

Functional Analysis of Epidermal Growth Factor Receptor (EGFR) Mutations and Potential Implications for EGFR Targeted Therapy

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Abstract Purpose: Epidermal growth factor receptor (EGFR) mutations in non-small cell lung cancer (NSCLC) might be predictive for clinical response to EGFR inhibitor treatment. However, retrospective analyses of EGFR mutations in clinical trials have shown inconclusive results and the effect of EGFR sequencing in NSCLC is still controversial. Because the vast majority of EGFR mutations described have not been functionally characterized, simple correlation of mutational status and treatment response may not provide reliable information about the predictive value of EGFR mutations. Thus, we aimed to characterize a comprehensive panel of clinically observed EGFR mutations.

Experimental Design and Results: A panel of 30 EGFR mutations was cloned and characterized for kinase activity and the ability to confer growth factor independence. Interestingly, 4 of 30 EGFR mutations showed no kinase activity even after ligand stimulation and were not able to confer growth factor independence. Ba/F3 cells expressing activating EGFR mutants were then used to test the efficacy of EGFR inhibitors in a cell proliferation assay. IC₅₀ values were calculated for gefitinib, erlotinib, and AEE788. We show that the sensitivity of EGFR mutations toward different inhibitors varies significantly, thus establishing a comprehensive sensitivity profile for each inhibitor.

Conclusions: EGFR mutations identified in NSCLC patients display distinct biological features. The variability in kinase activity, transforming potential, and sensitivity to EGFR inhibitors has to be considered in clinical studies aiming to correlate mutational status and drug response. The identification of comprehensive drug resistance profiles opens the opportunity to test alternative EGFR inhibitors *in vitro*.

Small-molecule inhibitors that target oncogenic tyrosine kinases have become an attractive treatment strategy in the therapy of several cancers. Recently, activating mutations in the epidermal growth factor receptor (EGFR) kinase domain were shown to sensitize a small subset of non-small cell lung cancer (NSCLC) patients to treatment with EGFR kinase inhibitors, such as gefitinib or erlotinib (1–3). However, consecutive studies did not find a correlation between mutation status and response to EGFR kinase inhibitor treatment (4). These conflicting results led to some uncertainty whether or how to use molecular diagnostics to predict responses to EGFR kinase

inhibitor treatment in NSCLC. Recently, growing experimental evidence indicates that individual EGFR mutations may show very distinct responses to EGFR kinase inhibitors. For example, it was shown that EGFR exon 20 insertions were relatively resistant to gefitinib compared with other mutations (5). A novel mutation EGFR-T790M was shown to cause secondary resistance to gefitinib (6, 7). Several studies reported additional, less frequent novel mutations (8–10). Because the majority of EGFR mutations reported were not functionally characterized (11, 12), we hypothesized that not all mutations may sensitize to treatment with EGFR kinase inhibitors. This would be of clinical significance because mere correlation of mutation status and drug response within clinical trials would not lead to meaningful conclusions.

Previous *in vitro* studies in chronic myelogenous leukemia, gastrointestinal stromal tumor, and acute myelogenous leukemia have shown that kinase inhibitor sensitivity significantly varies between different activating kinase mutations, as observed for Bcr-Abl, c-Kit, or Flt-3 (13–16). In addition, secondary mutations causing drug resistance were reported in several oncogenic kinases and switching kinase inhibitors has proven to be very effective in overcoming drug resistance in chronic myelogenous leukemia (17). Therefore, it may become increasingly important also in NSCLC to establish sensitivity profiles for different activating and resistance EGFR mutations, and this may even have implications for the selection of a specific EGFR inhibitor in the future. In the present study, we

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Translational Relevance

Activating mutations in epidermal growth factor receptor (EGFR) sensitize a small subset of non – small cell lung cancer patients to treatment with EGFR kinase inhibitors. However, consecutive studies did not always find a correlation between mutation status and treatment response. These led to uncertainty whether or how to use molecular diagnostics to predict responses to EGFR inhibitor treatment. Because the majority of EGFR mutations reported were not functionally characterized, we hypothesized that not all mutations may sensitize to treatment with EGFR inhibitors. Analysis of a panel of 30 mutations revealed that 4 mutations lacked kinase activity and transforming ability. Furthermore, the dose-response profiles for EGFR mutations show that the sensitivity of EGFR mutations toward different inhibitors varies significantly. This variability may in part be responsible for the discrepancies between correlative clinical studies. In addition, the identification of comprehensive drug resistance profiles will undoubtedly accelerate the development of second-generation EGFR kinase inhibitors.

aimed to test kinase activity and transforming ability of a comprehensive panel of EGFR mutations reported in NSCLC patients. In addition, we analyzed the sensitivity of EGFR mutations toward the EGFR inhibitors gefitinib, erlotinib, and AEE788.

