Emergence of Epidermal Growth Factor Receptor T790M Mutation during Chronic Exposure to Gefitinib in a Non–Small Cell Lung Cancer Cell Line

Atsuko Ogino,[1] Hiroyuki Kitao,[3] Seiki Hirano,[2] Akiko Uchida,[1] Masamichi Ishiai,[2] Toshiyuki Kozuki,[1] Nagio Takigawa,[1] Minoru Takata,[3] Katsuyuki Kiura,[1] and Mitsune Tanimoto[1]

[1]Department of Hematology, Oncology, and Respiratory Medicine, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences; [2]Department of Immunology and Molecular Genetics, Kawasaki Medical School, Kurashiki, Okayama, Japan; and [3]Department of Human Genetics, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan

Abstract

The epidermal growth factor receptor (EGFR)–specific tyrosine kinase inhibitor gefitinib may provide dramatic clinical responses in some patients with pulmonary adenocarcinoma carrying activating mutations of the EGFR. However, prolonged administration of gefitinib may eventually induce acquired resistance in such patients. To gain insight into the mechanisms of this phenomenon, we placed PC-9, a cell line derived from pulmonary adenocarcinoma that has a 15-bp deletion in EGFR exon 19, under the continuous selective pressure of low levels of gefitinib without any mutagen, and established a subline that was able to grow in the presence of 2 μmol/L of gefitinib (designated RPC-9). In this cell line, about half of the reverse transcription-PCR products from mutated EGFR also carried an additional mutation (T790M). In keeping with the proposed role of T790M in abrogating gefitinib binding with EGFR, gefitinib-treated RPC-9 hardly displayed any decrease in the constitutive phosphorylation of EGFR, Akt, or Erk1/2 unlike in PC-9 cells. Interestingly, transfection of the EGFR carrying only a 15-bp deletion reversed the resistance to gefitinib in RPC-9 cells. Thus, the balance of expression levels between gefitinib-sensitive or gefitinib-resistant EGFR may govern the response to gefitinib in lung cancer. [Cancer Res 2007;67(16):7807–14]

Introduction

The selective inhibitors of the epidermal growth factor receptor (EGFR) tyrosine kinase, gefitinib (Iressa, ZD1839) and erlotinib (Tarceva), prevent binding of ATP to the ATP-binding pocket of the EGFR in a competitive manner, thereby leading to the loss of catalytic activity (1, 2). They have provided dramatic clinical responses and even survival benefits for a subset of patients with pulmonary adenocarcinoma (3–10). Importantly, somatic mutations have been discovered in the kinase domain of EGFR in cancer cells of these non–small cell lung cancer (NSCLC) patients (3–5). Deletional mutations in exon 19 and substitution of leucine with arginine at codon 858 (L858R) account for ~90% of the mutations (2, 11, 12). Thus, these mutations seem to sensitize the cancer cells strongly to the growth-suppressive effects of the EGFR inhibitors. Of note, these patients are often never-smokers and females (9, 11, 12), and people with East Asian ethnicity carry the mutations in the lung cancer more often than Caucasians (13).

Not unexpectedly, the mutated EGFR is oncogenic, and is crucial for the maintenance of the tumor, as shown by transgenic mice studies (14, 15). The mutations render the EGFR tyrosine kinase constitutively active, although they still respond to ligands such as EGF (16, 17). In physiologic settings, activation of the EGFR is accompanied by ligand-induced homodimerization or heterodimerization with ErbB family members, leading to the active conformation of the kinase. Structural studies have indicated that EGFR normally remains in an autoinhibited inactive conformation, which is maintained by intramolecular interaction between the activation loop and the αC helix (18–20). Given the location of the mutations within the activation loop (L858R) or adjacent to αC helix (deletion), the autoinhibition is likely to be disrupted by the mutations, leading to the active conformation.

The dramatic clinical efficacy of the EGFR inhibitors in some NSCLC patients carrying the activating mutations tells us two things. First, the growth and survival of the NSCLC cells can be exquisitely dependent on the signal generated by EGFR, which is abrogated by the treatment. Second, the mutated EGFR is particularly susceptible to the inhibitors compared with wild-type EGFR. Indeed, gefitinib binds 20-fold more tightly to the L858R mutant EGFR compared with the wild-type receptor (20).

