Kinetic Analysis of Epidermal Growth Factor Receptor Somatic Mutant Proteins Shows Increased Sensitivity to the Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor, Erlotinib

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Abstract
We show that two commonly occurring epidermal growth factor receptor (EGFR) somatic mutations, L858R and an in-frame deletion mutant, Del(746-750), exhibit distinct enzymatic properties relative to wild-type EGFR and are differentially sensitive to erlotinib. Kinetic analysis of the purified intracellular domains of EGFR L858R and EGFR Del(746-750) reveals that both mutants are active but exhibit a higher Km for ATP and a lower Ki for erlotinib relative to wild-type receptor. When expressed in NR6 cells, a cell line that does not express EGFR or other ErbB receptors, both mutations are ligand dependent for receptor activation, can activate downstream EGFR signaling pathways, and promote cell cycle progression. As expected from the kinetic analysis, the EGFR Del(746-752) is more sensitive to erlotinib inhibition than the EGFR L858R mutant. Further characterization shows that these mutations promote ligand-dependent and anchorage-independent growth, and cells harboring these mutant receptors form tumors in immunocompromised mice. Analysis of tumor lysates reveals that the tumorigenicity of the mutant EGFR cell lines may be due to a differential pattern of mutant EGFR autophosphorylation as compared with wild-type receptor. Significant inhibition of tumor growth, in mice harboring wild-type EGFR receptors, is only observed at doses of erlotinib approaching the maximum tolerated dose for the mouse. In contrast, the growth of mutant tumors is inhibited by erlotinib treatment at approximately one third the maximum tolerated dose. These findings suggest that EGFR somatic mutations directly influence both erlotinib sensitivity and cellular transformation. (Cancer Res 2006; 66(16): 8163-71)

Introduction
Aberrant signaling from epidermal growth factor receptor (EGFR) is implicated in the pathogenesis of a number of different cancers including colorectal, head and neck, glioblastoma, lung, ovarian, and pancreatic cancer (1). Inhibitors of the EGFR signaling pathway include monoclonal antibodies directed to the extracellular domain of EGFR and small-molecule receptor kinase inhibitors such as erlotinib and gefitinib (1, 2). Erlotinib (OSI-774, Tarceva) is a selective, orally available competitive inhibitor for ATP binding to the EGFR tyrosine kinase domain. Erlotinib has potent antitumor activity in several xenograft animal model systems (3, 4) and has received Food and Drug Administration approval for use in the treatment of advanced non–small-cell lung cancer for patients whose disease progressed after one prior chemotherapy regimen (5).

Somatic mutations in the tyrosine kinase domain of EGFR have been identified in up to 10% of non–small-cell lung cancer patients, and >80% of these mutations are either an in-frame deletion mutation of exon 19 or a point mutation in exon 21 (6, 7). Several reports have correlated objective patient response to erlotinib or gefitinib therapy with the presence of EGFR kinase domain somatic mutations, suggesting that these mutations are a contributing factor for inhibitor efficacy (8–12). Retrospective analysis from several clinical trials suggests that other characteristics such as smoking history, tumor etiology, gender, and ethnicity may be better predictors of patient survival than kinase mutation status (13–18). Additional studies using cell lines indicate that cell background plays a significant role in determining response to EGFR tyrosine kinase inhibitors (19–22). Two of the most commonly occurring EGFR somatic mutations, EGFR L858R and EGFR Del(746-750), enhance downstream EGFR signaling pathways and influence cell survival (23, 24). However, when expressed in different heterologous cell systems, these mutations seem to be less active than wild-type EGFR or require heterodimerization with other ErbB family members to augment EGFR signaling (19, 25, 26). Thus, the role of EGFR somatic mutations in driving tumorigenesis and sensitivity to EGFR kinase domain inhibitors remains controversial.

The influence of somatic mutations in conferring EGFR tyrosine kinase inhibitor sensitivity and the role of these mutations in influencing EGFR signaling are unclear due to the wide range of cell systems and the possible influence of different cell backgrounds that have been used in previous studies. To directly assess the role of somatic mutations in the EGFR kinase domain, we have purified the intracellular domains of several EGFR mutant proteins and determined their steady-state kinetic parameters. To examine the effects of cell background on the signaling role of these mutations, we have developed a unique cell-based model system. We show that two of the most commonly observed EGFR somatic mutations, EGFR L858R and EGFR Del(746-750) or EGFR Del(746-752), display altered kinetic properties relative to the wild-type kinase, are more sensitive to erlotinib in a fibroblast transformation model both in vitro and in vivo, and augment EGFR-dependent signaling.