Materials and Methods

Constructs, cell lines, and reagents. All point mutations were introduced into pcDNA3.1/EGFR using the QuikChange Site-Directed Mutagenesis kit (Fermentas) according to the manufacturer's instructions. All constructs were confirmed by sequencing. Wild-type (WT) and mutant EGFR were cut with *Xho*I and *Eco*RV and subcloned into MSCV-YFP (MIY) using *Xho*I and *Hpa*I. EGFRvIII (a kind gift from Frank Furnari, Ludwig Institute for Cancer Research, San Diego, CA) was subcloned from pLERNL into MSCV-eGFP (MigRI). Point mutations were introduced into EGFRvIII/MigRI as described above.

NIH/3T3 and HEK293 cells were cultured in DMEM (Life Technologies) supplemented with 10% FCS (fetal bovine serum Gold; PAA Laboratories GmbH). Ba/F3 cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% FCS, glutamine, and interleukin-3 (IL-3; R&D Systems). Stable Ba/F3 cell lines expressing mutant EGFR were established by retroviral infection with either MIY-EGFR or MigRI-EGFRvIII followed by IL-3 withdrawal.

Recombinant human EGF was purchased from Chemicon. Gefitinib was kindly provided by AstraZeneca and AEE788 was a kind gift from Novartis Pharma AG. Erlotinib was purchased from a pharmacy. Each compound was dissolved in DMSO to make an initial stock solution of 10 mmol/L (gefitinib and AEE788) and 2.5 mmol/L (erlotinib).

Autophosphorylation and proliferation assays. NIH/3T3 cells were transiently transfected with WT or mutant EGFR/pcDNA3.1 constructs using Fugene HD reagent (Roche Diagnostics). Thirty-six hours after transfection, cells were serum starved for 12 h before stimulating with 50 ng/mL EGF for 5 min. HEK293 cells were transiently transfected with WT or mutant EGFRvIII/MigRI using Lipofectamine 2000 reagent (Invitrogen). Cells were then pelleted and used for biochemical analysis by Western blotting.

Cytokine-independent growth was studied by transducing Ba/F3 cells with mutant EGFR (in MIY) or EGFRvIII (in MigRI) constructs and analyzing cell growth on IL-3 withdrawal. A competitive growth assay was used to test the effect of EGF ligand on EGFR mutants. Briefly, Ba/F3 cells transduced with WT and mutant EGFRs (in MSCV-MIY) were cultured in 2 ng/mL IL-3. Cells were washed free of IL-3 and plated at a density of 0.25 million in 1 mL medium per well in a 24-well plate. Cells were either left untreated or treated with the indicated cytokine (2 ng/mL IL-3 or 50 ng/mL EGF). Preferential growth of EGFR-expressing cells (YFP positive) was measured by fluorescence-activated cell sorting (FACS) analysis.

For inhibitor analysis, cells (1×10^4 per well) were plated into 96-well plates, and inhibitors were added as indicated. Cell growth was measured at 48 h using the CellTiter96 Proliferation Assay (Promega) according to the manufacturer's instructions.

Western blotting and EGFR cell surface expression. Cells were lysed in lysis buffer containing 10 mmol/L Tris-HCl (pH 7.4), 5 mmol/L EDTA, 130 mmol/L NaCl, 1% Triton X-100, 20 mmol/L sodium phosphate (pH 7.5), 10 mmol/L sodium pyrophosphate (pH 7.0), 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L glycerolphosphate, and protease inhibitors (Roche Diagnostics). Samples were subjected to SDS-PAGE, and blotting was done on polyvinylidene fluoride membranes (Immobilon-P, Millipore). The following antibodies were used: phosphorylated EGFR-Tyr¹⁰⁶⁸ (Cell Signaling) and EGFR (Santa Cruz Biotechnology). Anti-phosphorylated signal transducer and activator of transcription 5 (Stat5; Tyr⁶⁹⁴) was obtained from Cell Signaling (New England Biolabs), and anti-Stat5 (G-2) was obtained from Santa Cruz Biotechnology. Rabbit polyclonal p44/42 mitogen-activated protein kinase [extracellular signal-regulated kinase (ERK) 1/ERK2] antibody and phosphospecific ERK1/ERK2 antibody were obtained from Cell Signaling. Other antibodies used were phosphospecific AKT antibody (Ser⁴⁷³) from Cell Signaling and goat polyclonal AKT1/2 from Santa Cruz Biotechnology. Bands were visualized using the enhanced chemiluminescence system (Amersham).

Cell surface expression of EGFR was analyzed by staining HEK293 cells with cetuximab. Forty-eight hours after transfection, 10^5 cells were washed with PBS and resuspended in FACS buffer (0.1% BSA in PBS). Cetuximab (1 μ g) was added to each sample for 30 min at 4°C. Cells were then washed twice with FACS buffer before adding Alexa Fluor 488 goat anti-human IgG (H+L) for 30 min at 4°C. Cells were then washed and analyzed by FACS.

Results

A panel of 30 EGFR kinase domain mutations that were recently reported in NSCLC patients was cloned and expressed for analysis of kinase activity, transforming potential, and drug sensitivity. These mutations affect the N-lobe (exons 18-20) and the C-lobe (exon 21) of the EGFR kinase domain as depicted in Fig. 1A.

