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## Acquired Resistance to EGFR Kinase Inhibitors Associated with a Novel T854A Mutation in a Patient with *EGFR*-Mutant Lung Adenocarcinoma

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### Abstract

**Purpose**—Somatic mutations in the tyrosine kinase domain of *EGFR* are associated with sensitivity of lung adenocarcinomas to the EGFR tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib. Acquired drug resistance is frequently associated with a secondary somatic mutation that leads to substitution of methionine for threonine at position 790 (T790M). We aimed to identify additional second-site alterations associated with acquired resistance.

**Experimental Design**—Tumor samples were obtained from 48 patients with acquired resistance. Tumor cell DNA was analyzed for *EGFR* kinase domain mutations. Molecular analyses were then performed to characterize biological properties of a novel mutant EGFR allele.

**Results**—A previously unreported mutation in exon 21 of *EGFR*, which leads to substitution of alanine for threonine at position 854 (T854A), was identified in one patient with a drug-sensitive *EGFR* L858R-mutant lung adenocarcinoma after long-term treatment with TKIs. The T854A mutation was not detected in a pretreatment tumor sample. Crystal structure analyses of EGFR suggest that the T854 side chain is within contact distance of gefitinib and erlotinib. Surrogate kinase assays demonstrate that the *EGFR* T854A mutation abrogates inhibition of tyrosine phosphorylation by erlotinib. Such resistance appears to be overcome by a new irreversible dual EGFR/HER2 inhibitor, BIBW 2992.

**Conclusions**—The T854A mutation is the second reported second-site acquired resistance mutation that is within contact distance of gefitinib and erlotinib. These data suggest that acquired

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**Statement of Translational Relevance:** The EGFR tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib, induce dramatic prolonged tumor responses in patients with EGFR mutant lung adenocarcinomas, but acquired resistance to the drugs invariably develops over time. Mechanisms of secondary resistance most commonly involve second-site EGFR mutations. Here, through molecular analysis of tumor cells from patients with acquired resistance, we report identification of a novel second-site EGFR T854A mutation. We also show that a second-generation EGFR inhibitor, BIBW2992, has potential to overcome acquired resistance mediated by this change. These data may facilitate the development of strategies to treat patients whose EGFR mutant lung cancers no longer respond to existing TKIs.

resistance to ATP-mimetic EGFR kinase inhibitors may often be associated with amino acid substitutions that alter drug contact residues in the EGFR ATP-binding pocket.

## Introduction

Lung adenocarcinomas sensitive to the epidermal growth factor receptor (EGFR) inhibitors, gefitinib and erlotinib, often harbor somatic mutations in exons encoding the tyrosine kinase domain of EGFR (1-3). Nearly 90% of these mutations occur as either multi-nucleotide in-frame deletions in exon 19 that eliminate four amino acids (LREA), or as single missense mutations that result in substitution of arginine for leucine at position 858 (L858R).

Unfortunately, patients with drug-sensitive *EGFR* mutations whose tumors initially respond to gefitinib or erlotinib eventually develop acquired resistance (4,5). In about half of cases, tumors biopsied after disease progression contain a second site mutation in the EGFR kinase domain (6-10). The most common (>90%) alteration involves a C→T change at nucleotide 2369 in exon 20, which results in substitution of methionine for threonine at position 790 (T790M). This substitution is analogous to the BCR-ABL T315I change found in patients with chronic myelogenous leukemias that have developed acquired resistance to imatinib (11-13). Based upon crystal structure analyses, the EGFR T790M substitution was predicted to impair binding of either gefitinib or erlotinib to the EGFR ATP-binding pocket (14). However, recent evidence suggests that the change could alter the relative affinity of ATP versus drug in the ATP-binding pocket (15).

Here we report identification of a second-site *EGFR* mutation, T854A, in a patient with *EGFR*-mutant lung adenocarcinoma treated with EGFR inhibitors for more than two years.

## Materials and Methods

### Tissue procurement

Tumor specimens were obtained through protocols approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center. All patients and/or families provided written informed consent.