Materials and Methods
Cell culture. The NR6 cell line was a gift from Mark Pegram and Dennis Slamon (University of California at Los Angeles; refs. 27, 28). All other cell lines used in this study were obtained from the American Type Tissue Culture Collection (Manassas, VA). NR6 cells were maintained in...
high-glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L l-glutamine, and 1% penicillin-streptomycin. Stable NR6 lines were maintained in growth medium with 50 μg/mL geneticin (Invitrogen, Carlsbad, CA). Cell lines were maintained at 37°C in a humidified incubator supplied with 5% CO₂.

Kinetic analysis of EGFR and EGFR mutations. EGFR kinase domains were expressed as fusion proteins in insect cells using recombinant baculovirus. One set of constructs contained residues 696 to 1,210 of human EGFR expressed as an NH₂-terminal glutathione S-transferase fusion protein with wild-type or point-mutated (L858R or L851Q) kinase domain sequences (purchased from Upstate, Inc., Charlottesville, VA). In addition, constructs comprising residues 695 to 1,022 of human EGFR were expressed as COOH-terminal His₆-tagged proteins containing an L858R mutation or a deletion mutation (lacking residues 746-750). Kinetic properties, including erlotinib inhibition characteristics, of the two L858R mutant proteins were directly compared and exhibited essentially identical kinetic properties. All proteins were purified using the appropriate affinity matrix and contained no significant phosphatase activity as judged by immunoblotting with an anti-phosphotyrosine antibody. Enzyme assays were done with an ELISA assay with poly(Glu:Tyr) as the substrate and product detection with an anti-phosphotyrosine antibody (PY20, Calbiochem) essentially as previously described (29). All kinetic analyses were done under conditions shown to maintain assay linearity throughout the incubation period.

DNA vectors, mutagenesis, and retroviral infection of NR6 cells. The kinase domain of EGFR was subcloned into pCR (Invitrogen) by PCR. The L858R point mutation and the Del(746-752) mutation were introduced into human EGFR by PCR mutagenesis. The cDNA encoding full-length wild-type and mutant versions of human EGFR were introduced into the EcoRI site of the retroviral vector pLXSN (BD Biosciences, San Jose, CA). Mutant versions of EGFR were subcloned from pCR into the XmnI-BgII site of pLXSN(EGFR). All constructs were sequenced to verify cloning and mutagenesis. Recombinant retrovirus was generated by transiently transfecting the Phoenix ecotropic packaging cell line with either pLXSN, pLXSN(EGFR), pLXSN(EGFR) L858R, or pLXSN(EGFR) Del(746-752) using LipofectAMINE 2000 according to the recommendations of the manufacturer. NR6 cells were infected with recombinant retrovirus and NR6 stable populations were selected for neomycin resistance. Cell populations were sorted for equivalent EGFR receptor expression by flow cytometry and equivalent receptor expression was confirmed by Western blotting.

Phosphorylation assays. Erlotinib (OSI-774, Tarceva) was prepared for in vitro and in vivo studies as previously described (30). NR6-EGFR, NR6-EGFR L858R, or NR6-EGFR Del(746-752) stable cell lines were serum starved for 6 hours in serum-free DMEM. One hour before stimulation, cells were preincubated with the indicated concentrations of erlotinib and then stimulated with 2 mmol/L transforming growth factor α (TGFα) for 10 minutes. Cells were lysed directly in the wells with reducing Laemmli buffer. Receptor phosphorylation, an index of EGFR receptor activation by growth factor stimulation, was detected by Western blotting with a horseradish peroxidase–conjugated anti-phosphotyrosine antibody (PY20, Oncogene Sciences, Cambridge, MA) or with specific phospho-EGFR antibodies described below. Receptor expression in each cell line assayed was evaluated using an antibody specific for the extracellular domain of human EGFR (AB1, MBL Laboratories, Nagoya, Japan). Level of receptor activation was evaluated from the Westerns using NIH image software. These data were then used to determine an IC₅₀ with a four-parameter fit function.

