Novel D761Y and Common Secondary T790M Mutations in Epidermal Growth Factor Receptor–Mutant Lung Adenocarcinomas with Acquired Resistance to Kinase Inhibitors

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Abstract

Purpose: In patients whose lung adenocarcinomas harbor epidermal growth factor receptor (EGFR) tyrosine kinase domain mutations, acquired resistance to the tyrosine kinase inhibitors (TKI) gefitinib (Iressa) and erlotinib (Tarceva) has been associated with a second-site *EGFR* mutation, which leads to substitution of methionine for threonine at position 790 (T790M). We aimed to elucidate the frequency and nature of secondary *EGFR* mutations in patients with acquired resistance to TKI monotherapy.

Experimental Design: Tumor cells from patients with acquired resistance were examined for secondary *EGFR* kinase domain mutations by molecular analyses.

Results: Eight of 16 patients (50% observed rate; 95% confidence interval, 25-75%) had tumor cells with second-site *EGFR* mutations. Seven mutations were T790M and one was a novel D761Y mutation found in a brain metastasis. When combined with a drug-sensitive L858R mutation, the D761Y mutation modestly reduced the sensitivity of mutant EGFR to TKIs in both surrogate kinase and cell viability assays. In an autopsy case, the T790M mutation was found in multiple visceral metastases but not in a brain lesion.

Conclusions: The T790M mutation is common in patients with acquired resistance. The limited spectrum of TKI-resistant mutations in *EGFR*, which binds to erlotinib in the active conformation, contrasts with a wider range of second-site mutations seen with acquired resistance to imatinib, which binds to ABL and KIT, respectively, in closed conformations. Collectively, our data suggest that the type and nature of kinase inhibitor resistance mutations may be influenced by both anatomic site and mode of binding to the kinase target.

Somatic activating mutations in exons encoding the epidermal growth factor receptor (EGFR) tyrosine kinase domain are found in ~10% and 25% of non–small-cell lung cancers (NSCLC) from the United States and East Asia, respectively (1). Nearly 90% of these mutations occur as either multinucleotide in-frame deletions in exon 19 or as single missense mutations that result in substitution of arginine for leucine at position 858 (L858R). Both mutations are associated with increased sensitivity to the selective EGFR kinase inhibitors gefitinib and erlotinib (2–4). Our initial analysis suggests that patients whose tumors harbor EGFR exon 19 deletion mutations have a longer median survival than those with EGFR L858R point mutation (5).

Patients with drug-sensitive *EGFR* mutations, whose tumors initially respond to gefitinib or erlotinib, develop acquired resistance after a median of about 12 months (5). We previously reported that in two of five NSCLC patients with such acquired resistance, tumors biopsied after disease progression contained a second-site mutation in the EGFR kinase domain, in addition to a drug-sensitive mutation (6). This C→T mutation at nucleotide 2,369 in exon 20 leads to substitution of methionine for threonine at position 790 (T790M). Whereas the amino acid

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change does not impair the catalytic activity of EGFR, it is predicted to block binding of either gefitinib or erlotinib to the EGFR ATP-binding domain. Others have reported similar findings collectively in drug-resistant tumors from five patients (7–10). Certain cases of inherited susceptibility to lung cancer may be associated with the T790M mutation (11), although somatic T790M mutations in patients who have never received gefitinib or erlotinib are very rare (6).

In this study, we aimed to elucidate the frequency and nature of secondary *EGFR* mutations in 16 more patients who progressed on either gefitinib or erlotinib monotherapy. Other exploratory studies were done to uncover additional potential mechanisms of acquired resistance to EGFR tyrosine kinase inhibitors (TKI).

Materials and Methods

Tissue procurement. Tumor specimens, including paraffin blocks, biopsies, and pleural effusions, were obtained through protocols approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center (protocols 92-055, 02-010, and 04-103). All patients provided informed consent. In total, 17 patients with acquired resistance were evaluated, but DNA from one patient was insufficient for analysis.

Mutational analyses. Genomic DNA was extracted from tumor specimens and primers for *EGFR* (exons 18-24) analyses were as published (4). PCR-RFLP assays for exon 19 deletions and L858R and T790M missense mutations were done as published (6, 12). All mutations were confirmed at least twice from independent PCR isolates and sequence tracings were reviewed in the forward and reverse directions by visual inspection.

Reverse transcription-PCR. *EGFR* cDNA was generated and examined as published (6).

Functional analyses of EGFR D761Y. 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 the full-length mutant EGFR L858R using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and cloned into the expression vector, pcDNA3.1(−), as described (4). The following primers were used to generate the D761Y mutation: D761Y F, 5′-GGAAATCCTCTATGAAGCCTA-3′; D761Y R, 5′-TAGGCTTCATAGAGGATTTCC-3′. The generation of the *EGFR L858R + T790M* cDNA was previously described (6).

