BRIEF REPORT

EGFR Mutation and Resistance of Non–Small-Cell Lung Cancer to Gefitinib

Susumu Kobayashi, M.D., Ph.D., Titus J. Boggon, Ph.D., Tajhal Dayaram, B.A.,
Pasi A. Jänne, M.D., Ph.D., Olivier Kocher, M.D., Ph.D.,
Matthew Meyerson, M.D., Ph.D., Bruce E. Johnson, M.D.,
Michael J. Eck, M.D., Ph.D., Daniel G. Tenen, M.D., and Balázs Halmos, M.D.

SUMMARY

Mutations of the epidermal growth factor receptor (*EGFR*) gene have been identified in specimens from patients with non–small-cell lung cancer who have a response to anilinoquinazoline EGFR inhibitors. Despite the dramatic responses to such inhibitors, most patients ultimately have a relapse. The mechanism of the drug resistance is unknown. Here we report the case of a patient with *EGFR*-mutant, gefitinib-responsive, advanced non–small-cell lung cancer who had a relapse after two years of complete remission during treatment with gefitinib. The DNA sequence of the *EGFR* gene in his tumor biopsy specimen at relapse revealed the presence of a second point mutation, resulting in threonine-to-methionine amino acid change at position 790 of EGFR. Structural modeling and biochemical studies showed that this second mutation led to gefitinib resistance.

From the Division of Hematology/Oncology (S.K., T.D., D.G.T., B.H.) and the Department of Pathology (O.K.), Beth Israel Deaconess Medical Center, Harvard Medical School; the Departments of Cancer Biology (T.J.B., M.J.E.), Medical Oncology (P.A.J., B.E.J., M.M.), and Pathology (M.M.), Dana–Farber Cancer Institute, Harvard Medical School; the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School (T.J.B., M.J.E.); and the Department of Medicine, Brigham and Women's Hospital, Harvard Medical School (P.A.J., B.E.J.) — all in Boston; and the Ireland Cancer Center, University Hospitals of Cleveland, Case School of Medicine, Cleveland (B.H.). Address reprint requests to Dr. Tenen at the Harvard Institutes of Medicine, HIM-954, 77 Louis Pasteur Ave., Boston, MA 02215, or at dtenen@bidmc.harvard.edu.

NON–SMALL-CELL LUNG CANCER IS THE LEADING CAUSE OF DEATH from cancer in both men and women in the United States.[1] Chemotherapy, the mainstay of treatment in advanced disease, is only marginally effective,[2,3] but gefitinib and erlotinib, which target the epidermal growth factor receptor (EGFR) pathway, show promise in the treatment of metastatic non–small-cell lung cancer.[4] Response rates are 10 to 20 percent when these ATP-competitive anilinoquinazoline inhibitors are used as second- or third-line treatment for advanced disease.[5-7]

Responsiveness to these drugs is a characteristic of distinct subgroups of patients: women, patients who have never smoked, patients with adenocarcinoma, and Asians.[8] In the majority of patients with highly responsive tumors, the tumor contains somatic mutations of the *EGFR* gene. These mutations are small deletions that affect amino acids 747 through 750 or point mutations (most commonly a replacement of leucine by arginine at codon 858 [L858R]).[9-11] These mutations mediate oncogenic effects by altering downstream signaling and antiapoptotic mechanisms.[12] Both types of mutation increase the sensitivity of the tumor to anilinoquinazoline inhibitors of EGFR, most likely by repositioning critical residues surrounding the ATP-binding cleft of the tyrosine kinase domain of the receptor, thereby stabilizing their interactions with both ATP and its competitive inhibitors.[9,10] Notwithstanding the success of these drugs in cases of non–small-cell lung cancer with activating *EGFR* mutations, it appears that all cases eventually progress despite such treatment.

CASE REPORT

A 71-year-old former smoker was found to have advanced, moderately differentiated adenocarcinoma of the lung in May 2001. The diagnostic transbronchial tumor-biopsy specimen had a deletion, delL747–S752,[13] identical to a previously described *EGFR* mutation that is associated with responsiveness to gefitinib. The disease progressed despite treatment with carboplatin, taxanes, and gemcitabine. However, the patient had a clinical and radiographic response to gefitinib monotherapy, which was started in August 2002.