Proliferation assays. Cells were seeded into 96-well dishes (Nunc, Rochester, NY) at a density of 10⁴ per well in quadruplicate in complete growth medium and allowed to adhere overnight. Cells were stimulated with TGFα with the indicated concentration of erlotinib in serum-free medium for 3 days. Alamdar Blue reagent (Trek Diagnostic Systems, CLEVELAND, OH) was added to the supernatant of the manufacturer, incubated for an additional 3 hours, and plates were read using a fluorometer. Proliferation is proportional to relative fluorescence units.

Soft-agar assays. Anchorage-independent growth assays were done essentially as described (30). Medium was formulated with or without 30 mmol/L TGFO and 100 mmol/L erlotinib as indicated. NR6 cell lines were lightly trypsinized and suspended in agarose-containing medium with indicated components and layered on top of 1% agarose–containing growth medium. All experiments were done in quadruplicate. Cells were allowed to grow for 3 weeks, with fresh medium layered on top every 5 days. Colonies were counted manually and each colony was designated as a single colony-forming unit.

Cell cycle analysis. NR6 cell lines were serum starved for 16 hours in low serum–containing medium to synchronize cells in G₀-G₁ phase of the cell cycle. Cells were treated with 30 mmol/L TGFO or left unstimulated with or without 50 mmol/L erlotinib. After 12 hours, cells were lightly trypsinized, washed twice with PBS, and resuspended in ice-cold PBS. Ethanol was added dropwise with constant vortexing to a final concentration of 70%. After an overnight fixation at 4°C, nuclei were stained with 50 μg/mL propidium iodide (Sigma, St. Louis, MO) with 100 units/mL DNase-free RNase (Sigma) and propidium iodide content was analyzed with a Becton Dickinson Elite XL flow cytometer. Data were analyzed using the Modfit software package (Verity Software, Topsham, ME).

Tumor growth studies. Female Nu/Nu mice (6-8 weeks of age, Charles Rivers Laboratories, Hollister, CA) were injected into their flank with 1×10⁶ NR6-EGFR, NR6-EGFR Del(746-752), NR6-EGFR L858R, or NR6 vector control cells s.c. in a volume of 0.2 mL. Tumors were allowed to establish and were then periodically measured in two dimensions using calipers. Tumor volume was expressed in cubic millimeters using the formula \( V = (a \times b^2) / 2 \), where \( a \) and \( b \) are the long and short diameters of the tumor, respectively. Data collected from each experimental group were analyzed using JMP software (SAS software systems, Raleigh, NC) and are expressed as mean ± SE. Tumor measurements were taken either once or twice weekly throughout the experiment. Mice were euthanized before tumor volumes reached 3,000 mm³ or when tumors showed signs of impending ulceration. In the dose-response studies, mice harboring tumors were randomized into groups of 10 with a mean tumor volume between 300 and 350 mm³. Erlotinib or vehicle (Capsitol) treatments were administered in 100 μL by gavage daily for 5 days. Erlotinib was given at 12, 25, 50, or 150 mg/kg. All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the NIH (NIH Publication 85-23, revised 1985). An Institutional Animal Care and Use Committee approved all animal protocols.

Western blotting from tumor cell lysates. Size-matched NR6-EGFR tumors were resuspended in lysis buffer with protease inhibitors [1% Triton X-100, 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 units/mL aprotinin, and 2 mmol/L Na₃VO₄] and homogenized using a polytron tissueizer (PT2100) on ice. Tumor lysates were cleared of insoluble material by centrifugation and total protein levels were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). A total of 50 μg of protein from tumor cell lysates were analyzed for each sample. Samples were separated on a 4% to 12% Tris-glycine gel and transferred to nitrocellulose membranes. Membranes were blocked in 5% bovine serum albumin/TBST and probed with the indicated antibodies, followed by a peroxidase-conjugated anti-mouse or rabbit secondary antibody as described. Blots were developed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Receptor expression was evaluated using a rabbit polyclonal antibody to EGFR (Cell Signaling Technology, Danvers, MA). Total cellular protein loading was evaluated using an antibody to actin (Santa Cruz Biotechnology, Santa Cruz, CA). Phospho-specific antibodies to EGFR were obtained from Cell Signaling Technology. Activated mitogen-activated protein kinase (MAPK) (Cell Signaling Technology), phosphoAkt-Ser473 (Cell Signaling Technology), total Akt (Cell Signaling Technology), total cdk4 (Cell Signaling Technology), and total extracellular signal–regulated kinase (Erk; Cell Signaling Technology) were used to detect activation of EGFR-dependent signaling.