### DNA sequencing

Genomic DNA was extracted from tumor specimens, and primers for *EGFR* (exons 18-24) analyses were as published (3). PCR-RFLP assays for exon 19 deletions, and L858R and T790M missense mutations were performed as published (7,16). All mutations were confirmed at least twice from independent PCR isolates, and forward and reverse sequence tracings were visually inspected.

### Functional analyses of EGFR T854A

Two numbering systems are used for *EGFR*. The first denotes the initiating methionine in the signal sequence as amino acid -24. The second, used here, denotes the methionine as amino acid +1. Mutations were introduced into full-length wildtype and L858R *EGFR* cDNAs using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and cloned into the expression vector, pcDNA3.1 (-) as described (3). The following primers were used to generate the T854A mutation: T854A F: 5'-TGTCAAGATCGCAGATTTTGG-3'; T854A R: 5'-CCAAAATCTGCGATCTTGACA-3'. The generation of the *EGFR* L858R T790M cDNA was previously described (7).

## Immunoblotting

See methods and supplementary methods in (3) for details on transient transfection of 293T cells, cell lysis, immunoblotting, and antibody reagents. The following antibodies were used: polyclonal rabbit anti-phospho-EGFR (Y<sup>1092</sup>) (Cell Signaling, #2234) and monoclonal mouse anti-total EGFR (BD Biosciences Pharmigen, #610017); and rabbit polyclonal anti-total actin (Sigma #A2066). At least two independent experiments were performed for all analyses.

## Cell growth inhibition assays

PC-9, H3255, and H1975 cells were grown in RPMI supplemented with 10% FBS and seeded into 96-well plates in sextuplicate at a density of  $4 \times 10^4$  cells/ml. Twenty-four hours after seeding, the cells were treated with various concentrations of erlotinib or BIBW 2992 for a total of 72 hours. Growth inhibition assays were performed with the CellTiter-Blue™ cell viability kit (Promega, Madison, WI), as per manufacturer's instructions. Levels of growth inhibition were calculated according to the CellTiter-Blue™-emitted fluorescence at 530nm (ex)/590nm (em), using a Fluoroskan Ascent FL plate reader (Thermo Electron Corporation, Waltham, MA). All curves were normalized to a DMSO-only control. All assays were performed three independent times and representative curves are shown. GI<sub>50</sub> values were calculated using BioDataFit 1.02<sup>1</sup>. Ba/F3 experiments were performed as previously published (9).

## Results

We previously reported analysis of tumor samples from a total of 21 patients with acquired resistance to gefitinib or erlotinib (7,9). To extend these data, we have obtained tumor materials from an additional 27 patients with lung adenocarcinomas that resumed growth after an initial response to the EGFR TKIs, erlotinib or gefitinib. Genomic DNA isolated from the tumor specimens was subjected to *EGFR* PCR-RFLP assays and sequencing of exons encoding the tyrosine kinase domain of EGFR. Of a total of 48 samples screened, all have harbored primary drug-sensitive mutations in *EGFR*: either a deletion in exon 19 or an exon 21 L858R missense mutation (data not shown). Twenty-five of the 48 samples (52%; C.I. 37%-67%) were found to contain a second site mutation in the *EGFR* kinase domain. Twenty-three (92%) of these 25 specimens contained the T790M mutation. One tumor we previously reported to have a D761Y mutation (9). One specimen from a separate patient, whose case report is detailed below, was found to harbor a novel second site mutation.

## Case report

The patient was a 69 year-old female former smoker who underwent a left upper lobectomy for a pathologic Stage IIB (T2N1) poorly differentiated adenocarcinoma. The patient did not receive adjuvant chemotherapy.

Thirteen months later, the patient underwent complete resection of a left frontal brain mass. The lesion was histologically similar to the primary lung tumor. Following resection, the patient began treatment with “adjuvant” temozolomide.

One month later, a bone scan showed increased radiotracer uptake within multiple ribs. The patient received gefitinib and a subsequent bone scan demonstrated resolution of the bone lesions. She remained on gefitinib for more than two years until the drug was discontinued following an episode of pneumonia.

<sup>1</sup><http://www.changbioscience.com/stat/ec50.html>

Two years later, a surveillance computed tomography scan revealed new lesions in the lungs, mediastinum, and pleura. The patient started treatment with erlotinib, but two months later developed severe thrombocytopenia. Erlotinib and temozolomide were discontinued. The patient's thrombocytopenia eventually resolved.