Immunoblotting. See methods and supplementary methods in ref. 4 for details on transient transfection of 293T cells, cell lysis, immunoblotting, and antibody reagents. At least three independent experiments were done for all analyses.

Generation of EGFR mutant-expressing Ba/F3 cells and growth inhibition analyses. Ba/F3 cells (a kind gift of Dr. C. Antonescu, Memorial Sloan-Kettering Cancer Center, New York, NY) were maintained in RPMI supplemented with 10% fetal bovine serum, 10 ng/mL interleukin-3 (IL-3; R&D Systems, Minneapolis, MN), and 10 units/mL penicillin and 10 μg/mL streptomycin, at 37°C and 5% CO_2. To generate polyclonal cell populations, Ba/F3 cells were electroporated (Amaxa, Gaithersburg, MD) with 2 μg each of the following plasmids: pcDNA3.1(−) empty vector, pcDNA3.1(−)-*EGFR L858R*, pcDNA3.1(−)-*EGFR L858R + D761Y*, and pcDNA3.1(−)-*EGFR L858R + T790M*. Following selection in 1.25 mg/mL G418 (Invitrogen, Carlsbad, CA) for 12 days in the presence of 10 ng/mL IL-3, G418-resistant cells were subjected to a second round of selection in the absence of IL-3 for 5 days and then maintained in the presence of 10 ng/mL IL-3 for further study.

Ba/F3 growth inhibition assays were done with the CellTiter-Blue cell viability kit (Promega, Madison, WI) as per instructions of the manufacturer. Briefly, Ba/F3 transfectants were washed with PBS twice and resuspended in RPMI medium containing 10% fetal bovine serum and 40 ng/mL EGF (Cell Signaling Technology, Danvers, MA). Cells were then seeded into 96-well plates in triplicate at a density of 100,000 per well and treated with various concentrations of EGFR inhibitors for 48 or 72 hours. Cell viability was 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, Waltham, MA). All assays were done at least three independent times.

EGFR amplification analysis by chromogenic* in situ *hybridization. Chromogenic *in situ* hybridization for *EGFR* was done according to the instructions of the manufacturer (Zymed Laboratories, Inc., South San Francisco, CA). Briefly, slides were incubated at 55°C overnight and then deparaffinized in xylene and graded ethanols. Heat pretreatment was carried out in the pretreatment buffer (Zymed Laboratories) at 98°C to 100°C for 15 minutes. Tissue was digested with pepsin for 10 minutes at room temperature. After application of Zymed SpotLight digoxigenin-labeled *EGFR* probe (Zymed Laboratories), the slide was coverslipped and edges sealed with rubber cement. The slide was heated at 95°C for 5 minutes followed by overnight incubation at 37°C using a moisturized chamber. Posthybridization washes were done the next day, followed by immunodetection using the chromogenic *in situ* hybridization polymer detection kit (Zymed Laboratories). Signal enumeration was done on a standard light microscope using a 40× objective. The total gene copy numbers in 30 tumor cell nuclei were counted for each sample, and the average gene copy number per nucleus was used as the chromogenic *in situ* hybridization result. The results of chromogenic *in situ* hybridization were interpreted as follows: <5 gene copies per nucleus, no amplification; 5 to 10 gene copies per nucleus, low-level amplification; and >10 gene copies per nucleus, high-level amplification.

Results

High prevalence of secondary EGFR T790M mutations in tumors from patients with acquired resistance to gefitinib or erlotinib. Clinical characteristics of patients studied are described in Table 1. All patients had initial radiographic response to TKI monotherapy. Tumor cells were obtained from individuals after documented disease progression on EGFR TKI, and direct Sanger sequencing analysis of *EGFR* exons 18 to 24 (which encode the kinase domain) was done. In addition, for all samples (except case no. 5; see footnote, Table 1), tumor DNA was screened for exon 19 deletions and L858R and T790M missense mutations by more sensitive PCR-RFLP assays, which can detect these specific mutations in specimens where DNA from mutant cell lines comprises only 5% to 10% of the entire sample (6, 12). Patients were on EGFR TKI treatment for a median of 13 months (range, 7-28 months) before documented progression. Patients were rebiopsied shortly after (range, 0-4 months) progression of disease.

Tumors from 7 of the 16 (44%) patients were found to have the previously reported T790M missense mutation; 5 of 7 (71%) were found in conjunction with an exon 19 deletion and 2 of 7 (43%) with the L858R missense mutation. In four cases of T790M-mediated resistance where there was adequate tissue pre and post drug treatment, the T790M mutation was not detected in the pretreatment specimen. This finding is consistent with the notion that a population of tumor cells harboring the T790M mutation emerged after treatment with TKI. One patient's tumor cells had a different second-site mutation (see below). No additional kinase domain mutations were detected in tumors from eight patients. The median

Table 1. Characteristics of NSCLC patients examined with acquired resistance to single-agent gefitinib or erlotinib