Results

Kinetic analysis of EGFR mutations. To evaluate the effect of clinically relevant EGFR kinase domain mutations on both the catalytic properties and erlotinib sensitivity of EGFR, the steady-state kinetic parameters of the intracellular kinase domain of
wild-type EGFR and three different EGFR kinase domain mutations [L858R, L861Q, and Del(746-750)] were characterized. Several kinetic parameters differ among the wild-type enzyme and mutant proteins examined (Fig. 1; Table 1). The L858R and Del(747-750) mutant EGFRs showed a higher $K_M$ for ATP, relative to the wild-type enzyme. In contrast, the L861Q mutant exhibited a lower $K_M$ for ATP. These data indicate that each EGFR mutant exhibits distinct kinetic properties that differ from those of the wild-type enzyme. We also observed significant differences between wild-type EGFR and mutant enzymes in sensitivity to inhibition by erlotinib. The apparent $K_i$ for wild-type EGFR was 17.5 nmol/L, a value that is consistent with previously published reports (29, 31); however, the apparent $K_i$ values measured for the L858R and Del(746-750) mutants were 6.3 and 3.3 nmol/L, respectively (Table 1). In contrast, the L861Q mutant had a higher $K_i$ value compared with wild-type EGFR. The ratio of the $K_i$ for erlotinib to the $K_M$ for ATP estimates the relative binding of erlotinib to each EGFR kinase domain at the respective $K_M$ concentration of ATP. The $K_i/K_M$ values measured for both L858R and Del suggest that these mutations are significantly more sensitive to erlotinib, whereas this does not seem to be the case for the L861Q mutant (Table 1). The effect of kinase domain mutations on EGFR inhibition by erlotinib was further examined by measuring IC_{50} values for each form of the enzyme across a range of ATP concentrations up to 100 µmol/L. Strikingly, at higher ATP concentrations, both the L858R and Del(746-750) mutant enzymes were considerably more sensitive to inhibition by erlotinib than the wild-type enzyme, whereas the L861Q mutant was comparable to wild-type EGFR (Fig. 1D). These kinetic data predict that the Del(746-750) and L858R mutants would be more sensitive to erlotinib treatment. This difference may also influence the magnitude of the objective clinical response observed in patients.
expressing different mutant EGFR proteins relative to those with tumors exclusively expressing wild-type tumors.

**Non–small-cell lung cancer EGFR mutations confer greater sensitivity to erlotinib in vitro.** To assess the significance of somatic mutations in the EGFR kinase domain, the two most common mutations observed clinically, a point mutation, L858R, and a deletion mutation, Del(746-752), were introduced into full-length EGFR, and stable cell lines that express equivalent levels of EGFR or mutant receptors were generated in the NR6 cell line. NR6 cells lack endogenous EGFR and do not detectably express other ErbB family members (27, 28, 32). Thus, this cell line provides a uniform background to investigate EGFR signaling in the absence of cross-talk with other ErbB receptor family members. All three cell lines, NR6-EGFR, NR6-EGFR L858R, and NR6-EGFR Del(746-752), are ligand dependent for receptor activation, lack detectable expression of other ErbB receptor members, and express equivalent amounts of EGFR (Fig. 2A).

EGFR activation and downstream signaling initiation is dependent on receptor phosphorylation and is a measure of kinase inhibitor potency. We initially investigated the erlotinib sensitivities of the NR6-EGFR, NR6-EGFR L858R, and NR6-EGFR Del(746-752) cell lines by analyzing erlotinib-mediated inhibition of ligand-dependent receptor phosphorylation. Erlotinib inhibits TGFα-dependent activation of the Del(746-752) and L858R mutant receptors with increased potency compared with wild-type receptor (Fig. 2B, top). Densitometry analysis of the Western blots allowed for an estimation of IC50 values for erlotinib inhibition of EGFR activation.