Cloning and expression of a panel of EGFR mutations. NIH/3T3 cells are devoid of endogenous EGFR and were therefore used for the analysis of autokinase activity of overexpressed mutant EGFR proteins (Fig. 1B). Autophosphorylation of overexpressed WT EGFR on EGF stimulation was taken as a positive control. Most of the EGFR mutations analyzed showed little or no autophosphorylation without EGF ligand after serum starvation. Addition of EGF resulted in autophosphorylation in the majority of EGFR mutants, except EGFR-L688P, EGFR-V851A, EGFR-I853T, and EGFR-E866K (Fig. 1B). Normal cell surface expression of these mutants was detectable and comparable with that of WT EGFR (Supplementary Fig. S1). This indicated that the amino acid changes in these mutations abrogated the kinase activity of EGFR. Analysis of EGFR

mutants was done in a second cell line (HEK293) with similar results ruling out cell type-specific phenomena (data not shown). These data suggested that some EGFR mutants reported in NSCLC patients lack kinase activity and thus may neither contribute to tumor growth nor serve as a rational target for EGFR kinase inhibitors.

Mutations EGFR-L688P, EGFR-V851A, and EGFR-I853T are kinase defective and are not able to confer growth factor independence. To test more directly whether the mutations

EGFR-L688P, EGFR-V851A, and EGFR-I853T interfere with the kinase activity of EGFR, we cloned these mutations into the background of a constitutively active EGFR mutation (EGFRvIII) and expressed the resulting constructs in HEK293 cells that do not express endogenous EGFR (18). As a positive control, we introduced the well-characterized activating mutation G719S in the EGFRvIII backbone (19). Phosphorylation of WT EGFR and its downstream target Stat5 was seen on EGF ligand stimulation (Fig. 2A). EGFRvIII and EGFRvIII-G719S showed constitutive