Five months later (more than five years after her original lung resection), imaging studies revealed multiple lesions in bone, mediastinum, and lung, as well as a new small left-sided pleural effusion. Biopsy of an iliac lesion documented metastatic lung adenocarcinoma. The brain tumor tissue was tested for *EGFR* mutations using PCR-based assays (16) and found to have the exon 21 L858R mutation. The patient underwent palliative radiation to spinal metastases and was re-started on erlotinib. Within a month, she was hospitalized due to worsening malignant left pleural effusion. A pleural catheter was placed, and pleural fluid cells were collected for DNA sequencing. Multiple imaging studies revealed progressive disease. The patient died one month later. No autopsy was performed.

### Identification of a novel *EGFR* T854A mutation

Sequencing of DNA extracted from pleural fluid cells revealed the presence of a heterozygous drug-sensitive *EGFR* L858R missense mutation (T→G at nucleotide 2573) in exon 21 and an additional peak at nucleotide 2560, representing a heterozygous A→G mutation also in exon 21 (Figure 1). This latter change leads to a substitution of alanine for threonine at position 854 (T854A).

The height of the additional peak at nucleotide 2560 was the same as the mutant G peak at nucleotide 2573, suggesting that both mutations were on the same allele. To investigate this possibility further, we amplified genomic DNA encompassing *EGFR* exon 21 from pleural fluid, cloned the PCR products, and analyzed 65 individual colonies for mutations (data not shown). Sequencing chromatograms of DNA from five clones showed both the 2560 A→G and 2573 T→G mutations. Nine clones showed only the L858R mutation, while the remaining 51 clones showed wildtype sequence. No clones showed the T854A mutation alone. These data confirm that both mutations were on the same allele.

Direct sequencing of DNA from the pre-treatment brain tumor specimen showed the L858R mutation but not the T854A mutation (Figure 1). No other mutations were found in *EGFR* exons 18-24 or in *KRAS* exon 2 in either the pre- or post-treatment specimens.

### Biochemical and physiological properties of *EGFR* T854A

To determine how the T854A amino acid change might affect wildtype and mutant L858R *EGFR*, we generated mutant *EGFR* alleles (3). Corresponding proteins (wildtype, T854A, L858R, L858R plus T854A) were then produced by transient transfection with expression vectors in 293T cells, which have very low levels of endogenous *EGFR*. Lysates from cells were analyzed by immunoblotting as previously described (7). As a surrogate gauge of kinase activity, we measured the levels of “autophosphorylated” tyrosine-1092 on *EGFR* in relation to levels of total *EGFR* protein, using densitometry. Addition of T854A to wildtype protein or the *EGFR* L858R mutant did not appear to alter appreciably baseline properties (Figure 2 and data not shown).

We next examined whether the T854A change would affect the sensitivity of wildtype or L858R *EGFR* to erlotinib. The TKI progressively inhibited the surrogate kinase activity of wildtype and L858R *EGFR*, as demonstrated by a reduction of Y<sup>1092</sup>-phosphorylated protein with increasing concentrations of drug (Figure 2). Corresponding mutants containing the T854A change displayed an obvious decrease in sensitivity (Figure 2). However, this difference was not as dramatic as that conferred by the T790M mutation, which we previously showed

abrogated inhibition of EGFR tyrosine autophosphorylation at gefitinib or erlotinib concentrations up to 10 micromolar (7). Consistent with these data, growth inhibition assays using transfected Ba/F3 cells demonstrated that cells harboring cDNAs encoding EGFR L858R plus T854A were ~3-fold less sensitive to erlotinib compared to those harboring EGFR L858R alone, while cells harboring L858R plus T790M were >100-fold less sensitive vs those with L858R alone (data not shown).