### Table 1. Kinetic characteristics of purified EGFR and EGFR mutant kinase domains

<table>
<thead>
<tr>
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<th>KM (μmol/L)</th>
<th>Ki (nmol/L)</th>
<th>Ki/KM Relative ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>5.0</td>
<td>17.5</td>
<td>3.50 × 10⁻³ 1</td>
</tr>
<tr>
<td>L858R</td>
<td>10.9</td>
<td>6.25</td>
<td>5.73 × 10⁻⁴ 6</td>
</tr>
<tr>
<td>Deletion</td>
<td>129.0</td>
<td>3.3</td>
<td>2.55 × 10⁻⁵ 137</td>
</tr>
<tr>
<td>L861Q</td>
<td>1.1</td>
<td>48.7</td>
<td>4.43 × 10⁻² 0.08</td>
</tr>
</tbody>
</table>

NOTE: KM values for ATP for the wild-type and mutant EGFR proteins were derived from the Lineweaver-Burke plots shown in Fig. 1. Ki values for erlotinib were determined from similar analyses done in the presence of varying concentrations of erlotinib. Relative ratio is calculated by dividing the value of Ki/KM derived for wild-type EGFR divided by the value of Ki/KM for each EGFR mutant.

![Figure 2](image-url)

**Figure 2.** EGFR mutations are more sensitive to erlotinib than EGFR wild-type receptor in vitro. A, NR6 cells lack endogenous expression of EGFR or other ErbB family receptors. NR6-EGFR and NR6-EGFR L858R or NR6-EGFR Del(746-752) stable cell lines were serum starved overnight and stimulated with 2 nmol/L TGFα for 10 minutes or left untreated. Receptor activation was evaluated using an anti-phosphotyrosine antibody and an EGFR phospho-specific antibody to Y1068. B, TGFα-dependent EGFR activation is inhibited by erlotinib. Equivalent numbers of NR6-EGFR, NR6-EGFR L858R, and NR6-EGFR Del(746-752) cells were grown in low serum–containing growth medium for 12 hours, preincubated with the indicated amounts of erlotinib for 1 hour before stimulation, and stimulated with 2 nmol/L TGFα for 10 minutes. Erlotinib-dependent inhibition of EGFR phosphorylation or total EGFR expression was evaluated by Western blotting. C, phosphotyrosine EGFR blots were scanned by densitometry and these data were used to calculate an IC50 for erlotinib inhibition (legend) of ligand-dependent receptor activation for each of the NR6-EGFR cell lines. Points, mean decrease in receptor phosphorylation from three independent experiments for each cell line; bars, SE. D, NR6-EGFR and mutant cell lines are equally responsive to TGFα-induced cell proliferation. Each cell line was grown in 96-well plates in quadruplicate for 3 days with different indicated concentrations of TGFα and assayed for increased cell growth as indicated by an increase in relative fluorescence units. The EC50 for TGFα-dependent EGFR activation is indicated for each cell line in the legend. E, inhibition of TGFα-induced proliferation by erlotinib. NR6 cell lines were grown in 96-well plates in quadruplicate in normal growth media. Points, mean relative fluorescence units from three independent experiments; bars, SE. An IC50 for erlotinib for each cell line was calculated from the slope of the curves generated using a four-parameter fit function and is shown in the legend.
EGFR phosphorylation for each of the cell lines (Fig. 2C). All EGFR-expressing NR6 cell lines exhibited similar sensitivity to TGFα in a proliferation assay, suggesting equivalent responsiveness to growth factor–dependent activation of receptor signaling (Fig. 2D). These data reveal that ligand concentrations used for all experiments are at or above the experimentally determined EC₅₀ for EGFR when expressed in NR6 cells for all experiments. Again, as expected from the aforementioned kinetic data, erlotinib inhibited TGFα-induced cellular proliferation with an IC₅₀ of 12.9 ± 1.4 nmol/L for the NR6-EGFR Del(746-752) cell line, 40.5 ± 5.9 nmol/L for the NR6-EGFR L858R cell line, and 50.4 ± 7.8 nmol/L for the NR6-EGFR cell line (Fig. 2E). Both the phosphorylation assay and the proliferation assay indicate that the Del(746-752) mutant EGFR kinase domain and, to a somewhat lesser extent, the L858R mutant domain are more sensitive to erlotinib than wild-type EGFR, consistent with the kinetic characterization of the mutant and wild-type EGFR proteins. Because of the common background of the NR6 expression system, which lacks endogenous ErbB family members, these altered sensitivities are likely to be due to the mutations themselves and independent of other cellular specific factors such as heterodimerization with wild-type EGFR or other ErbB family members.