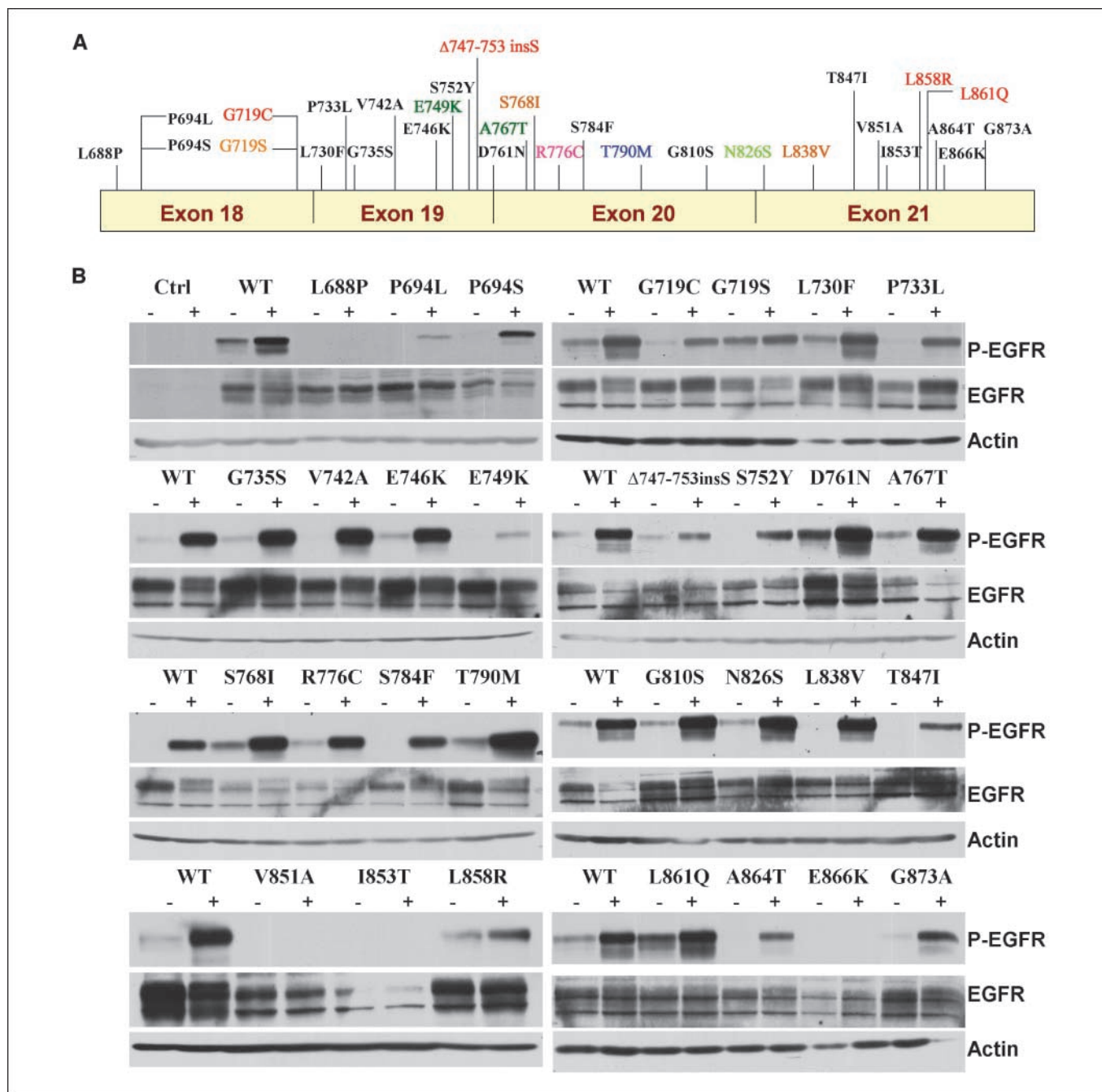


Fig. 1. Analysis of autophosphorylation identifies kinase domain mutations that have lost autokinase activity. **A**, all kinase domain mutations analyzed in this study are represented (not drawn to scale). Mutations were indicated according to the following references: red (1), black (4), orange (2), green (10), pink (3), brown (9), light green (8), and blue (6). **B**, NIH/3T3 cells transiently overexpressing WT or mutant EGFR were serum starved for 12 h before stimulating with EGF for 5 min. Autokinase activity was analyzed using phosphorylated EGFR (P-EGFR) antibody.

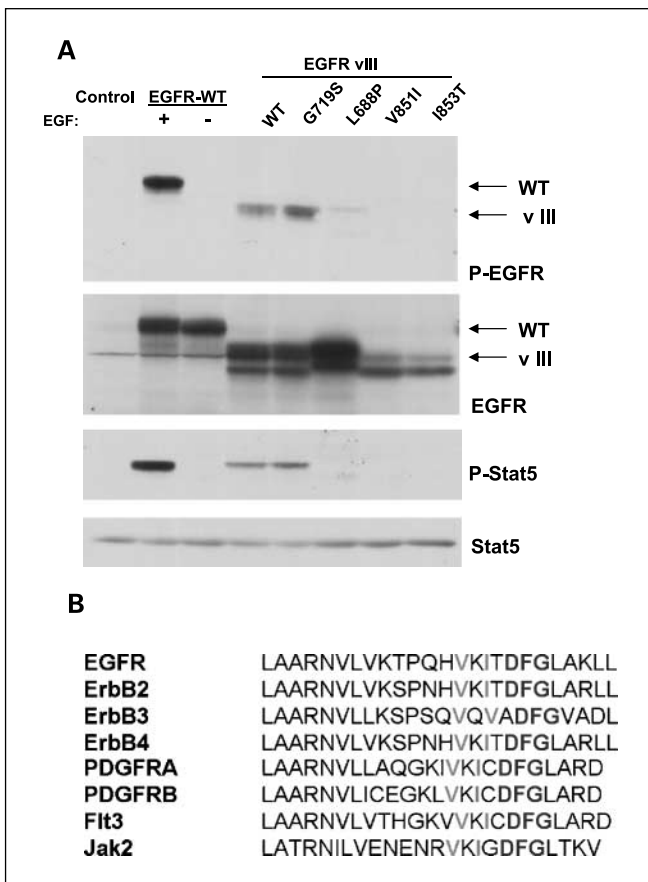


Fig. 2. Kinase-dead mutations abrogate oncogenic activity of EGFRvIII. **A**, indicated kinase domain mutations in the EGFRvIII backbone were transiently overexpressed in HEK293 cells and analyzed for autokinase activity. Because EGFRvIII has no extracellular ligand binding domain and thus cannot bind EGF, cells transfected with EGFRvIII constructs were neither serum starved nor stimulated with EGF. As a control, WT EGFR (full length) was transfected, serum starved for 12 h, and stimulated with human EGF for 5 min. Immunoblotting was done using indicated antibodies. **B**, part of the kinase domain containing the DFG motif from different kinases was aligned. Residues, which rendered EGFR kinase defective, are highlighted in red.

autophosphorylation and phosphorylation of Stat5. In contrast, mutations at L688P, V851A, and I853T largely reduced (L688P) or abrogated (V851A and I853T) the kinase activity of constitutively activated EGFRvIII as evidenced by phosphorylated EGFR and phosphorylated Stat5 levels (Fig. 2A). We then tested if the observed kinase-dead mutations would have any effect on the oncogenic potency of the constitutively active EGFRvIII. EGFRvIII and EGFRvIII-G719S induced IL-3-independent growth of Ba/F3 cells. In contrast, EGFRvIII-L688P, EGFRvIII-V851A, and EGFRvIII-I853T failed to induce growth factor-independent proliferation of Ba/F3 cells, suggesting that these point mutations indeed result in the loss of the oncogenic potential of EGFRvIII (Table 1). V851 and I853 are conserved among several receptor tyrosine kinases as shown in Fig. 2B and located in the near vicinity of the DFG motif, which is indispensable for kinase activity. It is interesting to note that these residues are conserved in all kinase-active receptors such as EGFR, Her2, and Her4 but absent in the kinase-defective receptor Her3. Thus, mutations in these conserved residues probably destroy the catalytic core of the EGFR. Identification

of EGFR mutations in NSCLC patients that abrogate kinase activity indicates that EGFR mutants in these cases do not serve as a molecular target for EGFR kinase inhibitors.