### Location of the EGFR T854A mutation

To support the interpretation of putative kinase domain mutations, we previously created a mutation interpretation tool for tyrosine kinases (TKs), called “Mutagrator”<sup>2</sup> (17), which takes curated mutation data from the literature and displays it in the context of a master protein (chosen by the user) and a protein-registered TK multiple domain alignment. Using this tool, we found that T854 in EGFR is not well-conserved among kinases (Figure 3A). Moreover, a mutation analogous to T854A has not been reported in other tyrosine kinases, even among kinases (e.g. ABL, KIT, PDGFR) that develop second-site mutations after prolonged exposure to another kinase inhibitor, imatinib (Figure 3A).

Crystal structure analyses of EGFR indicates that T854 is at the “bottom” of the ATP-binding site, on the C-lobe (18) (Figure 3B). The T854 side chain is within contact distance of gefitinib in the active structure as well as lapatinib in the inactive structure (19).

### Sensitivity of T854A to an irreversible EGFR kinase inhibitor, BIBW 2992

We next tested the sensitivity of the T854A mutant against BIBW 2992, a promising new irreversible dual EGFR and HER2 tyrosine kinase inhibitor (Figure 4A). Enzymatic assays using recombinant human wildtype EGFR and HER2 indicate that the concentrations of drug needed to inhibit 50% activity (IC<sub>50</sub>) are 0.5 and 14 nM, respectively (20, 21). In our own in vitro cellular assays, the concentration of BIBW 2992 needed to inhibit the growth by 50% (GI<sub>50</sub>) of PC-9 cells, a lung cancer cell line with a drug-sensitive exon 19 deletion (E746-A750), was ~0.4 nM. H3255 cells, a lung cell line with a drug-sensitive exon 21 L858R mutation, were inhibited with a GI<sub>50</sub> value of ~0.5 nM (Figure 4B). By contrast, erlotinib was significantly less potent against these cells, with GI<sub>50</sub> values of ~10 nM for PC-9 and ~99 nM for H3255 cells. H1975 cells, a lung cancer cell line with both a drug-sensitive L858R mutation and a second-site resistance mutation, T790M, were completely resistant to inhibition by erlotinib at concentrations up to 1 μM (GI<sub>50</sub> ~10 μM) but were sensitive to BIBW 2992 (GI<sub>50</sub> ~100 nM) (Figure 4B).

Consistent with the cellular data, surrogate kinase assays demonstrated that BIBW 2992 inhibited the activity of wildtype and L858R EGFR in the 1-10 nanomolar range (Figure 4C). Furthermore, BIBW 2992 was able to overcome the resistance conferred by both T790M and T854A. The drug inhibited the activity of both L858R plus T790M EGFR and L858R plus T854A EGFR in the 10-100 nanomolar range (Figure 4C). By contrast, nanomolar concentrations of erlotinib were unable to completely overcome the resistance conferred by T854A to L858R EGFR (Figure 2, Figure 4D).

## Discussion

We report the identification of a novel second-site exon 21 *EGFR* mutation, T854A, in tumor cells from a patient with *EGFR* mutant lung adenocarcinoma and acquired resistance to EGFR TKIs. The patient initially received gefitinib for more than two years. After a drug hiatus during which the disease progressed, she was rechallenged with erlotinib, which had no effect.

<sup>2</sup><http://cbio.mskcc.org/~lash/mutagrator/>

Consistent with the notion of the T854A mutation being associated with acquired resistance, the T854A mutation was found in her post-treatment pleural fluid cells but not in her pre-treatment lesion. Biochemical and physiological evidence suggest that the T854A mutation reduces sensitivity of L858R mutant EGFR to EGFR TKIs by ~3-fold.

