovirus encoding EGFR mutants showed that the mutant receptors are constitutively activated and able to induce cell transformation in the absence of exogenous EGF (32). We examined the activation status of endogenous EGFRs in the six NSCLC cell lines that harbor EGFR mutations. The H1650, PC-9, and Ma-1 cell lines, all of which harbor the same exon 19 deletion, showed different patterns of EGFR autophosphorylation in the COOH-terminal region of the protein. EGFR autophosphorylation was ligand dependent in H1650 cells, which are negative for EGFR amplification, whereas Y1068 (but not Y845 and Y1173) was constitutively phosphorylated in PC-9 and Ma-1 cells, both of which manifest EGFR amplification. These results suggest that both EGFR mutation and amplification may be required for constitutive activation of EGFR in NSCLC cells that harbor the exon 19 deletion. In contrast, NSCLC cell lines (H1975, KT-2, and KT-4) that harbor the L858R mutation exhibited constitutive phosphorylation of EGFR at Y845, Y1068, and Y1173, regardless of the absence or presence of EGFR amplification. It is thought that EGFR mutations result in repositioning of critical residues surrounding the ATP-binding cleft of the tyrosine kinase domain of the receptor and thereby stabilize the interactions with ATP and EGFR-TKIs, leading to increased tyrosine kinase activity and EGFR-TKI sensitivity (15, 23, 24). The differential activation of EGFR mutants observed in the present study may result from distinct conformational changes within the catalytic pocket caused by the different types of EGFR mutation. NSCLC patients with exon 19 deletions were recently shown to manifest longer overall survival than did those with the exon 21 point mutation after treatment with EGFR-TKIs, supporting the notion that the two major types of mutant receptors have different biological properties (46, 47).

Ligand-induced receptor dimerization underlies the activation of receptor tyrosine kinases (4, 5). Chemical cross-linking revealed that EGF binding to EGFR induced receptor dimerization in A549 cells, which express only the wild-type form of the receptor. In contrast, endogenous EGFRs in NSCLC cells harboring either the exon 19 deletion or the point mutation in exon 21 of EGFR were found to dimerize in the absence of ligand, suggesting that the constitutive activation of the mutant receptors is attributable to ligand-independent dimerization. EGFR dimerization was shown to be induced by interaction of quinazolines with the ATP-binding site of the tyrosine kinase domain of the receptor and thereby stabilize the interactions with ATP and EGFR-TKIs, leading to increased tyrosine kinase activity and EGFR-TKI sensitivity (15, 23, 24). The differential activation of EGFR mutants observed in the present study may result from distinct conformational changes within the catalytic pocket caused by the different types of EGFR mutation. NSCLC patients with exon 19 deletions were recently shown to manifest longer overall survival than did those with the exon 21 point mutation after treatment with EGFR-TKIs, supporting the notion that the two major types of mutant receptors have different biological properties (46, 47).

Figure 6. Mechanism of constitutive activation of EGFR in NSCLC cell lines. A, effect of neutralizing antibodies (Neut Ab) on EGFR phosphorylation. Serum-deprived NSCLC cells (A549, PC-9, or KT-4) were incubated for 3 h with a combination of neutralizing antibodies to EGF, TGF-α, and EGFR and then for 15 min in the additional absence or presence of EGF (100 ng/mL). Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to the pY1068-phosphorylated form of EGFR or to total EGFR. B, EGFR dimerization. Serum-deprived cells were incubated for 15 min in the absence or presence of EGF (100 ng/mL), exposed to a chemical cross-linker, lysed, and subjected to immunoblot analysis with antibodies to EGFR. Representative of three independent experiments.
of ligand-independent dimerization. Cells with the two most common types of EGFR mutation also manifest different patterns of EGFR autophosphorylation. Prospective studies are required to determine the potential for exploitation of these EGFR alterations in the treatment of advanced NSCLC.

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