After 24 months of complete remission, during which he continued to take gefitinib monotherapy, his symptoms worsened and computed tomography revealed progressive lung abnormalities consistent with the occurrence of a relapse. At this point, gefitinib was stopped, the patient provided written informed consent, and a transbronchial aspirate and transbronchial biopsy specimen were obtained. Both cytologic and pathological analysis confirmed the presence of recurrent, moderately differentiated adenocarcinoma (Fig. 1A) against a background of normal tissue, with approximately 30 percent of the specimen made up of tumor cells. We hypothesized that the patient's relapse may have been due to an acquired, second mutation in the *EGFR* gene that conferred resistance to gefitinib, and therefore, we resequenced the *EGFR* tyrosine kinase domain in the second biopsy specimen. Since the relapse, the patient has been receiving various salvage therapies for advanced lung cancer.

METHODS

SEQUENCING OF THE *EGFR* GENE

Genomic DNA was extracted from both tumor specimens from the patient, and exons 18 through 21 (the area of the *EGFR* gene coding for the tyrosine kinase domain) were amplified and sequenced as previously described.[9] Sense and antisense sequences were obtained from the products of the same amplification reactions. All polymerase-chain-reaction (PCR) assays were repeated twice. The presence of the second mutation was confirmed by isolating RNA from the tumor specimen and sequencing the resultant complementary DNA (cDNA). Total RNA was extracted from paraffin-embedded tumor samples with the use of an RNA isolation kit (Optimum FFPE, Ambion Diagnostics). Then, 800 ng o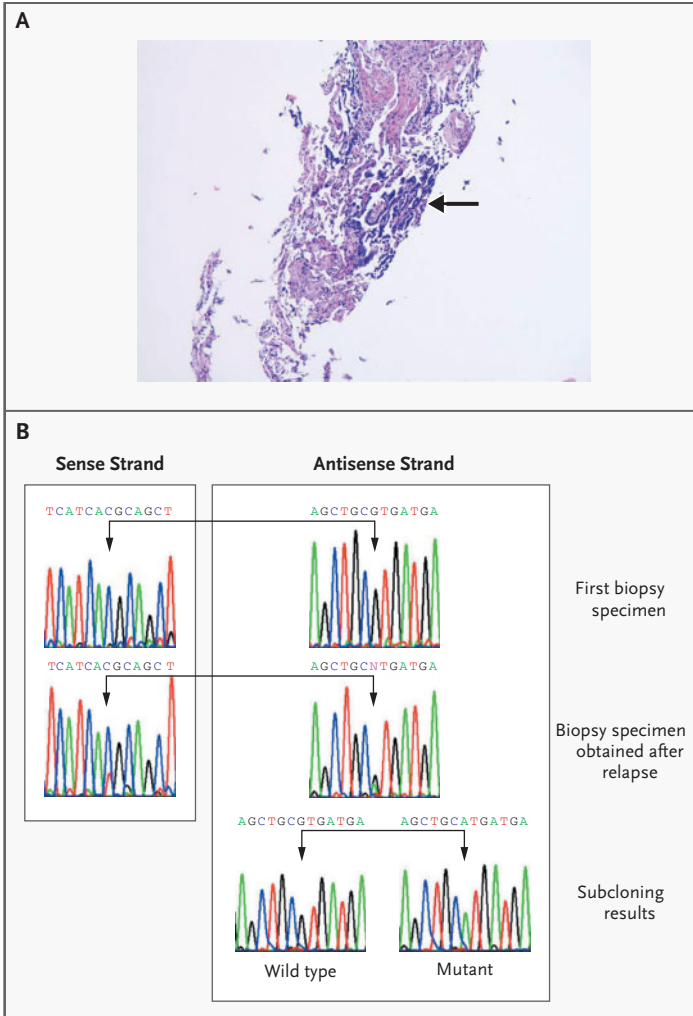f total RNA was subjected to reverse-transcription PCR with random hexamer primers. Amplification of the cDNA (sense primer: 5'GTTAAAATTCCCGTCGCTATC3', and antisense primer: 5'GGACATAGTCCAGGAGGCAG3') yielded two fragments, a 196-bp (wild-type) fragment and a 178-bp (deletion product) fragment. PCR products were subcloned into the pGEM-T easy cloning vector (Invitrogen) and sequenced.