Oncogenic potential of EGFR mutations. Only a few activating mutations in the kinase domain of EGFR were tested for their potential to confer a growth advantage *in vitro* thus far (5, 7, 20, 21). Therefore, a panel of kinase domain mutations of EGFR was introduced into Ba/F3 cells and tested whether they are able to induce IL-3-independent growth (Table 2). As previously reported, EGFR-L858R and EGFR-G719S induced IL-3-independent growth in Ba/F3 cells (21). This was also observed for the majority of other EGFR mutations tested, indicating that these additional EGFR mutations lead to a growth advantage *in vitro*. In contrast, EGFR-L688P, EGFR-V851A, EGFR-I853T, and EGFR-E866K again failed to induce IL-3-independent growth, presumably due to the absent catalytic activity. In addition, EGFR-G719C also failed to induce IL-3-independent growth. In addition, we tested selected EGFR mutations for their ability to confer growth in the presence of EGF ligand (Supplementary Fig. S2). As expected, cells expressing EGFR-WT and the constitutive activated EGFR-G719S grew in the presence of EGF, whereas cells expressing kinase-dead EGFR-E866K failed to grow. Interestingly, also cells expressing EGFR-G719C did not grow in the presence of EGF. This mutant is kinase active as shown in Fig. 1B and the reason for its lacking growth potential is unclear at the moment.

Biochemical analysis of the transformed cell lines showed constitutive autophosphorylation of the EGFR mutants and revealed activation of key prosurvival and proliferation pathways, such as ERK, Stat5, and AKT, in all cell lines tested (Fig. 3). Ba/F3-EGFR-WT showed activation of EGFR and downstream key signaling molecules only on stimulation with EGF ligand (Fig. 3).

Sensitivity toward EGFR kinase inhibitors varies significantly between different activating EGFR mutations. The small-molecule EGFR kinase inhibitors gefitinib and erlotinib were shown to be effective in inhibiting the most frequent activating mutations EGFR-L858R and EGFR- Δ 747-753insS (1). However, there is growing experimental and clinical evidence that erlotinib and gefitinib may show differential activity toward specific EGFR activating and resistance mutations (5, 22, 23). We therefore tested the sensitivity of a comprehensive panel of transforming EGFR mutations toward the EGFR inhibitors gefitinib, erlotinib, and AEE788 (Fig. 4A). Again, we used Ba/F3 cells as readout because these cells do not express endogenous

Table 1. Oncogenic ability of EGFRvIII mutants

Mutation	Growth factor independence
EGFRvIII	Yes
EGFRvIII + G719S	Yes
EGFRvIII + L688P	No
EGFRvIII + V851A	No
EGFRvIII + I853T	No

NOTE: Ba/F3 cells stably expressing WT or mutant EGFRvIII were analyzed for cytokine-independent growth on IL-3 withdrawal and followed up to 3 wk for survival.

Table 2. IC₅₀ values of EGFR mutants for the indicated EGFR inhibitors

Mutation	Exon	Growth factor independence of Ba/F3	IC ₅₀ value (nmol/L)		
			Gefitinib	Erlotinib	AEE788
L858R	21	Yes	12	6	6
Δ747-753insS	19	Yes	7	5	5
L688P	18	No	—	—	—
G719C	18	No	—	—	—
G719S	18	Yes	68	16	13
V742A	19	Yes	65	21	37
D761N	19	Yes	104	75	37
S768I	20	Yes	315	250	125
R776C	20	Yes	110	47	36
S784F	20	Yes	193	95	43
T790M	20	Yes	>2,000	>2,000	>2,000
G810S	20	Yes	96	57	30
N826S	21	Yes	505	505	348
L838V	21	Yes	187	160	70
V851A	21	No	—	—	—
I853T	21	No	—	—	—
L861Q	21	Yes	170	103	51
A864T	21	Yes	75	49	40
E866K	21	No	—	—	—

NOTE: Cellular IC₅₀ values for gefitinib, erlotinib, and AEE788 for various EGFR mutants were calculated using the data from Fig. 4.