Previously identified second-site *EGFR* resistance mutations found in lung adenocarcinomas after TKI treatment include the common T790M mutation (accounting for >90% of second-site *EGFR* mutations and 50% of TKI-resistant EGFR-mutant lung tumors) (6-10), D761Y (9), and L747S (22). The T790M mutation occurs at a critical “gatekeeper” residue in the ATP-binding pocket of EGFR, analogous to the T315I mutation in imatinib-resistant BCR-ABL (12). This change in EGFR was thought to impair binding of erlotinib or gefitinib (14), but recent data suggests that it may alter the binding affinity of drug versus ATP (15). The T790M mutation by itself has also been shown to increase kinase activity and oncogenic potential (23), and its expression in mouse lung epithelia can induce the formation of lung adenocarcinomas (24). The D761Y mutation (9), by contrast, is predicted to occur in the  $\alpha$ -C-helix of EGFR, adjacent to residues involved in the formation of a salt bridge that interacts with  $\alpha$ - and  $\beta$ -phosphates when ATP is bound (14). While mutations within the  $\alpha$ -C-helix of other kinases have been associated with acquired resistance to other TKIs, such as D276G in BCR-ABL (25), no resistance mutation has been reported at the analogous residue in ABL. How the D761Y mutation affects the EGFR kinase domain remains to be determined. Finally, the L747S mutation occurs at the start of the loop between the  $\beta$ 3 strand and the  $\alpha$ -C-helix (14,22). L747 lies toward the rear of the catalytic cleft, and mutations in the analogous residue of ABL1 (L273M) and ErbB2 (L755S/P) have been detected in imatinib-resistant CML and untreated gastric, breast, and lung tumors, respectively (26-28).

The T854 residue, located at the “bottom” of the ATP-binding site on the C-lobe, is not conserved among other kinases, and no analogous mutations have yet been reported. Notably, the side chain of T854 is within contact distance of erlotinib/gefitinib in the active structure (14,18) and lapatinib in the inactive structure (19). Thus, the T854A substitution could result in loss of contacts and thus binding affinity to these inhibitors. Although the T854 side chain is not within contact distance of bound ATP, it is possible that it affects ATP binding, but this remains to be established (N. Pavletich, personal communication). Another possibility is that the T854A mutation causes a local conformational change in the kinase. Consistent with this, recent work using a cell-based *in vitro* random mutagenesis screen to identify *EGFR* mutations that confer resistance to the irreversible EGFR kinase inhibitor, CL-387,785, found novel mutations at 14 residues in EGFR (29). One mutation identified, H773L, occurs at a residue known to form a hydrogen bond with the carbonyl oxygen at the adjacent V851 residue (29).

While this analysis was being performed, another group identified the T854A change in an EGFR resistance mutation screen with erlotinib (30). Similar to our data, that study showed that Ba/F3 cells expressing EGFR L858R plus T854A were 3.3-fold less sensitive to drug than cells expressing EGFR L858R alone. This *in vitro* work further supports the notion that the T854A change found in the patient we describe constitutes a bona fide resistance mutation to EGFR TKIs.

While the T854A mutation confers a substantial degree of resistance to erlotinib, our work here using surrogate kinase assays suggests that a novel irreversible EGFR kinase inhibitor, BIBW 2992, can overcome such resistance at nanomolar concentrations. Presumably, the covalent binding of BIBW 2992 to Cys 797 in EGFR is not strongly disturbed by the T854A change. Further, while there could be an effect on ATP binding by T854A, the fact that the T854 side chain is not within contact distance of bound ATP suggests that any effect on ATP affinity is not strong enough to offset binding of the irreversible inhibitor. Since the T854A change is less resistant to BIBW 2992 than the T790M mutation, the former mutation could have less (if

any) effect on ATP-binding affinity than the latter. BIBW 2992 thus appears to have potential for overcoming acquired resistance to gefitinib or erlotinib that is mediated by second-site kinase domain mutations.

Developing new therapeutic strategies to overcome acquired resistance to EGFR TKIs remains a challenge and a priority for the treatment of *EGFR* mutant lung cancers. Identifying all the mechanisms by which tumors develop resistance to these drugs, whether by second-site mutations or by activation of other kinases such as MET (31,32), is of utmost importance for the success of future targeted therapies against *EGFR* mutant lung cancers.