Figure 1. Identification of a Second *EGFR* Mutation.

Panel A shows the patient's transbronchial biopsy specimen obtained at the time of relapse. Nests of moderately differentiated adenocarcinoma cells (arrow) are intermixed with normal stromal and epithelial elements. In Panel B, sequencing of exon 20 demonstrates a novel C-to-T (antisense G-to-A) base-pair change (arrows). Raw sequences of the original biopsy specimen and the biopsy specimen obtained on relapse demonstrate the appearance of this mutation, and further subcloning confirms the presence of both the wild-type and mutant sequences.

EGFR EXPRESSION CONSTRUCTS

Constructs with the full-length wild-type *EGFR*, as well as the common mutations L858R and delL747–P753insS, were kindly provided by Dr. Kwok-Kin Wong. The delL747–S752 mutation (the initial *EGFR* mutation identified in our patient) and a second mutation in which methionine was substituted for threonine at position 790 (T790M — the second *EGFR* mutation identified in our patient) were introduced by means of a site-directed mutagenesis kit (QuikChange XL, Stratagene). All fragments containing point mutations or deletions, or both, were swapped with the corresponding sequence in wild-type *EGFR* that had been subcloned into plasmid DNA (pcDNA3.1). A hemagglutinin tag was added to the 3′ end of the *EGFR* coding region. The resulting constructs were confirmed by sequencing.

TRANSFECTION AND WESTERN BLOTTING

For transient-transfection experiments, COS-7 or NIH-3T3 cells were plated at a concentration of 5×10^4 cells per well in six-well plates. The following day, these cells were transfected with 1 μg of the expression constructs with the use of Fugene 6 (Roche), incubated for 12 hours in serum, and then incubated in serum-free medium for an additional 12 hours. The cells were then stimulated with 100 ng of epidermal growth factor (EGF) per milliliter for 15 minutes (Sigma). To determine whether the mutant receptors were inhibited by gefitinib (AstraZeneca), AG1478 (Calbiochem), cetuximab (commercial supply), erlotinib (commercial supply), or CL-387,785 (Calbiochem), each drug was added to the culture medium three hours before the addition of EGF. Whole-cell extracts were separated on 8 percent sodium dodecyl sulfate–polyacrylamide gels, transferred to nitrocellulose or polyvinylidine difluoride membranes, and analyzed with the use of a chemiluminescence reagent (Western Lightning, PerkinElmer Life Science). Autophosphorylation of EGFR was detected with antibody against phosphotyrosine at position 1068 (1:1000 dilution; Cell Signaling Technology), and total protein expression was measured with the use of antibody against EGFR (1:1000 dilution; Santa Cruz Biotechnology).

STRUCTURAL MODELING

The crystallographic structure of the EGFR tyrosine kinase domain, solved in complex with erlotinib, was used as a model for the prediction of kinase-inhibitor binding (Protein Data Bank accession code 1M17).[14] The inhibitor and solvent were stripped from the model. We used the AutoDock program, version 3.0,[15] to predict binding, first using a model of erlotinib, made by means of the JME molecular-editing feature of the online resource PRODRG.[16] The erlotinib test yielded a model for ligand binding highly similar to that seen in the crystal structure. Using the AutoDockTools interface, we used a grid spacing of 0.375Å and 60×50×40 points centered around the catalytic cleft of the enzyme for docking and adopted the genetic algorithm with local search using default settings. Gefitinib and CL-387,785 were then docked with the use of the same protocol. To illustrate potential inhibitor clashes with the T790M mutant, we prepared figures in which threonine at position 790 (T790) is mutated to methionine. We then chose the lowest free-energy cluster that overlapped in the quinazoline moiety with the crystallographic coordinates found for erlotinib binding.

