Acquired Resistance to Epidermal Growth Factor Receptor Kinase Inhibitors Associated with a Novel T854A Mutation in a Patient with \textit{EGFR}-Mutant Lung Adenocarcinoma

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Abstract

Purpose: Somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (\textit{EGFR}) gene are associated with sensitivity of lung adenocarcinomas to the \textit{EGFR} tyrosine kinase inhibitors, gefitinib and erlotinib. Acquired drug resistance is frequently associated with a secondary somatic mutation that leads to the 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 \textit{EGFR} kinase domain mutations. Molecular analyses were then done to characterize the biological properties of a novel mutant \textit{EGFR} allele.

Results: A previously unreported mutation in exon 21 of \textit{EGFR}, which leads to substitution of alanine for threonine at position 854 (T854A), was identified in one patient with a drug-sensitive \textit{EGFR} L858R\textendash{}mutant lung adenocarcinoma after long-term treatment with tyrosine kinase inhibitors. The T854A mutation was not detected in a pretreatment tumor sample. The crystal structure analyses of \textit{EGFR} suggest that the T854 side chain is within contact distance of gefitinib and erlotinib. Surrogate kinase assays show that the \textit{EGFR} T854A mutation abrogates the inhibition of tyrosine phosphorylation by erlotinib. Such resistance seems to be overcome by a new irreversible dual \textit{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 resistance to ATP-mimetic \textit{EGFR} kinase inhibitors may often be associated with amino acid substitutions that alter drug contact residues in the \textit{EGFR} ATP-binding pocket.

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

Unfortunately, patients with drug-sensitive \textit{EGFR} mutations whose tumors initially respond to gefitinib or erlotinib eventually develop acquired resistance (4, 5). In about half of the cases, tumors biopsied after disease progression contain a second-site mutation in the \textit{EGFR} kinase domain (6–10). The most common (>90\%) alteration involves a C\textendash{}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 on crystal structure analyses, the \textit{EGFR} T790M substitution was predicted to impair the binding of either gefitinib or erlotinib to the \textit{EGFR} ATP-binding pocket (14). However, recent evidence suggests that the change could alter the relative affinity of ATP versus drug (15).

Here, we report the identification of a second-site \textit{EGFR} mutation, T854A, in a patient with \textit{EGFR}-mutant lung adenocarcinoma treated with \textit{EGFR} inhibitors for \textgreater{}2 years.
Translational Relevance

The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, 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. The mechanisms of secondary resistance most commonly involve second-site EGFR mutations. Here, through the molecular analysis of tumor cells from patients with acquired resistance, we report the 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 tyrosine kinase inhibitors.

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 done 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 -2. The second, used here, denotes the methionine as amino acid +1. Mutations were introduced into full-length wild-type and L858R EGFR cDNAs using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) and cloned into the expression vector, pcDNA3.1(+) as described (3). The following primers were used to generate the T854A mutation: T854A forward, 5′-TGTCAAGATCG-CAGATTITGC-3′ and T854A reverse, 5′-CCAAAATCTGCGATCTTGACA-3′. The generation of the EGFR L858R T790M cDNA was previously described (7).

Immunoblotting. See Methods and Supplementary Methods in Pao et al. (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 (Y1068; Cell Signaling), monoclonal mouse anti-total EGFR (BD Biosciences Pharmingen), and rabbit polyclonal anti-total actin (Sigma). At least two independent experiments were done for all analyses.

Cell growth inhibition assays. PC-9, H3255, and H1975 cells were grown in RPMI supplemented with 10% fetal bovine serum and seeded into 96-well plates in sextuplicate at a density of 4 × 10³ cells/mL. Twenty-four hours after seeding, the cells were treated with various concentrations of erlotinib or BIBW2992 for a total of 72 h. Growth inhibition assays were done with the CellTiter-Blue cell viability kit (Promega), as per manufacturer’s instructions. Levels of growth inhibition were calculated according to the CellTiter-Blue–emitted fluorescence at 530 nm (ex)/590 nm (em), using a Fluoroskan Ascent FL plate reader (Thermo Electron Corporation). All curves were normalized to a DMSO-only control. All assays were done three independent times, and representative curves are shown. Values to inhibit the growth by 50% (GI₅₀) were calculated using BioDataFit 1.02.3 Ba/F3 experiments were done as previously published (9).

Results

We previously reported the 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 tyrosine kinase inhibitors, 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%; confidence interval, 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 IIIB (T₃N₁) 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 showed resolution of the bone lesions. She remained on gefitinib for >2 years until the drug was discontinued following an episode of pneumonia.

Two years later, a surveillance computed tomography scan revealed new lesions in the lungs, mediastinum, and pleura. The patient started treatment with erlotinib but developed severe throbocytopenia 2 months later. Erlotinib and temozolomide were discontinued. The patient’s thrombocytopenia eventually resolved.

Five months later (>5 years after her original lung resection), imaging studies revealed multiple lesions in the 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 restarted on erlotinib. Within a month, she was hospitalized because of a worsening malignant left pleural effusion. A pleural catheter was placed, and pleural fluid cells were

http://www.changbioscience.com/stat/ec50.html
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 (Fig. 1). This latter change leads to 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 the 2560 A → G and 2573 T → G mutations. Nine clones showed only the L858R mutation, whereas the remaining 51 clones showed wild-type sequence. No clones showed the T854A mutation alone. These data confirm that both mutations were on the same allele.

The direct sequencing of DNA from the pretreatment brain tumor specimen showed the L858R mutation but not the T854A mutation (Fig. 1). No other mutations were found in EGFR exons 18 to 24 or in KRAS exon 2 in either the pre- or posttreatment specimens. The primary lung lesion was unavailable for analysis.