Although the tumors carrying these mutations display response rates to gefitinib as high as 80%, the cancer cells eventually become resistant to the treatment, and the median duration of response is typically 9 to 10 months (21, 22). This acquired resistance has been associated with a secondary mutation, T790M, in EGFR exon 20 (23–25). This situation is analogous to those observed in Bcr-Abl in imatinib-resistant chronic myelogenous leukemia (26). EGFR carrying both activating mutation and T790M is resistant to inhibition by gefitinib (25, 27), and gefitinib-sensitive NSCLC cell lines can be rendered resistant to gefitinib when introduced with EGFR carrying T790M (28). Furthermore, structural modeling suggests that T790M can abrogate the binding of gefitinib or erlotinib with the ATP-binding pocket of the EGFR kinase domain (28).

However, it is currently unclear whether the T790M mutation provides a universal explanation for the secondary resistance to gefitinib or erlotinib. Not all tumors that acquired resistance have been shown to harbor T790M-positive cells, and it is often difficult to detect a small number of cells with the T790M mutation (24).

Note: Current address for S. Hirano: Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DS, United Kingdom.
Current address for T. Kozuki: Cleveland Clinic, Cleveland, OH 44195.
Requests for reprints: Katsuyuki Kiura, Department of Hematology, Oncology, and Respiratory Medicine, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayaka 700-8558, Japan. Phone: 81-86235-7225; E-mail: kkiura@md.okayama-u.ac.jp or Minoru Takata, Department of Human Genetics, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan. Phone: 81-82257-5828; E-mail: minorut@hiroshima-u.ac.jp.

doi:10.1158/0008-5472.CAN-07-0681

The biological meaning of the T790M mutation is further complicated by the observation that some cancer cells might have the mutation even before the treatment (29, 30), and that a family with multiple cases of NSCLC is found to carry this mutation in the germ line (31). Furthermore, many factors other than T790M have been reported to affect gefitinib sensitivity of the cancer cells. For example, activation of downstream signaling by loss of PTEN (32), or expression of activated Ras (33), phosphoinositide-3-kinase (PI3K) or Akt (28) may bypass the requirement of EGFR signaling, leading to gefitinib resistance.

To gain insight about potential mechanisms of acquired resistance to EGFR inhibitors, we established a gefitinib-resistant NSCLC cell line termed RPC-9 from PC-9 cells (34), which are highly gefitinib-sensitive and have a 15-bp deletion in EGFR exon 19 (35). We found the T790M mutation in RPC-9 cells, and consistent with this, gefitinib treatment could not significantly decrease the levels of phosphorylation of EGFR as well as downstream effectors Akt and Erk1/2 in RPC-9. We also found diminished expression of ErbB3 in RPC-9 cells together with an altered signal transduction pathway to PI3K, however, this is unlikely to contribute to resistance because ectopic expression of ErbB3 did not revert the phenotype. Interestingly, introduction of the EGFR carrying only a 15-bp deletion restored sensitivity to gefitinib in RPC-9 cells. Finally, we provide evidence that a minor population with the T790M mutation gradually became dominant during a 2-month course of *in vitro* gefitinib treatment.

Materials and Methods

Establishment of the gefitinib-resistant subline from PC-9 cells. Human NSCLC cell line PC-9 was derived from an untreated Japanese patient with pulmonary adenocarcinoma that carried an in-frame deletion in EGFR exon 19 (delE746-A750; ref. 36). PC-9 cells were cultured at 37.0°C with 5% CO_2 in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. To establish a gefitinib-resistant subline, the cells were treated with 0.01 μmol/L of gefitinib, which was lower than the IC_{50} of PC-9, and the concentration was increased in a stepwise manner. After eight passages (~2 months), the cells were able to grow in 1 μmol/L of gefitinib. The cells had been continuously subcultured with 1 to 2 μmol/L of gefitinib for an additional 6 months, and then we did a single-cell cloning and established the gefitinib-resistant cell line (RPC-9).

Antibodies. Rabbit antisera against EGFR, phospho-specific EGFR (pY1068), Erk1/2, phospho-Erk (pT202/pY204), phospho-specific Akt ($pSer^{473}$), and total Akt were purchased from Cell Signaling Technology. Another polyclonal anti-EGFR (SC-03) antibody was obtained from Santa Cruz Biotechnology. Polyclonal antibodies against ErbB2, or PI3K p85α, and the anti-phosphotyrosine 4G10 or anti-ErbB3 monoclonal antibodies were purchased from Upstate Biotechnology. Anti-FLAG M2 monoclonal antibody was from Sigma.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Growth inhibition was measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cell Counting Kit-8; Dojindo). Briefly, the cells were plated on 96-well plates at a density of ~3,000 cells per well, and exposed to gefitinib for 96 h. Each assay was done in quadruplicate, and the mean and the SD were calculated.