Case	Sex	Smoking	Drug	Duration (mo)	Prior chemo	Prior RT	Tumor sites examined	Primary mutation	Secondary mutation
1	M	Never	G	13	Y	Y	Lung	del L747-S752	T790M
2	M	Oligo	G	11	Y	Y	Omentum	del E746-A750	T790M
3	F	Never	G	15	Y	N	Lung, pericardial fluid	del L747-P753insS	T790M
4	F	Never	E	10	N	N	Pleural fluid	del E746-T751insA	T790M
5	F	Oligo	→E*	n/a	Y	N	Lung	del E746-A750	T790M†
6	F	Never	G	15	N	N	Lung	L858R	T790M
7	F	Never	G	13	N	N	Lung	L858R	T790M‡
8	F	Never	G	13	Y	Y	Brain	L858R	D761Y
9	F	Oligo	G	19	Y	N	Ascites	del E746-A750	None
10	F	Never	G	8	N	N	Pleura	del E746-A750	None
11	F	Never	E	10	Y	N	Lung	del E746-A750	None
12	M	Never	E	9	N	N	Pleural fluid	del E746-A750	None
13	F	Oligo	G	7	Y	N	Cervix	del‡	None
14	M	Former	G	12	Y	Y	Inguinal lymph node	del‡	None
15	F	Oligo	G	28	N	N	Lung	del‡	None
16	M	Never	G→E	19	Y	Y	Pleural fluid	L858R	None

NOTE: Smoking, smoking history; never, smoked <100 cigarettes in a lifetime; oligo, smoked 100 or more cigarettes but <15 pack-years total; former, smoked >15 pack-years and quit <1 year before diagnosis of lung cancer. Drug, gefitinib (G) or erlotinib (E). Duration, months on drug until documented progression. Prior chemo, patient did (Y) or did not (N) receive prior systemic chemotherapy. Prior RT, patient did (Y) or did not (N) receive prior radiation therapy to non-CNS sites. Mutation, amino acids affected in EGFR; primary indicates drug-sensitive mutation; del, exon 19 deletion; secondary indicates drug-resistant mutation. For all tumor specimens, exons 18 to 24 of *EGFR*, which encode the tyrosine kinase domain, were examined by direct Sanger sequencing; DNA was additionally examined for exon 19 deletions and the L858R and T790M missense mutations by more sensitive PCR-RFLP assays. None, no mutation observed.

*Patient was initially on a clinical trial involving ZD6474 versus gefitinib for nearly 12 months; following disease progression, she received erlotinib for another 6 months until further disease progression.

†Mutation analysis reported by Genzyme Genetics.

‡Mutation detected by PCR-RFLP only.

time-to-progression in patients with and without a second-site *EGFR* mutation seemed to be similar (13 versus 11 months), and there seemed to be no correlation with prior chemotherapy or radiation therapy.

Identification of a novel EGFR D761Y mutation in a metastatic brain lesion. In one TKI-resistant tumor, a novel second-site *EGFR* kinase domain mutation was identified (case 8, Table 1). The patient, a 72-year-old female East Asian "never smoker" with stage IV NSCLC involving pleura, liver, and bone, was initially treated with chemotherapy and radiation to the spine. She then had a partial radiographic response to gefitinib, but progressed 13 months later with tumor growth in the right pleura. Shortly thereafter, she developed aphasia and was found to have a large left frontal lobe mass by magnetic resonance imaging of the brain. This mass was resected and pathology showed metastatic NSCLC.

Analysis of DNA from the brain tumor revealed the presence of a dominant heterozygous drug-sensitive L858R missense mutation (T→G at nucleotide 2,573) in exon 21 and an additional peak at nucleotide 2,281, suggesting a heterozygous G→T mutation at the 3′-end of exon 19 (Fig. 1). This mutation leads to substitution of tyrosine for aspartic acid at position 761 (D761Y) and is predicted to occur in the α-C-helix of EGFR (Fig. 2A). The height of the additional peak at nucleotide 2,281 was the same as the mutant G peak at nucleotide 2,573, suggesting that both mutations were on the same allele. To

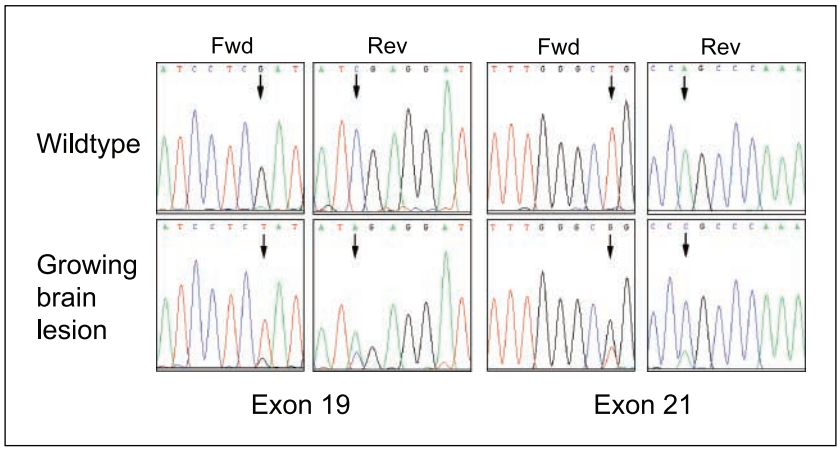

Fig. 1. Sequencing chromatograms with the *EGFR* D761Y exon 19 and L858R exon 21 mutations detected in a metastatic brain lesion. In the pretreatment pleural biopsy specimen, the L858R mutation was detected only by a more sensitive PCR-RFLP technique (data not shown).