RESULTS

Exons 18 through 21 of the *EGFR* gene were sequenced from DNA isolated from both the original diagnostic biopsy specimen and the biopsy specimen obtained at relapse. These exons encompass most of the tyrosine kinase binding domain of *EGFR* and all activating *EGFR* mutations described thus far. The original diagnostic biopsy specimen contained a small deletion mutation, delL747–S752, consistent with other, commonly identified *EGFR* mutations. Examination of the sequences of exon 19 confirmed the persistence of the original delL747–S752 mutation in the second biopsy specimen.

Comparison of the DNA sequences from the original diagnostic biopsy specimen and the second biopsy specimen demonstrated the presence of a new, double peak in exon 20, which was confirmed by re-amplification and sequencing in both sense and antisense directions (Fig. 1B). The product of exon 20 amplification was subcloned, and multiple subclones were sequenced. Whereas 13 of 17 subclones demonstrated wild-type sequences, 4 contained an identical single base-pair change from cytosine to thymidine (C to T) (Fig. 1B), confirming that the new peak was caused by a base-pair change at this position (position 164208; GenBank accession number AY588246).
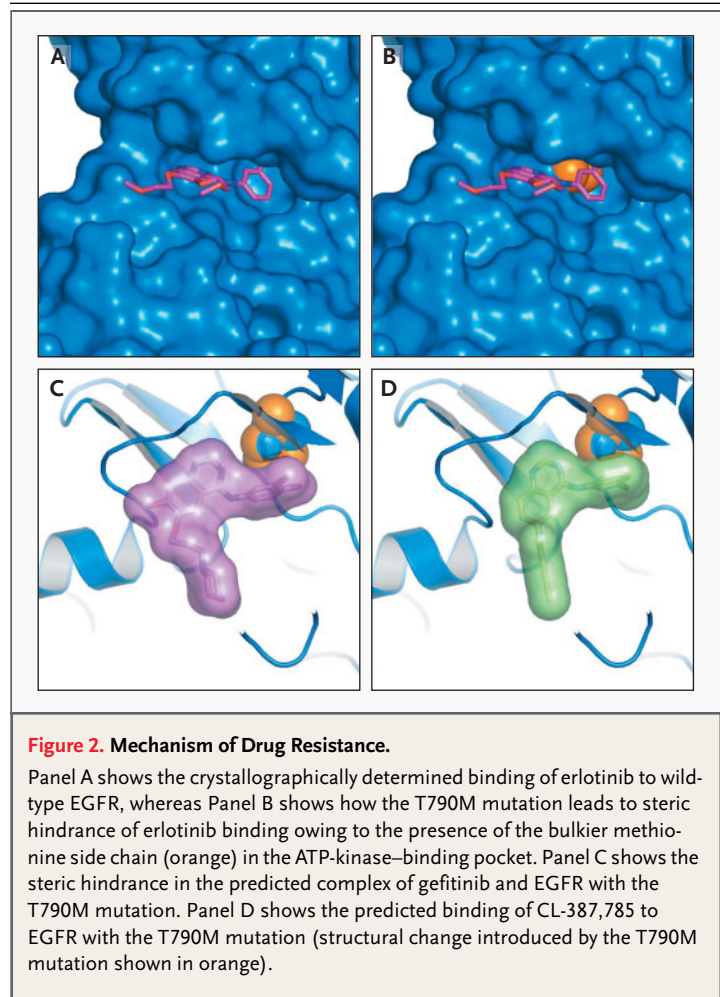
To obtain further evidence of the presence of a second mutation, cDNA was generated from RNA isolated from the paraffin block of the biopsy specimen obtained at the time of relapse. The cDNA

was amplified, and the C-to-T base-pair change was confirmed in 14 of 40 subclones. Interestingly, the C-to-T base-pair change was consistently observed with either wild-type or delL747–S752 sequences, suggesting that the mutation is either biallelic or that the tumor has two distinct populations of cells.