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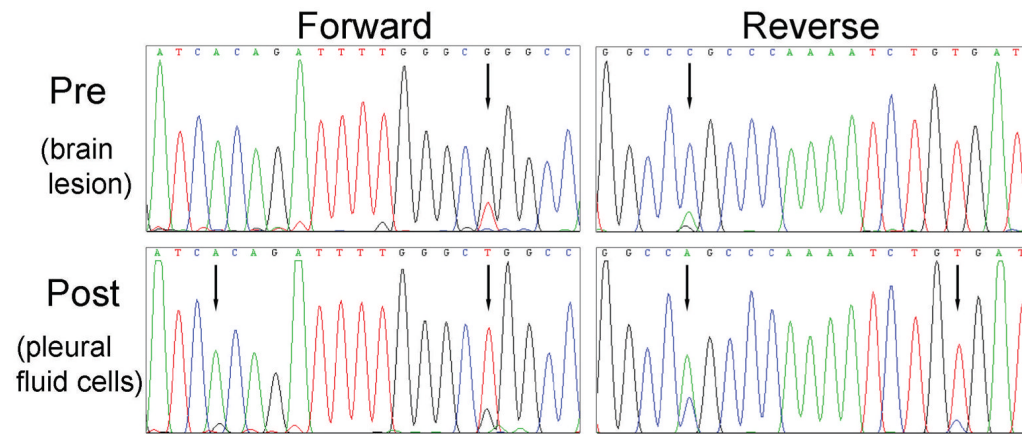
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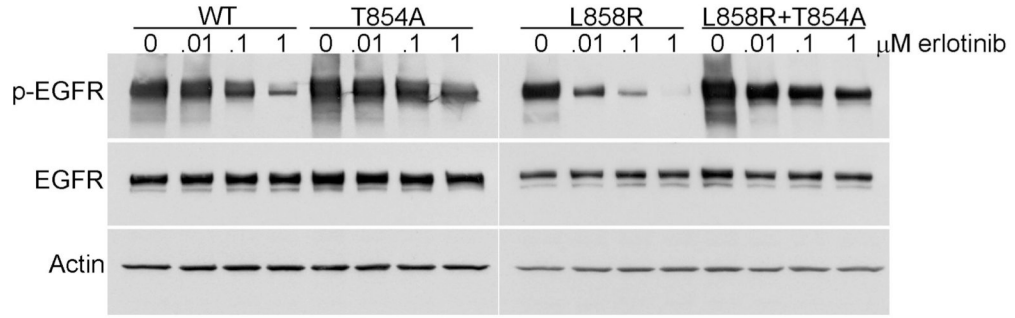


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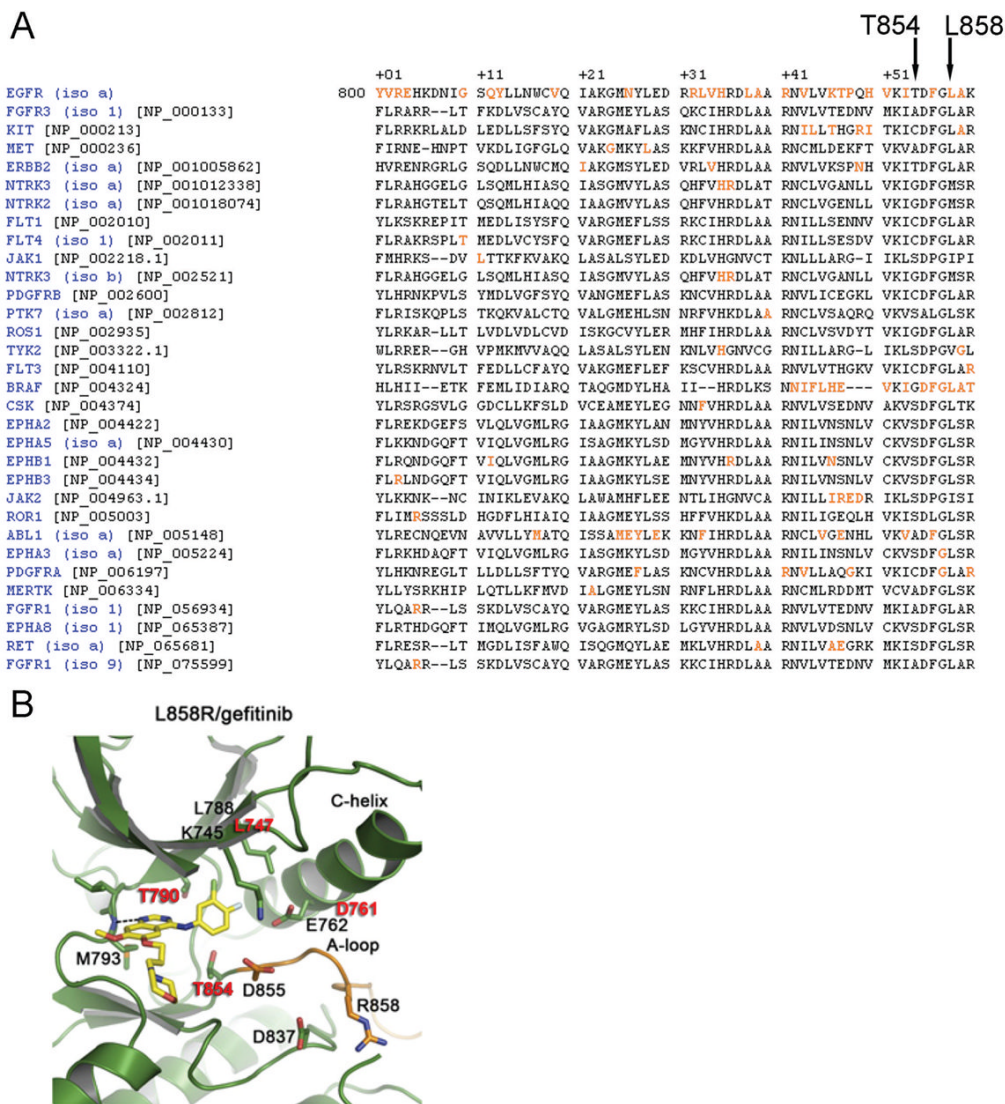