investigate this possibility further, we did reverse transcription-PCR on mRNA derived from an independent fragment of the same brain tumor specimen to generate cDNAs that spanned exons 19 through 21. PCR products were then cloned and 20 individual colonies were analyzed for *EGFR* mutations. Sequencing chromatograms of DNA from 13 of 13 clones showed both the 2281G→T and 2573T→G mutations, confirming that both mutations were on the same allele (data not shown). The remaining seven clones showed only wild-type sequence. The D761Y and L858R mutations were not found in the patient's peripheral blood DNA (data not shown), indicating that they were somatic.

Direct sequencing of DNA from a pretreatment pleural biopsy, which showed infiltrating adenocarcinoma but was predominantly composed of stroma (>80%), did not show either the D761Y or L858R mutations. However, the L858R mutation was detected using the more sensitive PCR-RFLP assay (data not shown). Lack of remaining DNA from this specimen precluded further analysis by a more sensitive PCR-RFLP assay to detect the D761Y mutation.

Biochemical and physiologic properties of EGFR D761Y. To determine how the D761Y amino acid change would affect the mutant L858R EGFR, we introduced the secondary mutation into *EGFR* cDNAs (4). Corresponding proteins (L858R, L858R

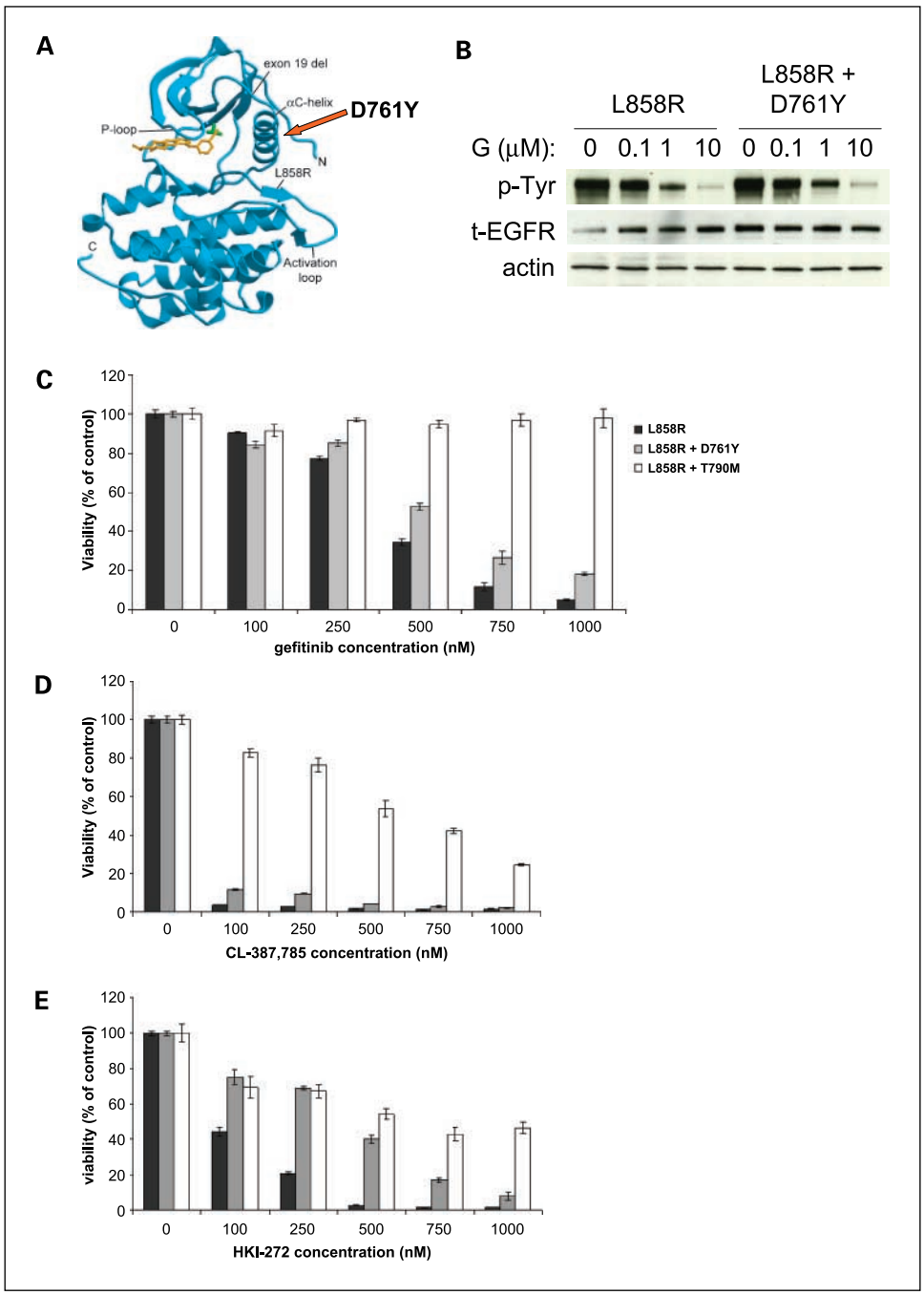

Fig. 2. EGFR mutants containing the D761Y amino acid change are less sensitive to inhibition by EGFR TKIs. *A,* crystal structure of erlotinib bound to EGFR (29), with landmarks depicted as shown. The D761Y change occurs in the middle of the α-C-helix. *B,* immunoblotting analysis of lysates from 293T cells transiently transfected with mutant *EGFR* cDNAs and treated with various concentrations of gefitinib (*G*) were done as in Materials and Methods. p-Tyr, phosphotyrosine; t-EGFR, total EGFR. *C* to *E,* Ba/F3 cells stably expressing EGFR L858R, EGFR L858R + D761Y, and EGFR L858R + T790M displayed different sensitivities to gefitinib (*C*), CL-387,785 (*D*), and HKI-272 (*E*). Cells were seeded into 96-well plates in triplicates at a density of 100,000 per well and treated with various concentrations of one of the above EGFR inhibitors for 72 hours (*C*) or 48 hours (*D* and *E*). Bars, SD ($n = 3$). Representative of three independent experiments.

plus D761Y) 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 (6). Surrogate kinase assays showed that addition of D761Y to the EGFR L858R mutant did not abolish or seem to augment its kinase activity (Fig. 2B).