EGFR and none of the drugs showed toxicity against these cells at concentrations of up to 2 μmol/L (Fig. 4B-D). EGFR-L858R and EGFR-Del 747-753insS were extremely sensitive to all three kinase inhibitors with IC₅₀ values in the low nanomolar range with no significant differences in IC₅₀ values between gefitinib, erlotinib, and AEE788. In contrast, EGFR-T790M was completely resistant to all drugs tested with IC₅₀ values >2 μmol/L. This mutation is already known to be associated with kinase inhibitor resistance (Fig. 4B-D; ref. 6). In addition, EGFR-N826S required high concentrations of all three EGFR kinase inhibitors of ~500 nmol/L for complete inhibition. Interestingly, EGFR-N826S was detected in a NSCLC patient who did not respond to gefitinib treatment and this lack of response might be explained by the high IC₅₀ value (8). All other EGFR

mutations showed IC₅₀ values for the kinase inhibitors tested in the range of 10 to 300 nmol/L (Fig. 4B-D; Table 2). Several EGFR mutations displayed varying IC₅₀ values depending on the kinase inhibitor. EGFR-G719S was relatively more resistant to gefitinib (IC₅₀ = 68 nmol/L) than EGFR-L858R (IC₅₀ = 12 nmol/L) and this finding is in line with a recent report (19). However, EGFR-G719S was very sensitive to erlotinib (IC₅₀ = 16 nmol/L) and AEE788 (IC₅₀ = 13 nmol/L), comparable with EGFR-L858R (IC₅₀ for erlotinib and AEE788 = 6 nmol/L). Another frequent mutation EGFR-L861Q was quite insensitive to both gefitinib and erlotinib with IC₅₀ values above 100 nmol/L but with a lower IC₅₀ value for AEE788 (IC₅₀ = 51 nmol/L). Similarly, mutations EGFR-V742A, EGFR-R776C, and EGFR-S784F were more sensitive to erlotinib than gefitinib,

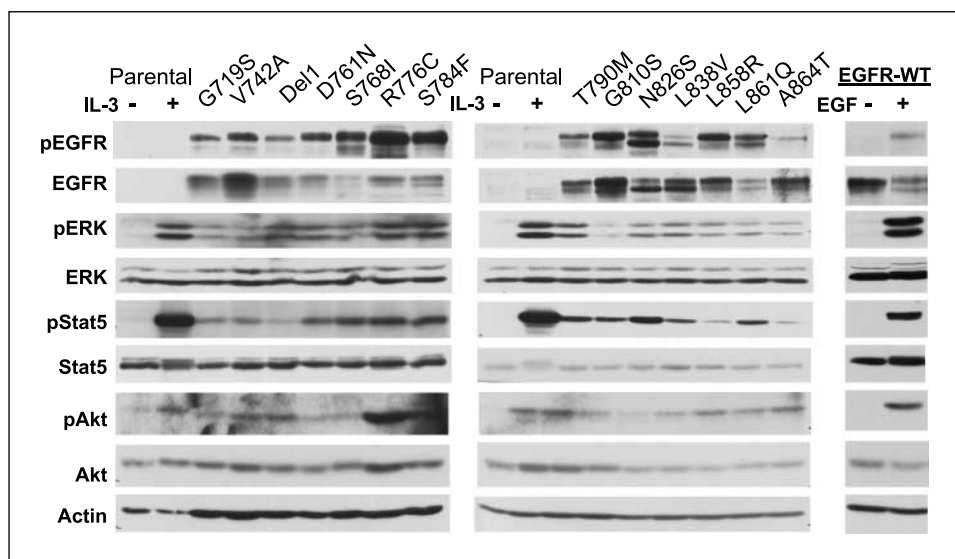


Fig. 3. Mutations in the EGFR kinase domain activate prosurvival and proliferation pathways. Ba/F3 cell lines stably expressing mutant EGFRs were established and analyzed. Ba/F3 cells stably expressing WT EGFR were serum starved for 12 h and left unstimulated or stimulated for 5 min with 50 ng/mL EGF before analysis. Activation of prosurvival and proliferation pathways was done by immunoblotting using the indicated antibodies. As a control, parental Ba/F3 cells were grown in the absence of IL-3 for 12 h before stimulating with IL-3 for 1 h before analysis.