**EGFR somatic mutations are transforming.** Previously published reports have shown that the L858R and Del(746-752) mutations enhance ligand-dependent EGFR activation of Akt and signal transducer and activator of transcription (STAT) signaling pathways and confer a survival advantage to cells harboring these mutations (23). Although we have not observed sustained activation of Akt or STAT signaling pathways in the NR6-EGFR mutant cell lines in vitro, we tested the potential for these mutations to facilitate anchorage-independent growth. Interestingly, all cell lines expressing EGFR were able to grow in an anchorage-independent manner. In each instance, anchorage-independent growth was significantly enhanced when EGFR-expressing cell lines were grown in the presence of TGFα (Fig. 3A). The number of colonies for both the NR6-EGFR Del(746-752) and L858R mutant cell lines was higher relative to NR6-EGFR cells, both in the presence or absence of TGFα. In each case, colony formation was augmented by the addition of TGFα. No colony formation was observed with the NR6 parental cells. These data are consistent with previously published reports showing the transforming potential of EGFR (32–35). Transformation was ligand (EGF/TGFα) dependent, suggesting that NR6 cells likely produce ligand in an autocrine manner. Notably, the...
The number of colonies for both NR6-EGFR mutants was significantly higher relative to NR6-EGFR cells in the presence or absence of TGFα. Growth of NR6-EGFR and NR6-EGFR mutant cell lines in soft agar was completely inhibited by 50 nmol/L erlotinib (Fig. 3A), showing that colony formation is dependent on EGFR activity.

Because the NR6-EGFR cell lines were able to grow in an anchorage-independent manner, we assessed the ability of NR6, NR6-EGFR, and NR6-EGFR mutant cell lines to form tumors in athymic nude mice. Palpable tumor growth was detectable within 3 weeks of inoculation for the NR6-EGFR, NR6-EGFR L858R, and NR6-EGFR Del(746-752) cell lines, but not for the parental NR6 cell line (Fig. 3B). Interestingly, the growth rate of the NR6-EGFR Del(746-752) cell line was more aggressive in vivo than either the NR6-EGFR L858R or NR6-EGFR cell line.

Tumor lysates were prepared from size-matched NR6-EGFR, NR6-EGFR L858R, and NR6-EGFR Del(746-752) tumors and were examined for similarities and differences in EGFR receptor phosphorylation by Western blotting. Whereas there were no differences in the Y845, Y992, Y1068, and Y1173 phosphorylation sites, both NR6-EGFR L858R and NR6-EGFR Del(746-752) tumors showed significant decreases in the level of two EGFR phosphorylation sites, Y1045 and Y1148, compared with NR6-EGFR tumors (Fig. 3C). Reduced Y1148 and Y1045 phosphorylation may decrease the association of the adaptors Shc and Cbl with the mutant receptors and affect EGFR downstream signaling. Differences in EGFR phosphorylation patterns have been reported in other systems and were associated with enhanced activation of EGFR signaling (23, 24). Despite these differences, we were not able to detect any differences in the degree of Akt or Erk activation between the NR6-EGFR and NR6-EGFR mutant tumors (Fig. 3D). This may be due to the lack of any detectable difference between the NR6-EGFR and NR6-EGFR mutant tumors in Y1173 or Y1068 phosphorylation. EGFR autophosphorylation at the Y1173 site results in the recruitment of Shc to initiate MAPK/Erk activation and phosphorylated Y1068 can recruit Gab1 to EGFR, initiating the phosphatidylinositol 3-kinase/Akt pathway (36, 37).

Increased proliferation of NR6-EGFR mutant cell lines in vivo and in vitro. To assess the influence of kinase domain mutations on EGFR-dependent proliferation, cell cycle analysis was done. NR6 vector–transfected cells showed no change in DNA content when stimulated with ligand or when treated with erlotinib (Fig. 4B, top row). All three NR6-EGFR cell lines showed a shift of cells into S phase and G2-M when stimulated with ligand (Fig. 4B, center). Both NR6-EGFR L858R and NR6-EGFR Del(746-752) cells have a higher percentage of cells in S phase and in G2-M than the NR6-EGFR cell line. However, the difference between ligand-induced S + G2-M fractions for the NR6-EGFR and NR6-EGFR Del(746-752) cell lines is marginal whereas this fraction is higher for the NR6-EGFR Del(746-752) cell line. Erlotinib inhibited TGFα-stimulated cell cycle progression of NR6-EGFR and NR6-EGFR mutant cell lines (Fig. 4B, right). These data suggest that the increased proliferative ability of the NR6-EGFR mutations augments cell cycle progression and contributes to the tumorigenicity of these mutations.