We next examined whether the D761Y change would affect the sensitivity of EGFR L858R to gefitinib by performing immunoblotting on lysates from 293T cell transfectants that were serum starved and pretreated with gefitinib. Gefitinib progressively inhibited the activity of L858R EGFR with increasing concentrations of drug, as shown by a reduction of tyrosine-phosphorylated proteins (Fig. 2B), whereas the L858R plus D761Y mutant displayed a modest (<10-fold) decrease in sensitivity (Fig. 2B). This slight difference was in sharp contrast to the T790M mutation, which abrogated inhibition of tyrosine autophosphorylation at gefitinib concentrations up to 10 μmol/L (6).

To determine whether a small change in sensitivity might confer a survival advantage to cells expressing the D761Y mutation, we generated stable polyclonal populations of Ba/F3 cells carrying cDNAs for L858R alone, L858R plus D761Y, and L858R plus T790M, and assessed the ability of gefitinib to inhibit their proliferation *in vitro*. Ba/F3 cells are normally an IL-3-dependent murine hematopoietic cell line, but they can be rendered IL-3-independent by introduction of transforming tyrosine kinases including mutant EGFR L858R (13). Kinase-dependent Ba/F3 cells grown in various concentrations of kinase inhibitors can also be used to estimate the IC_{50}; indeed, Ba/F3 cells have previously been used to show that the T790M mutation confers resistance to drug-sensitive EGFR mutants (14).

Ba/F3 cells transfected with D761Y plus L858R were ~2-fold less sensitive to growth inhibition by gefitinib than L858R alone (Fig. 2C), consistent with the immunoblotting data from 293T cell lysates. The D761Y-containing cells were much less resistant to gefitinib than cells with L858R plus T790M. A similar trend was seen for cells grown in the presence of erlotinib (data not shown). Collectively, these data show that the D761Y amino acid change can confer a modest survival advantage to cells with an existing drug-sensitive L858R mutation in the presence of reversible EGFR TKIs.

We also assessed the survival of L858R plus D761Y–containing Ba/F3 cells in the presence of the irreversible EGFR inhibitors CL-387,785 (Fig. 2D) and HKI-272 (Fig. 2E), which we and others have shown can overcome T790M-mediated resistance *in vitro* (10, 14, 15). Again, Ba/F3 cells with EGFR L858R plus D761Y showed intermediate sensitivity compared with cells with L858R alone or L858R plus T790M. In comparison to results with gefitinib (Fig. 2C), the cells with EGFR L858R plus D761Y seemed to be relatively less sensitive to inhibition by HKI-272 (Fig. 2E). This finding suggests that different EGFR mutants may have differential sensitivity to the reversible and irreversible EGFR TKIs.