The C-to-T base-pair change is predicted to change threonine to methionine at position 790 (T790M) in the catalytic cleft of the *EGFR* tyrosine kinase domain. Structural modeling was performed on the basis of a cocrystallization model of the binding of erlotinib to the EGFR tyrosine kinase domain.[14] Using the coordinates from this model, we found that T790 appears to be critical for the binding of erlotinib to EGFR and is in juxtaposition to the acetylene side chain of the aniline group (Fig. 2A). Because the methionine substitution introduces a bulkier amino acid side chain than does threonine at this position, the resulting steric hindrance may interfere with the binding of erlotinib (Fig. 2B). Moreover, high-affinity binding of erlotinib by means of water-mediated hydrogen bonding could not take place with the methionine side chain, whereas the hydroxyl group of T790 likely contributes to high-affinity binding of erlotinib.
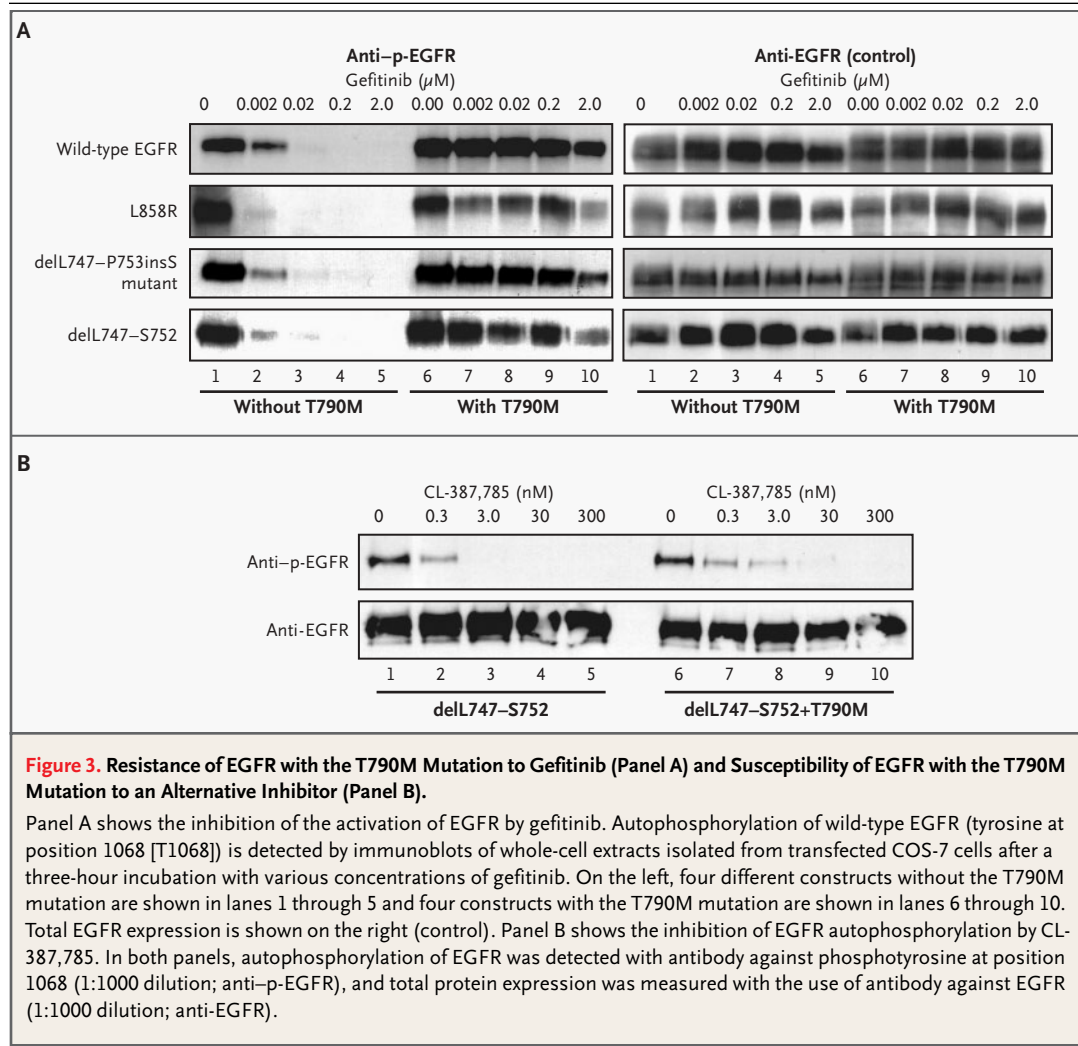
Although a crystal structure of the gefitinib–EGFR complex has not been published, a model of this complex suggested that, in a conformation similar to the erlotinib–EGFR complex, the chloride at the 3 position of the aniline group lies in juxtaposition to the T790 moiety. A T790M substitution is predicted to lead to a similar steric clash with the gefitinib molecule (Fig. 2C). This amino acid change is not expected to interfere with ATP-binding itself and, therefore, is not expected to alter the activity of the kinase on ligand stimulation.