**Figure 1. Identification of a novel *EGFR* mutation in a patient with acquired resistance to *EGFR* inhibitors**

Sequencing chromatograms showing presence of the *EGFR* T854A mutation along with the L858R mutation in pleural fluid cells collected from the index patient after prolonged treatment with gefitinib and erlotinib. Only the L858R mutation was detected in a pretreatment metastatic brain tumor specimen.



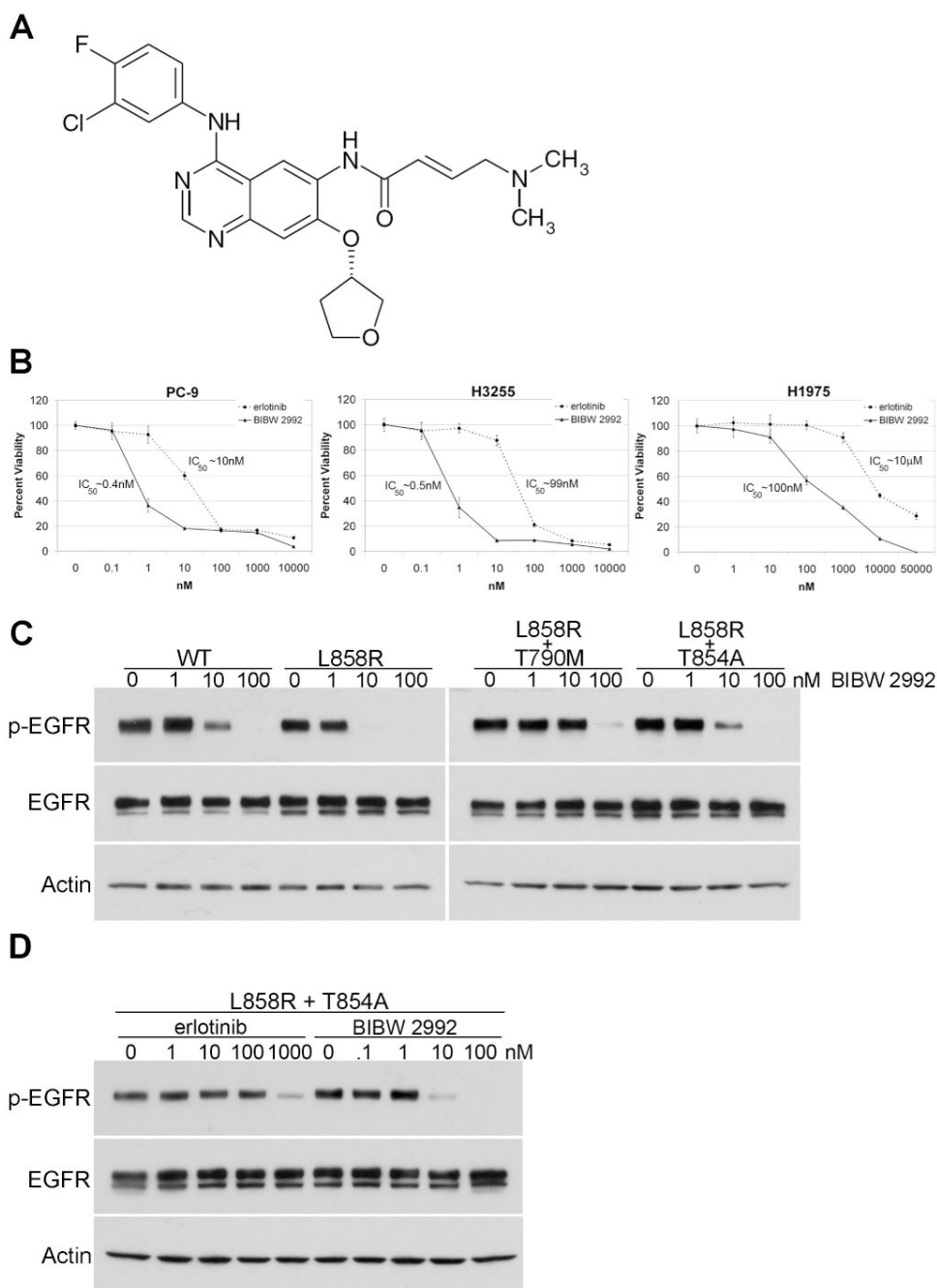
**Figure 2. T854A mutation reduces inhibition of autophosphorylation of EGFR by erlotinib**  
Lysates from 293T cells transiently transfected with cDNAs encoding either EGFR WT, T854A, L858R, or L858R plus T854A and treated with different concentrations of erlotinib were immunoblotted for phospho-EGFR (Y<sup>1092</sup>), total EGFR, and Actin. T854A reduced the degree of inhibition of phospho-EGFR by erlotinib relative to WT or L858R EGFR. Actin is shown as a loading control.