Analysis of progressive lesions in an autopsy case. To begin to assess whether analysis of DNA from a single lesion within an individual with acquired resistance to EGFR TKIs was representative of all tumors within a given patient, we examined multiple tumors from one patient at autopsy. This patient had been reported to have a T790M mutation in tumor cells at the time of disease progression (6). Three years after originally initiating EGFR TKI therapy, she was found to have brain metastases, with a dominant left frontal lobe mass. She died 2 months later.

At autopsy, this patient had multiple visceral metastases, involving right and left lungs, spine, adrenal gland, liver, and brain, all histologically adenocarcinoma. Sequencing of *EGFR* exons 18 to 24 using DNA extracted from seven different sites showed the same exon 19 deletion (L747-E749;A750P) in all lesions. Whereas the drug-resistant T790M mutation was found in all six visceral sites examined, this mutation was not detected in the metastatic brain lesion, even with the more sensitive PCR-RFLP assay (Fig. 3). Collectively, the presence of the D761Y mutation in the previously described brain lesion and the lack of T790M in the metastatic brain lesion from this case suggest that the selection pressure for resistant tumor cells that grow in the presence of EGFR TKIs may be different within the central nervous system (CNS) and the periphery.

EGFR copy number analysis in progressing lesions. In patients with chronic myelogenous leukemia, a related TKI, imatinib (Gleevec), targets cells harboring the activated *BCR-ABL* oncogene. One mechanism of resistance to imatinib in the absence of second-site ABL mutations involves amplification of *BCR-ABL* itself, although this scenario seems to be infrequently encountered (16, 17). To assess the role of *EGFR* amplification in acquired resistance to EGFR TKIs, we analyzed eight available tumor specimens from a total of 21 patients (16 from this study and 5 from our previous study) for *EGFR* copy number by chromogenic *in situ* hybridization. As shown in studies of *HER2* in breast cancer (18–20), chromogenic *in situ* hybridization is nearly 100% concordant with fluorescence *in situ* hybridization for the detection of amplification and has the added benefits of morphology-based scoring (nonneoplastic nuclei are not scored, unlike fluorescence *in situ* hybridization). Based on our own studies and the literature, chromogenic *in situ* hybridization results were interpreted as follows: <5 gene copies per nucleus, no amplification; 5 to 10 gene copies per nucleus, low-level amplification; and >10 gene copies per nucleus, high-level amplification.

All available (eight of eight) "resistant" tumor specimens showed amplification of *EGFR*, with copy numbers exceeding 5 (Table 2). Comparison of *EGFR* copy number pre- and

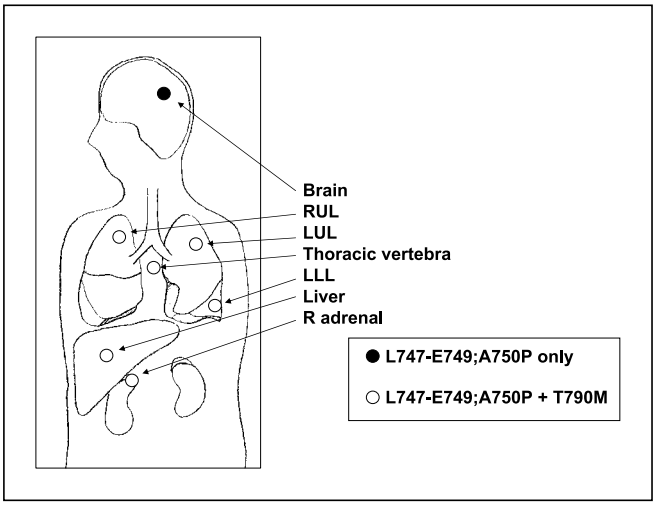

Fig. 3. Analysis of multiple metastatic sites within a patient for EGFR kinase domain mutations. At autopsy, the same exon 19 deletion (L747-E749;A750P) was detected in seven sites of disease (*RUL,* right upper lobe; *LUL,* left upper lobe; *LLL,* left lower lobe). All but the brain lesion contained a secondary T790M mutation.

Table 2. *EGFR* gene copy analysis in patients with acquired resistance to gefitinib or erlotinib

Sample	Primary mutation	Secondary mutation	*EGFR* copy number
1*	del L747-E749;A750P	T790M	5.7
2	del E746-A750	T790M	5.1→6.3
3	del L747-P753insS	T790M	9.6→11
4	L858R	D761Y	6.0
5*	del E746-A750	None	2.9→6.1
6	del E746-A750	None	8.4
7	del E746-A750	None	5.7
8	del E746-A750	None	7.2

NOTE: Tumor specimens pre- or post-EGFR TKI treatment were analyzed for *EGFR* copy number by chromogenic *in situ* hybridization (CISH) as per Materials and Methods. CISH values represent the average of signals for 30 nuclei per specimen examined. Pretreatment to posttreatment changes are indicated by an arrow; if only one value is listed, it was obtained from the posttreatment specimen.
*Patient previously reported [Pao et al. (6)].

post-treatment was possible in only three individuals. In one patient without a T790M mutation, the value changed from 2.9 to 6.1. Two other patients with the T790M mutation had slight increases from 5.1 to 6.3 and 9.6 to 11. The clinical significance of these changes is uncertain.