Biochemical and physiologic properties of EGFR T854A. To determine how the T854A amino acid change might affect wild-type and mutant L858R EGFR, we generated mutant EGFR alleles (3). Corresponding proteins (wild-type, 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 wild-type protein or the EGFR L858R mutant did not seem to alter appreciably baseline properties (Fig. 2 and data not shown).

We next examined whether the T854A change would affect the sensitivity of wild-type or L858R EGFR to erlotinib. The tyrosine kinase inhibitor progressively inhibited the surrogate kinase activity of wild-type and L858R EGFR, as shown by a reduction of Y1092-phosphorylated protein with increasing concentrations of drug (Fig. 2). Corresponding mutants containing the T854A change displayed an obvious decrease in sensitivity (Fig. 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 μmol/L (7). Consistent with these data, growth inhibition assays using transfected Ba/F3 cells showed that cells harboring cDNAs encoding EGFR L858R plus T854A were ~3-fold less sensitive to erlotinib compared with those harboring EGFR L858R alone, whereas cells harboring L858R plus T790M were >100-fold less sensitive versus those with L858R alone (data not shown).

Identification of a novel EGFR T854A mutation. To support the interpretation of putative kinase domain mutations, we previously created a mutation interpretation tool for tyrosine kinases, called Mutagrator (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 tyrosine kinase multiple domain alignment. Using this tool, we found that T854 in EGFR is not well conserved among kinases (Fig. 3A). Moreover, a mutation analogous to T854A has not been reported in other tyrosine kinases, even among kinases (e.g., ABL, KIT, PDGFR) associated with second-site mutations after prolonged exposure to another kinase inhibitor, imatinib (Fig. 3A).

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

Fig. 1. Identification of a novel EGFR exon 21 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.
EGFR and HER2 tyrosine kinase inhibitor (Fig. 4A). Enzymatic assays using recombinant human wild-type EGFR and HER2 indicate that the IC\textsubscript{50} values are 0.5 and 14 nmol/L, respectively (20, 21). In our own in \textit{vitro} cellular assays, the concentration of BIBW 2992 needed to inhibit the growth by 50\% (GI\textsubscript{50}) of PC-9 cells, a lung cancer cell line with a drug-sensitive exon 19 deletion (E746-A750), was \~{}0.4 nmol/L. H3255 cells, a lung cell line with a drug-sensitive exon 21 L858R mutation, were inhibited with a GI\textsubscript{50} value of \~{}0.5 nmol/L (Fig. 4B). By contrast, erlotinib was significantly less potent against these cells, with GI\textsubscript{50} values of \~{}10 nmol/L for PC-9 and \~{}99 nmol/L for H3255 cells. H1975 cells, a lung cancer cell line with a drug-sensitive L858R mutation and a second-site resistance mutation, T790M, were completely resistant to inhibition by erlotinib at concentrations up to 1 \mu mol/L (GI\textsubscript{50}, \~{}10 nmol/L; Fig. 4B).

Consistent with the cellular data, surrogate kinase assays showed that BIBW 2992 inhibited the activity of wild-type and L858R EGFR in the 1 to 10 nmol/L range (Fig. 4C). Furthermore, BIBW 2992 was able to overcome the resistance conferred by both T790M and T854A. The drug inhibited the activity of L858R plus T790M EGFR and L858R plus T854A EGFR in the 10 to 100 nmol/L range (Fig. 4C). By contrast, nanomolar concentrations of erlotinib were unable to completely overcome the resistance conferred by T854A to L858R EGFR (Figs. 2 and 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 tyrosine kinase inhibitors. The patient initially received gefitinib for >2 years. After a drug hiatus during which the disease progressed, she was rechallenged with erlotinib, which had no effect. Consistent with the notion of the T854A mutation being associated with acquired resistance, the T854A mutation was found in her posttreatment pleural fluid cells but not in her pretreatment lesion. Biochemical and physiologic evidence suggest that the T854A mutation reduces sensitivity of L858R-mutant EGFR to EGFR tyrosine kinase inhibitors by \~{}3-fold.

Previously identified second-site EGFR resistance mutations found in lung adenocarcinomas after tyrosine kinase inhibitor treatment include the common T790M mutation (accounting for \~{}90\% of second-site EGFR mutations and 50\% of tyrosine kinase inhibitor–resistant EGFR-mutant lung tumors; refs. 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). Although mutations within the \(\alpha\)-C-helix of other kinases have been associated with acquired resistance to other tyrosine kinase inhibitors, 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 chronic myelogenous leukemia 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 or gefitinib in the active structure (14, 18) and lapatinib in the inactive structure (19). 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. Another possibility is that the T854A mutation causes a local conformational change in the kinase. Consistent with this, recent work using a cell-based \textit{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 done, 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 tyrosine kinase inhibitors.

Fig. 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 reveals no other identified mutations (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 ref. 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. Adopted with permission from Cancer Cell, Vol 11, C.-H. Yun, T.J. Boggon, Y.Li et al. Structures of lung cancer-derived mutants and inhibitor complexes: Mechanism of activation and insights into differential inhibitor sensitivity, pp 209–11, copyright 2007, with permission from Elsevier.

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Although 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. Furthermore, although 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. Because 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 seems 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 tyrosine kinase inhibitors 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 the 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.

**Disclosure of Potential Conflicts of Interest**

V. Miller has received honoraria from Abbott, Boehringer-Ingelheim, Genentech, Genmab, and Imclone; V. Miller and G. Riely are consultants for Genentech and Boehringer-Ingelheim; V. Miller is also a consultant for Abbott, Genmab, and Imclone; and G. Riely is also a consultant for Hoffman-LaRoche. The rights to a patent application on the testing of EGFR T790M mutations have been licensed by MSKCC on behalf of V. Miller, W. Pao, and others to Molecular MD.
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