**Figure 3. T854A mutation is not analogous to other known kinase mutations and is located at a drug contact site**

**A)** Alignment of the kinase domain of EGFR with other tyrosine kinases adapted from the Mutagrator Tool<sup>3</sup> reveals no other identified mutations (highlighted in orange) at analogous residues. Overall the EGFR T854 position is not highly conserved among other kinases. **B)** Crystal structure of the L858R EGFR mutant bound to gefitinib (adapted from (18)) reveals the T854 residue is at the “bottom” of the ATP-binding site, on the C-lobe. Residues with known mutations associated with acquired resistance in patients are shown in red.

<sup>3</sup><http://cbio.mskcc.org/~lash/mutagrator/>



**Figure 4. BIBW 2992 more potently inhibits both sensitive and resistant *EGFR* mutants, including T854A**

**A)** Chemical structure of BIBW 2992. Adapted from (21). **B)** Cell growth inhibition assays of three *EGFR* mutant cell lines treated with various doses of erlotinib (dashed line) or BIBW 2992 (solid line). PC-9 cells have a drug-sensitive exon 19 deletion in *EGFR*, H3255 cells have the drug-sensitive L858R mutation, and H1975 cells have both L858R and the T790M resistance mutation. All plots are relative to a DMSO-only control and error bars indicate one standard deviation from six replicates. All assays were performed three independent times and representative plots are shown. IC<sub>50</sub> values were calculated using BioDataFit 1.02. **C)** Lysates from 293T cells transiently transfected with cDNAs encoding either *EGFR* WT, L858R, L858R

plus T790M, or L858R plus T854A and treated with different concentrations of BIBW 2992 were immunoblotted for phospho-EGFR, total EGFR, and actin. Actin is shown as a loading control. **D)** Lysates from 293T cells transiently transfected with *L858R* plus *T854A EGFR* cDNA and treated with different concentrations of erlotinib or BIBW 2992 were immunoblotted for phospho-EGFR, total EGFR, and actin.