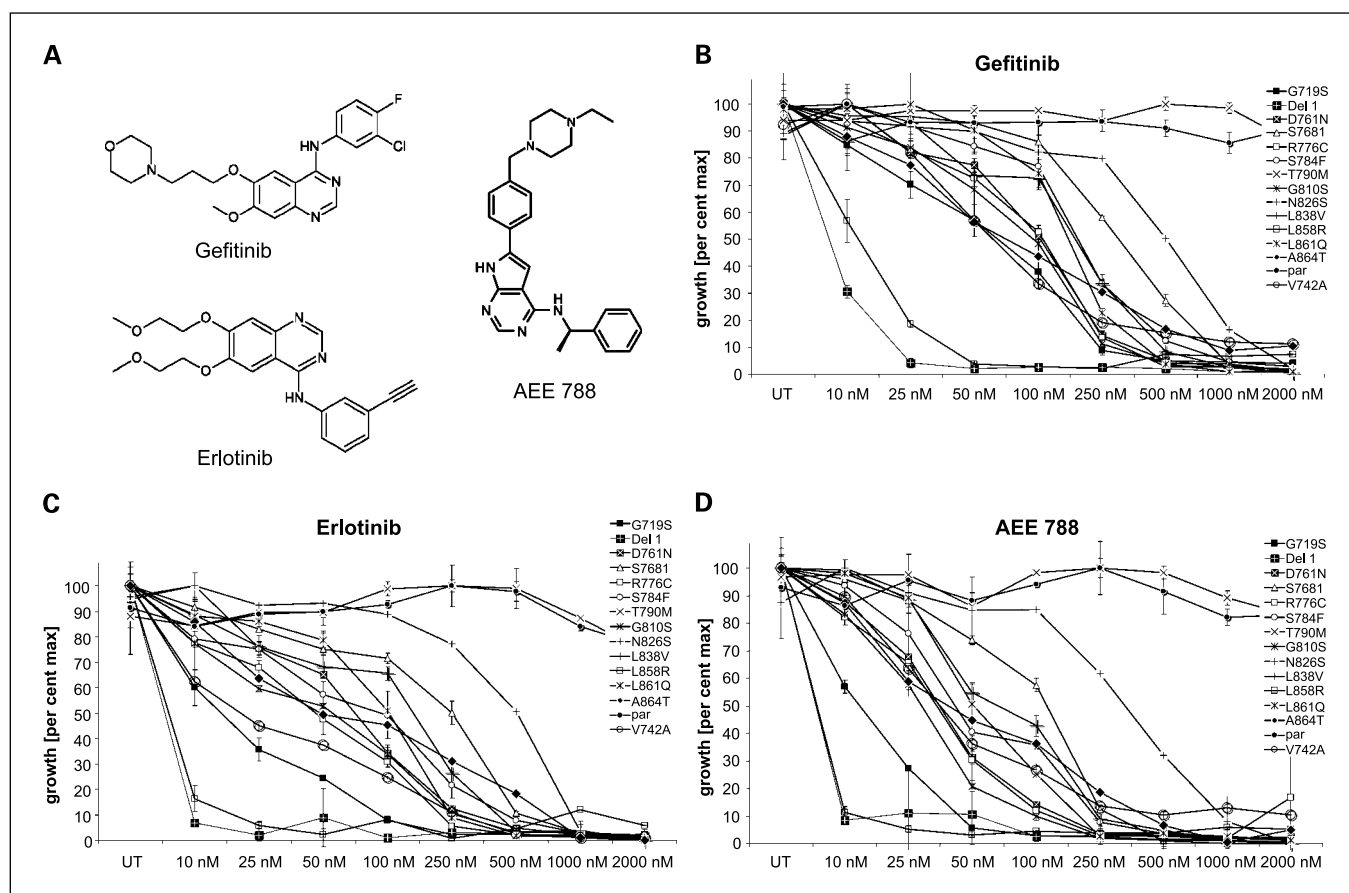


Fig. 4. Sensitivity toward tyrosine kinase inhibitors varies between different EGFR mutants. **A**, chemical structures of the kinase inhibitors used in this study. Parental Ba/F3 cells and cells stably overexpressing EGFR mutants were incubated with increasing concentrations of gefitinib (**B**), erlotinib (**C**), and AEE788 (**D**). Cell viability was determined after 48 h. Data are presented as percentage of untreated cells and represent values \pm SD of triplicates. One representative of at least two independent experiments is shown.

with at least two times lower IC_{50} values. EGFR mutations EGFR-D761N, EGFR-S768I, EGFR-S784F, and EGFR-L838V were more sensitive to AEE788 compared with both gefitinib and erlotinib (Table 2). Gefitinib and erlotinib are anilinoquinazolines, whereas AEE788 is a pyrrolopyrimidine compound (Fig. 4A). These differences in the chemical structures may account for the distinct responses observed with all three drugs. Thus, the sensitivity of activating EGFR mutations toward different EGFR kinase inhibitors varies significantly and this may have implications for the sequential and potential combinational use of this compound in EGFR-mutated NSCLC patients.

Discussion

Identification of activating mutations in the kinase domain of EGFR in NSCLC patients that sensitize the receptor to small-molecule kinase inhibitors led to retrospective analysis of several clinical trials to confirm a correlation between EGFR mutational status and treatment response to kinase inhibitors such as erlotinib and gefitinib. One of the largest studies conducted identified several novel EGFR mutations but failed to find a correlation of mutational status and treatment response and thus concluded that the mutational status of

EGFR in NSCLC patients is not a predictive factor for erlotinib response (4). Hence, molecular analysis to predict treatment response was not recommended. Numerous additional studies conducted with both gefitinib and erlotinib produced conflicting results about the mutational status as a predictive factor of drug responsiveness (4, 8, 24, 25). It is important to note that most of the less frequent mutations reported were not functionally characterized thus far, making it difficult to draw meaningful conclusions from correlative studies comprising only the mutational status but not the type of mutation. Moreover, additional EGFR mutations were reported that do not sensitize but cause resistance toward EGFR inhibitors, making correlative studies even more complicated (5, 7, 20, 22, 26). Differences in sequencing techniques, interpretation of the results, and probably potential differences in the sensitivity of EGFR mutations toward different EGFR inhibitors sparked a controversy about whether EGFR sequencing analysis has an important role in guiding clinical use of EGFR inhibitors in NSCLC patients (27, 28).