To confirm the functional effects predicted by our structural model, the T790M substitution was introduced into the sequence of the wild-type EGFR, delL747–P753insS mutant EGFR (a frequently identified deletion mutant), the L858R mutant EGFR (the most common point mutation), and the delL747–S752 mutant. We performed transient-transfection experiments in COS-7 and NIH-3T3 cells using all four construct pairs (original construct vs. original construct with the T790M mutation). All four construct pairs demonstrated identical levels of expression of total EGFR and phosphorylated EGFR (phosphorylated EGFR corresponds to activated EGFR tyrosine kinase), suggesting that the presence of the T790M mutation does not substantially alter the production, degra-

Figure 2. Mechanism of Drug Resistance.

Panel A shows the crystallographically determined binding of erlotinib to wild-type EGFR, whereas Panel B shows how the T790M mutation leads to steric hindrance of erlotinib binding owing to the presence of the bulkier methionine side chain (orange) in the ATP-kinase–binding pocket. Panel C shows the steric hindrance in the predicted complex of gefitinib and EGFR with the T790M mutation. Panel D shows the predicted binding of CL-387,785 to EGFR with the T790M mutation (structural change introduced by the T790M mutation shown in orange).

dation, activation, or deactivation of the scaffold EGFR molecule. In contrast, when transiently transfected COS-7 cells were treated with increasing concentrations of gefitinib before EGF stimulation, the original constructs were fully inhibited at a concentration of 20 nM, whereas all four constructs carrying the T790M amino acid substitution demonstrated high-level resistance, with persistent generation of phosphorylated EGFR at concentrations of gefitinib as high as 2 μM (Fig. 3A). Identical results were obtained in NIH-3T3 cells (data not shown).

To determine whether the T790M mutation leads to resistance to EGFR inhibitors that have different molecular structures and mechanisms, we screened four commercially available EGFR inhibitors (AG1478, cetuximab, erlotinib, and CL-387,785) using cells that were transiently transfected with the delL747–S752 construct and the delL747–S752+

Figure 3. Resistance of EGFR with the T790M Mutation to Gefitinib (Panel A) and Susceptibility of EGFR with the T790M Mutation to an Alternative Inhibitor (Panel B).

Panel A shows the inhibition of the activation of EGFR by gefitinib. Autophosphorylation of wild-type EGFR (tyrosine at position 1068 [T1068]) is detected by immunoblots of whole-cell extracts isolated from transfected COS-7 cells after a three-hour incubation with various concentrations of gefitinib. On the left, four different constructs without the T790M mutation are shown in lanes 1 through 5 and four constructs with the T790M mutation are shown in lanes 6 through 10. Total EGFR expression is shown on the right (control). Panel B shows the inhibition of EGFR autophosphorylation by CL-387,785. In both panels, autophosphorylation of EGFR was detected with antibody against phosphotyrosine at position 1068 (1:1000 dilution; anti–p-EGFR), and total protein expression was measured with the use of antibody against EGFR (1:1000 dilution; anti-EGFR).