Transfected EGFR mutants in NR6 are more sensitive to erlotinib in vivo. NR6-EGFR cell lines were transplanted s.c. into
the flank of nude mice and tumors were allowed to grow to a mean volume of 300 mm³. Tumor-bearing mice were treated with vehicle or the indicated doses of erlotinib by gavage daily for a total of 5 days. The results from these studies are shown in Fig. 5A to F. Significant growth inhibition of NR6-EGFR tumors was observed only at the highest dose tested, 150 mg/kg (Fig. 5A). As previously reported, this is the maximum tolerated dose of erlotinib in mice (4). In contrast, NR6-EGFR L858R and NR6-EGFR Del(746-752) tumors are more sensitive to erlotinib treatment (Fig. 5C and E). NR6-EGFR Del(746-752) tumor inhibition is observed for doses as low as 12 mg/kg/d and for the NR6-EGFR L858R line as low as 50 mg/kg/d.

Tumor-bearing mice were treated with erlotinib for a short duration to enable comparison of tumor regrowth characteristics between the different NR6-EGFR cell lines. The effect of erlotinib on tumor regrowth was different relative to vehicle-treated control tumors for both NR6-EGFR L858R and NR6-EGFR Del(746-752) tumors at 50 mg/kg (P < 0.001), whereas tumor regrowth reduction was only observed at the highest dose, 150 mg/kg, for NR6-EGFR tumors (Fig. 5B, D, and F). The NR6-EGFR Del(746-752) mutation was also more sensitive to erlotinib at lower doses, with reductions in tumor size at dose levels as low as 12 mg/kg (P < 0.05) and 25 mg/kg (P < 0.001). The mean time to tumor doubling for NR6-EGFR tumors in the vehicle-treated group was 6 days whereas the NR6-EGFR Del(746-752) group doubled in 2.5 days, suggesting a significantly greater growth rate for the EGFR Del(746-752) tumors relative to the EGFR wild-type tumors. The mean change in tumor volume from day 0 to day 5 was also significantly greater for both mutations in the vehicle-treated group at day 5 compared with EGFR wild-type tumors (Fig. 5B, D, and F). These data suggest that, in addition to greater sensitivity to erlotinib, mutations in the kinase domain also provide a distinct growth advantage to these transformed fibroblasts.

**Discussion**

EGFR somatic mutations exhibit altered properties and downstream signaling. We determined the steady-state kinetic parameters for three different EGFR somatic mutations that were previously identified in patients with non–small-cell lung cancer. Analysis of two of the most commonly occurring mutations, L858R and Del(746-750), revealed a lower Ki for erlotinib than wild-type receptor and a profound difference in sensitivity to erlotinib at higher ATP concentrations, which may be an accurate reflection of the physiologic effect of EGFR mutations due to the relatively high intracellular ATP concentration. These observations are consistent with clinical data showing that patient tumors harboring either the L858R point mutation in exon 21 or a deletion mutation in exon 19 of EGFR exhibit a greater objective response rate to erlotinib and gefitinib (7). Of note, the L861Q mutant does not display increased sensitivity to erlotinib compared with wild-type EGFR, suggesting that not all mutations are more sensitive to erlotinib relative to wild-type receptor. Because these experiments were done using the purified intracellular domain of each EGFR mutant, these findings suggest that the L858R and Del(746-750) mutations directly influence EGFR tyrosine kinase inhibitor sensitivity.

The structural basis for the enhanced sensitivity mediated by these mutations is not apparent from published reports of crystal structure data of EGFR tyrosine kinase inhibitors with the kinase domain of EGFR (31, 38). Members of the 4-anilinoquinazoline class...
of compounds can competitively compete for ATP binding, yet modeling of either mutation onto the published structures does not reveal any clear mechanism to explain sensitivity. Moreover, the findings of this study suggest that L858R and deletion mutations may affect sensitivity by different mechanisms. It is possible that repositioning of critical residues of the tyrosine kinase domain as a consequence of deleting residues in the α-helix may account for the greater sensitivity of the deletion mutant relative to the L858R mutant. The lower $K_d$ value for erlotinib observed for the deletion mutant suggests the possibility that the off-rate for erlotinib may be slower, thus prolonging the duration of erlotinib binding. Further insights may be gained by the characterization of a cocrystral structure of erlotinib with the L858R and Del(746-750) mutants.