Discussion

In patients with metastatic lung adenocarcinomas that harbor drug-sensitive *EGFR* kinase domain mutations, mechanisms of acquired resistance to gefitinib and erlotinib could remain mutant EGFR dependent or become mutant EGFR independent (assuming no alterations in EGFR TKI pharmacokinetics). In the former case, disease progression would be associated with secondary changes in *EGFR*, such as second-site mutations and/or gene amplification. Altered EGFR trafficking in the absence of mutation has also been postulated as a possible EGFR-dependent mechanism of resistance, at least *in vitro* (10). For EGFR-independent mechanisms, acquired resistance could be associated with changes in other molecules (e.g., mutations in oncogenes or tumor suppressor genes). We show through analysis of tumor DNA from patients progressing on EGFR TKI therapy that second-site *EGFR* kinase domain mutations are common after disease progression [8 of 16 in this series and 2 of 5 in our previous series (6); collectively 10 of 21 (48%)]. This finding indicates that the mutant EGFR is a bona fide *in vivo* target of gefitinib or erlotinib in human lung tumors and suggests that, in at least half of patients with acquired resistance, tumors remain dependent on mutant EGFR for survival. The mechanism(s) underlying the other ~50% of cases remains to be elucidated.

It remains a possibility that substantially more than half of tumors are EGFR mutant-dependent after developing resistance. The amount of tissue obtained from progressing lesions, usually by a core needle biopsy, was often limited, and previous studies have suggested by clonal analysis that not all cells within a growing tumor contain the T790M mutation (10). Thus, sampling bias could have occurred at the time of biopsy. In addition, the methods used here (direct Sanger sequencing and PCR-RFLPs) have technical limitations in their ability to detect mutations. We hope in future studies to use more sensitive mutation detection methods, such as those based on high-performance liquid chromatography (21), tandem mass-spectrometry (22), or high-density picoliter reactors (23).

A second mechanism of EGFR-dependence could be accounted for by second-site *EGFR* mutations that occur outside of the kinase domain (exons 2-17, 25–28). We have made a preliminary effort to detect such mutations in exons 2-17, 25–28 samples with sufficient tissue for analysis (n = 4) but have not identified any somatic variants (data not shown).

EGFR amplification could also contribute to EGFR-dependent acquired resistance. Our initial studies suggest that resistant tumors (eight of eight) have amplified *EGFR*. One patient with acquired resistance without a second-site mutation did have an increase in copy number pre- and post-treatment, but how frequent an increase in gene copy number alone mediates acquired resistance clinically remains to be determined.

Among the 26 patients examined for mechanisms of acquired resistance to gefitinib or erlotinib now published in the literature (6–10), 14 of 15 with second-site mutations have had the T790M change. The skewed frequency of this "gatekeeper" mutation (24) stands in contrast to imatinib-resistant second-site ABL and KIT mutations, observed in chronic myelogenous leukemia and gastrointestinal stromal tumors, respectively, where the spectrum of amino acid changes seems to be much broader (Table 3). For example, the analogous mutation in ABL, T315I, is found in only ~20% of resistant cases, with the remaining mutations scattered throughout the kinase domain (25). For patients with KIT-mutant gastrointestinal stromal tumors, the analogous T670I mutation has been found in 27% of patients with acquired resistance to imatinib (26–28). One possible explanation for this difference is that erlotinib binds to the EGFR kinase domain in the active conformation (29), as opposed to imatinib, which binds to both ABL (30) and KIT in the inactive conformations (31). For imatinib, any mutation in BCR-ABL or KIT, which disrupts the stability of the inactive

Table 3. Frequency of clinically relevant gatekeeper resistance mutations found in various kinases targeted by kinase inhibitors

Disease	Target	Mutation	% Clinical cases	Conformation for TKI binding	References
CML	ABL	T315I	~20	Inactive	(25)
GIST	KIT	T670I	~27	Inactive	(26–28)
NSCLC	EGFR	T790M	~50	Active	This study

Abbreviations: CML, chronic myelogenous leukemia; GIST, gastrointestinal stromal tumor; NSCLC, non–small-cell lung cancer.