T790M construct. We consistently found that CL-387,785, a specific and irreversible anilinoquinazoline EGFR inhibitor,[17] strongly inhibited EGF-induced phosphorylation of both the delL747–S752 construct (apparent 50 percent inhibitory concentration [IC_{50}] of 0.3 nM) and the delL747–S752+T790M double-mutant construct (apparent IC_{50} of 3.0 nM) (Fig. 3B). When tested with the double-mutant construct, the CL-387,785 inhibitor demonstrated 1/10th its potency against the delL747–S752 construct, whereas the other tested inhibitors were ineffective against the double-mutant construct (data not shown). A model was generated with CL-387,785 and EGFR (Fig. 2D). The 3-bromine side chain in this model points away from T790, as opposed to its orientation with respect to erlotinib and gefitinib, thereby potentially reducing

the steric hindrance to binding of the mutated protein. The sensitivity of the delL747–S752+T790M construct to CL-387,785 might be explained by either its altered binding to the kinase domain or its covalent binding to EGFR.

DISCUSSION

We identified a novel, second mutation of the *EGFR* gene in a tumor with the delL747–S752 mutation of *EGFR*. The delL747–S752 mutation is associated with susceptibility of non–small-cell lung cancer to gefitinib, and in our patient, the tumor was highly responsive to the drug. After a two-year remission while still receiving gefitinib, however, the patient had a relapse, and analysis of a biopsy of the gefitinib-resistant tumor revealed a second mutation

in the tyrosine kinase domain of *EGFR*. Insertion of this mutation into test cells rendered them resistant to gefitinib in vitro. The development of a second mutation in the *EGFR* gene that confers resistance to gefitinib suggests that the tumor cells remain dependent on an active EGFR pathway for their proliferation.

In chronic myeloid leukemia and gastrointestinal stromal tumors, the two main mechanisms of resistance to imatinib are point mutations or, less commonly, amplification of the *BCR-ABL* gene.[18-20] Knowledge of these mechanisms has led to the development of second-generation BCR-ABL inhibitors.[21] Interestingly, one of the most common imatinib resistance mutations in *BCR-ABL* replaces threonine at position 315 (the amino acid structurally corresponding to T790 of EGFR) with isoleucine in the *ABL* tyrosine kinase domain (T315I), leading to a structural change very similar to that observed with EGFR T790M.[18] In fact, on the basis of the structural similarity between ABL and EGFR tyrosine kinases, the T790M change has been introduced into wild-type EGFR; this altered EGFR has high-level resistance to anilinoquinazoline inhibitors.[22] Daily oral administration of gefitinib at recommended doses results in mean steady-state plasma concentrations of 0.4 to 1.4 μM.[23] Since at these levels the T790M mutation still allows the activation of EGFR, the presence of such a mutation might result in clinical resistance.

Although the T315I substitution in *BCR-ABL* confers high-level resistance to all tested inhibitor compounds, the corresponding T790M mutation of EGFR does not seem to confer such universal resistance, given the fact that in the context of the delL747–S752 mutation, it can effectively be inhibited by CL-387,785. Our results should motivate the development of alternative EGFR inhibitors or inhibitors of downstream targets of EGFR such as phosphatidylinositol 3′-kinase or STAT5 for patients with EGFR-mutant tumors with acquired resistance to anilinoquinazoline inhibitors.

Our findings, and results in patients with chronic myeloid leukemia and gastrointestinal stromal tumors, suggest that when a relapse occurs in patients with anilinoquinazoline-responsive lung cancer with a drug-susceptibility mutation, the tumor cells will contain other mutations in *EGFR* that confer resistance to the drug. Our work also underscores the need to consider incorporating repeated biopsies into clinical studies of novel targeted therapies, such as those involving mutant tyrosine kinases. Such information may guide the selection of second-line EGFR-inhibitor therapy.

REFERENCES

1. Jemal A, Tiwari RC, Murray T, et al. Cancer statistics, 2004. CA Cancer J Clin 2004; 54:8-29.

2. Schiller JH, Harrington D, Belani CP, et al. Comparison of four chemotherapy regimens for advanced non–small-cell lung cancer. N Engl J Med 2002;346:92-8.