Therefore, we aimed to do a comprehensive analysis of a large panel of published EGFR mutations with respect to kinase activity, transforming potential, and sensitivity toward different EGFR kinase inhibitors. Surprisingly, 4 of 30 EGFR mutations studied were defective in kinase activity even after EGF

stimulation. Because EGFR kinase activity is indispensable for the activation of oncogenic signaling pathways, it seems unlikely that these kinase-dead mutations contribute to tumor development. However, a recent study has reported that inhibition of EGFR kinase activity alone does not result in cytotoxicity in tumor cells (29). Kinase-defective EGFR expression was sufficient to maintain basal glucose levels and tumor cell survival. Thus, kinase-dead mutations identified in patient samples may have a role in tumor maintenance. In any case, however, usage of EGFR inhibitors will have no effect.

The kinase-dead mutation EGFR-V851A was identified in a large retrospective study, which concluded that there is no correlation between EGFR mutation status and response to erlotinib treatment (4). Interestingly, an EGFR mutation with a different exchange at the same position (EGFR-V851A) has been reported in two patients who were not responsive to gefitinib (30, 31). Because V851 is critical for the catalytic activity of EGFR, this mutation may not contribute to tumor growth and survival in these cases. It was suggested that the identification of novel EGFR mutations in NSCLC patients may result from PCR artifacts due to the use of formalin-embedded tissue in some cases (27). Such artifacts include C→T/G→A and A→G/T→C transitions (28), which are present in the kinase-dead mutations identified in this study (4). On the other hand, EGFR-V851A was independently reported in patients by different investigators (30, 31). The reason for the detection of kinase-dead EGFR mutation in NSCLC is unclear at the moment and these mutations may also present so-called passenger or bystander mutations as reported previously in lung cancer (32, 33). Nevertheless, the variability of kinase activity and sensitivity to EGFR kinase inhibitors may in part be responsible for the discrepancies between clinical studies aiming to correlate mutational status and drug response.

Sensitivity toward different kinase inhibitors can vary significantly between individual activating and resistance mutations, as it has been shown for Bcr-Abl, c-Kit, or Flt-3 (14, 34). This prompted us to establish drug sensitivity profiles for a comprehensive panel of EGFR mutations toward three EGFR kinase inhibitors. From these studies, we have identified four sets of mutations based on their drug sensitivity profiles: (a) mutations that are very sensitive to all three drugs tested with IC₅₀ values in the low nanomolar range (L858R and Del 747-753insS), (b) mutations that are less sensitive to gefitinib (IC₅₀ > 100 nmol/L) but sensitive to both erlotinib and AEE788 (G719S, V742A, and R776C; IC₅₀ < 100 nmol/L), (c) mutations that are less sensitive to both gefitinib and erlotinib but sensitive to AEE788 (D761N, S768I, S784F, L838V, and

L861Q), and (d) mutations that are resistant to all three drugs tested (N826S and T790M).

Does such a dose-response profile for EGFR mutations have any effect on the clinical management of NSCLC and do differences of IC₅₀ values below or above 100 nmol/L constitute a clinically significant difference? With both gefitinib and erlotinib, mean plasma concentrations well above 1 μmol/L can be achieved, and this is well above the *in vitro* concentrations at which most of the EGFR mutants can effectively be inhibited. However, mean plasma concentrations do not provide information about drug concentration within a tumor cell and whether the EGFR target is efficiently inhibited. Two recent articles describe secondary EGFR mutations in gefitinib- and erlotinib-resistant patients. In one article, it was shown that erlotinib treatment could overcome gefitinib resistance in a NSCLC patient caused by a EGFR-L858R+L747S mutation (22). *In vitro* IC₅₀ values for this mutant were 200 and 80 nmol/L for gefitinib and erlotinib, respectively. Similarly, in a second article, erlotinib resistance caused due to an EGFR-L858R+E884K mutation could be overcome by gefitinib treatment. Again, *in vitro* data suggested IC₅₀ differences in the 100 nmol/L range (23). This indicates that in a clinical setting, IC₅₀ values for a particular EGFR mutant above and below 100 nmol/L might well be important for whether a patient responds to EGFR kinase inhibitor treatment and that *in vitro* sensitivity profiles could be used to improve treatment strategies.

In summary, our results suggest that not all EGFR mutations reported to date are of pathophysiologic relevance for NSCLC development and maintenance and underscore the need of functional characterization of every new EGFR mutation discovered in NSCLC patients as it has been done in other malignancies such as chronic myelogenous leukemia.

Identification of comprehensive drug resistance profiles opens the opportunity to test alternative EGFR inhibitors *in vitro* such as AEE788. Other recent studies have shown that irreversible inhibitors of EGFR kinase were effective to overcome the resistance caused by reversible inhibitors such as gefitinib and erlotinib (20). Such preclinical investigations will undoubtedly accelerate the development of second-generation EGFR kinase inhibitors. However, as it is the case for chronic myelogenous leukemia, several resistance mechanisms, including the switch to alternate oncogenic pathways, will add complexity to the resistance issue (35).

Disclosure of Potential Conflicts of Interest

J. Duyster, consultant, Novartis.

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