We have developed a unique cell line system that allows for the assessment of wild-type and EGFR mutants in an identical cellular context that lacks expression of endogenous EGFR or other ErbB family members or ligands. Our system allowed for a baseline assessment of receptor activation and the characterization of the tumor biology of two frequently observed EGFR somatic mutations in non–small-cell lung cancer patients, L858R and Del(746-752). Both NR6-EGFR L858R and NR6-EGFR Del(746-752) cell lines require ligand for activation of receptor phosphorylation and downstream signaling in agreement with previously published reports using more complex systems (8, 9, 23, 25). Consistent with the kinase assay data, both mutations are more sensitive to erlotinib than wild-type EGFR. Furthermore, mice bearing tumors derived from these mutant cell lines are sensitive to erlotinib at doses significantly lower than those for wild-type tumors. Because NR6 cells are deficient in the expression of ErbB family members, these data indicate that enhanced sensitivity to erlotinib does not require the expression of other ErbB family members as has been previously proposed (20, 26). These findings suggest that enhanced erlotinib sensitivity of either the L858R or the Del(746-752) mutation in NR6 fibroblasts is predominantly dependent on the direct effects of these mutations on the properties of the enzyme.

**EGFR somatic mutations promote tumorigenesis in NR6 cells.** In this report, we show for the first time that NR6 cells expressing either the L858R or the Del(746-752) mutation grow in an anchorage-independent manner and are tumorigenic when transplanted into immunocompromised mice. Similar to the differences in the kinetic properties and erlotinib sensitivity for each mutant, there are also differences between the two mutations in conferring a growth advantage to the NR6 cell line. However, in each case, these growth advantages and the enhanced signaling properties of the L858R and Del(746-750) mutants are inhibited by erlotinib. Thus, these tumors remain dependent on EGFR for both proliferation and survival, consistent with the notion that tumors bearing these mutations may be “addicted” to EGFR signaling (39).

The findings in this report are consistent with recent reports showing that EGFR kinase domain mutations influence EGFR autophosphorylation and enhance downstream signaling (23, 40). However, our NR6 system displays differences in EGFR autophosphorylation patterns and EGFR signaling compared with the findings of another published report (23). Tumors derived from either the NR6-EGFR L858R or the NR6-EGFR Del(746-752) cell line show a decrease in the degree of phosphorylation of the Y1045 and Y1148 sites. Reduction of Y1045 phosphorylation would reduce Cbl association, resulting in decreased EGFR cell surface down-regulation through receptor ubiquitylation, thereby prolonging receptor activation and enhancing downstream signaling (41, 42). This may account for the enhanced cell cycle dynamics and growth rate of these mutants. The Y1148 EGFR autophosphorylation site has been shown to recruit the adaptor protein Shc and regulate Erk signaling (43). However, there are no detectable differences in the level of Erk activation or Akt signaling in this system. A possible explanation for the discrepancy could be that cellular context may influence EGFR coupling to downstream signaling pathways or the interaction with other ErbB family members. This notion is consistent with the results of a recent study showing that ErbB3 expression was necessary for the EGFR L858R mutant receptor-dependent sustained activation of Akt (26). These data suggest that whereas EGFR mutations seem to increase tumor response rates with EGFR inhibitors, cellular context in epithelial-derived tumors can play an important role in dictating the efficacy of EGFR inhibitors on patient long-term survival (21, 22).

**The relationship of erlotinib dose to tumor responsiveness.** Retrospective analysis of several clinical trials suggests that non–small-cell lung cancer patients whose tumors harbor EGFR kinase mutations frequently exhibit dramatic objective responses to either gefitinib or erlotinib therapy (11, 12, 44, 45). In contrast, only erlotinib, which is administered near its maximum tolerated dose, shows a survival benefit as a single agent in second- and third-line non–small-cell lung cancer patients (16). The data presented in this report offer possible insights to these clinical observations. In our system, the growth of NR6-EGFR wild-type tumors was inhibited only by the highest doses of erlotinib whereas NR6-EGFR mutant tumors are inhibited at much lower doses, which is consistent with the increased objective response rate observed in patients with mutant EGFR tumors treated with gefitinib or erlotinib. These data also suggest that higher drug exposure levels observed in patients treated with erlotinib, in contrast to those treated with gefitinib, may be required to achieve optimal clinical benefit in patients whose tumors are wild type for EGFR (46–49).

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**References**


Kinetic Analysis of Epidermal Growth Factor Receptor Somatic Mutant Proteins Shows Increased Sensitivity to the Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor, Erlotinib

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