3. Scagliotti GV, De Marinis F, Rinaldi M, et al. Phase III randomized trial comparing three platinum-based doublets in advanced non-small-cell lung cancer. J Clin Oncol 2002;20:4285-91.

4. Mendelsohn J, Baselga J. Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. J Clin Oncol 2003;21:2787-99.

5. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (the IDEAL 1 Trial). J Clin Oncol 2003;21:

2237-46. [Erratum, J Clin Oncol 2004;22: 4811.]

6. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA 2003; 290:2149-58.

7. Perez-Soler R, Chachoua A, Hammond LA, et al. Determinants of tumor response and survival with erlotinib in patients with non-small-cell lung cancer. J Clin Oncol 2004;22:3238-47.

8. Miller VA, Kris MG, Shah N, et al. Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non-small-cell lung cancer. J Clin Oncol 2004;22:1103-9.

9. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness

of non–small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129-39.

10. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497-500.

11. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 2004;101:13306-11.

12. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305:1163-7.

13. Cho D, Kocher O, Tenen DG, et al. Unusual cases in multiple myeloma and a dramatic response in metastatic lung cancer: case 4: mutation of the epidermal growth factor receptor in an elderly man with ad-

vanced, gefitinib-responsive, non-small-cell lung cancer. J Clin Oncol 2005;23:235-7.

14. Stamos J, Sliwkowski MX, Eigenbrot C. Structure of the epidermal growth factor receptor kinase domain alone and in complex with a 4-anilinoquinazoline inhibitor. J Biol Chem 2002;277:46265-72.

15. Morris GM, Goodsell DS, Halliday RS, et al. Automated docking using a Lamarckian genetic algorithm and empirical binding free energy function. J Comput Chem 1998; 19:1639-62.

16. Schuettelkopf AW, van Aalten DMF. PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. Acta Crystallogr D Biol Crystallogr 2004;60: 1355-63.

17. Discafani CM, Carroll ML, Floyd MB Jr, et al. Irreversible inhibition of epidermal growth factor receptor tyrosine kinase with in vivo activity by N-[4-[(3-bromophenyl)amino]-6-quinazolinyl]-2-butynamide (CL-387,785). Biochem Pharmacol 1999; 57:917-25.

18. Gorre ME, Mohammed M, Ellwood K, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science 2001;293:876-80.

19. Chen LL, Trent JC, Wu EF, et al. A missense mutation in KIT kinase domain 1 correlates with imatinib resistance in gastrointestinal stromal tumors. Cancer Res 2004; 64:5913-9.

20. Azam M, Latek RR, Daley GQ. Mechanisms of autoinhibition and STI-571/imatinib resistance revealed by mutagenesis of BCR-ABL. Cell 2003;112:831-43.

21. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding imatinib resistance with a novel ABL kinase inhibitor. Science 2004;305:399-401.

22. Blencke S, Ullrich A, Daub H. Mutation of threonine 766 in the epidermal growth factor receptor reveals a hotspot for resistance formation against selective tyrosine kinase inhibitors. J Biol Chem 2003;278: 15435-40.

23. Albanell J, Rojo F, Averbuch S, et al. Pharmacodynamic studies of the epidermal growth factor receptor inhibitor ZD1839 in skin from cancer patients: histopathologic and molecular consequences of receptor inhibition. J Clin Oncol 2002;20:110-24.

Copyright © 2005 Massachusetts Medical Society.

FULL TEXT OF ALL *JOURNAL* ARTICLES ON THE WORLD WIDE WEB

Access to the complete text of the *Journal* on the Internet is free to all subscribers. To use this Web site, subscribers should go to the *Journal*'s home page (**www.nejm.org**) and register by entering their names and subscriber numbers as they appear on their mailing labels. After this one-time registration, subscribers can use their passwords to log on for electronic access to the entire *Journal* from any computer that is connected to the Internet. Features include a library of all issues since January 1993 and abstracts since January 1975, a full-text search capacity, and a personal archive for saving articles and search results of interest. All articles can be printed in a format that is virtually identical to that of the typeset pages. Beginning six months after publication, the full text of all Original Articles and Special Articles is available free to nonsubscribers who have completed a brief registration.