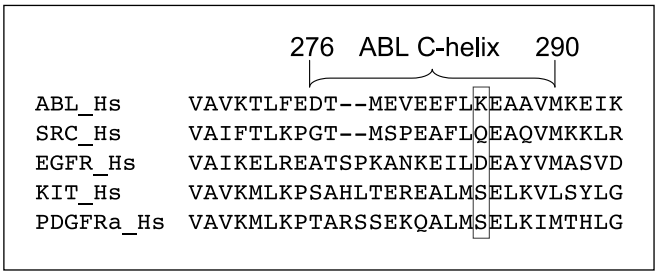

Fig. 4. Amino acid alignments of kinases targeted by clinically available kinase inhibitors. The α-C-helix of ABL is depicted as shown. The EGFR D761 residue is boxed to show the analogous residues in the other kinases, ABL, SRC, KIT, and PDGFRα. Hs, human isoform.

conformation, could lead to improper binding of the drug to the kinase domain and subsequent clinical resistance. However, for gefitinib or erlotinib, resistance would occur predominantly through mutations in residues that would lead to improper binding of the drug to the ATP-binding pocket in the active kinase domain.

A dominant type of resistance mutation may make it easier to identify agents that overcome acquired resistance. Our studies with erlotinib analogues suggest that the substituted methionine at position 790 exerts a profound stereoelectronic effect that cannot be overcome by modifying aniline moieties on erlotinib itself (see Supplementary data). Thus, newer agents that inhibit EGFR in a different manner need to be developed. Potential candidates include irreversible EGFR inhibitors (e.g., HKI-272) that covalently bind to EGFR at Cys^{797} (32) or heat shock protein 90 inhibitors (e.g., geldanamycin) that destabilize mutant EGFRs (33).

We also found a novel second-site exon 19 *EGFR* mutation (D761Y) in one patient and provide biochemical and physiologic evidence that it decreases sensitivity of the EGFR L858R mutant to EGFR TKIs. Preliminary studies indicate that Ba/F3 cell transfectants expressing EGFR D761Y alone become IL-3 independent in the presence of EGF (data not shown); further *in vitro* studies that characterize "gain-of-function" properties of this mutant are still under investigation. Nevertheless, in tumors from patients not treated with either gefitinib or erlotinib, the 2281G→T mutation occurring in codon 761 seems to be extremely rare. We have not identified this in sequence tracings of exon 19 from 155 previously analyzed tumors, and among nearly 3,000 lung cancers in which analysis of *EGFR* exons 18 to 21 has been done, none have been reported to harbor this mutation.[7]

From analysis of the crystal structure of the EGFR kinase domain bound to erlotinib, the D761Y mutation is predicted to occur in the α-C-helix of EGFR (Fig. 2A), adjacent to a well-conserved glutamic acid residue at position 762 (E762), which, together with Lys^{745}, forms a salt bridge that interacts with the α- and β-phosphates when ATP is present (29). Mutations within the α-C-helix of other kinases targeted by kinase inhibitors have been reported in patients who developed acquired resistance, most notably D276G in BCR-ABL with imatinib (34). No clinically relevant resistance mutation affecting the analogous amino acid in ABL (K285; Fig. 4) has yet been described, but a K285N mutation was recovered from a saturated mutagenesis screen for resistance of BCR-ABL against both imatinib and an alternative ABL inhibitor, PD166326 (35). In that study, the K285N mutation reduced the cellular IC_{50} of Ba/F3 transfectants 2.3-fold versus wild-type BCR-ABL, analogous to our data on the effect of D761Y on the EGFR L858R mutant. The ABL D276G mutation has been predicted to disrupt interactions that help anchor the activation loop in the inactive Src-like conformation (36). How the D761Y mutation affects the EGFR kinase domain remains to be determined, although it is not predicted to lead to bulky steric clash as with the T790M change.[8]

It is noteworthy that the EGFR D761Y mutation was found in a brain lesion in a patient who developed CNS metastases late in the course of gefitinib treatment. In a separate patient who developed CNS metastases late in the course of gefitinib therapy, a T790M mutation was found in multiple metastatic sites but not in the brain. It has been previously documented that there is a high incidence of disease recurrence in the brain and leptomeninges in patients with NSCLC after response to gefitinib, perhaps due to incomplete drug penetrance into the CNS (37). Consistent with this hypothesis, the mean predicted steady state trough concentration of drug following a 250-mg dose in patients has been measured at 584 nmol/L (95% confidence interval, 197-1,732 nmol/L; ref. 38). By contrast, levels of gefitinib measured in cerebrospinal fluid in a patient on 500 mg daily were only 6.15 nmol/L.[9] Although findings from only two patients are presented in this report, the molecular data do support the notion that selection pressure in the CNS is different from that in the rest of the body. If verified in analyses of other patients, this observation suggests that patients with initial response to gefitinib may benefit from higher levels of drug in the CNS or other prophylactic measures (e.g., whole brain radiation) to suppress the development of resistant tumor cells in the CNS.

Acquired resistance is a significant problem for patients with lung cancer, whose tumors harbor *EGFR* kinase domain mutations that are sensitive to gefitinib and erlotinib. Despite experiencing often dramatic initial responses, these patients develop disease progression. Further understanding of the mechanisms underlying this process will facilitate ways to both overcome and suppress the development of acquired resistance.

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