Cell cycle analysis. To analyze the cell cycle profile, trypsinized cells were fixed in 70% ethanol overnight, and stained with propidium iodide. Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

Sequencing of EGFR gene and mutant-enriched PCR assay for T790M. The exons encoding the intracellular domain of EGFR were amplified from genomic DNA and directly sequenced. Alternatively, cDNA was synthesized and part of the kinase domain was amplified and sequenced. In some cases, the PCR products were cloned, and inserts were amplified from colonies and directly sequenced. The mutant-enriched PCR analysis was done to detect low-frequency T790M mutation as described in ref. (30), with some modifications. Briefly, PCR was carried out using 100 ng of genomic DNA and primers (forward, 5′-ACTGACGTGCCTCTCCCTCC-3′; reverse, 5′-CGAAGGGCATGAGCCGC-3′), then the products were digested with *Bst*UI to get rid of the wild-type products. After the second round of PCR using forward primer (5′-CCTCCAGGAAGCCTACGTGA-3′) and the reverse primer (the same one used in the first round of PCR), the products were directly sequenced.

Western blot analysis and immunoprecipitation. Cells were lysed in radioimmunoprecipitation assay buffer [1% Triton X-100, 0.1% SDS, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β-glycerol-phosphate, 10 mmol/L NaF, 1 mmol/L Na-orthovanadate, containing protease inhibitor tablet (Roche)] and were briefly sonicated. For immunoprecipitation, the cells were lysed in 0.1% Triton buffer [0.1% Triton X-100, 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β-glycerol-phosphate, 10 mmol/L NaF, 1 mmol/L Na-orthovanadate, containing protease inhibitors], subsequently sonicated and incubated with an appropriate antibody overnight at 4°C. The immunoprecipitates were collected using Protein G beads (Pierce). Proteins were separated by electrophoresis on polyacrylamide gels, transferred to nitrocellulose membranes, and probed with specific antibodies followed by detection with enhanced chemiluminescence plus (GE Healthcare Biosciences).

Ectopic expression of EGFR and ErbB3. For retroviral transduction, human EGFR carrying the 15-bp deletion tagged with COOH-terminal FLAG epitope or human ErbB3 (kindly provided by Dr. Shigeki Higashiyama, Ehime University) was subcloned into pMMP-IRES-puro (kindly provided by Dr. Toshiyasu Taniguchi, Fred Hutchinson Cancer Center) or pMX-GFP vector (kindly provided by Dr. Toshio Kitamura, University of Tokyo), respectively. To make ecotropic retrovirus, the retroviral plasmid was transfected into PlatE cells (kindly provided Dr. Toshio Kitamura) using LipofectAMINE 2000 (Invitrogen). After 48 h, the culture supernatants were transferred to the target cell culture, which had been transiently transfected with MCAT-1 (the receptor for ecotropic retrovirus) expression vector (kindly provided by Dr. James Cunningham, Harvard Medical School) 1 day before the transduction. Transduced cells were selected using puromycin (2 μg/mL) or the GFP-positive cell population was sorted using FACSAria (Becton Dickinson).

EGFR copy number. Comparative genomic hybridization (CGH) was carried out using bacterial artificial chromosome (BAC) microarray GSP-Array530 (GSP Lab Inc.). Genomic DNA was extracted from PC-9 and RPC-9, and was compared with normal human genomic DNA by CGH.

Results

Gefitinib-resistant PC-9 cells emerged during chronic gefitinib treatment. To create a model system for studying mechanisms of acquired gefitinib resistance in NSCLC, we established a resistant subline designated RPC-9 from PC-9 cells as described in Materials and Methods. The IC_{50} was ~8 μmol/L in RPC-9 cells for cell growth inhibition as measured by MTT assay (Fig. 1*A*), which was a 400-fold decrease in gefitinib sensitivity compared with parental PC-9 cells (IC_{50}, 0.02 μmol/L). The decreased sensitivity did not recover even in cells that were kept in culture for >1 year without gefitinib (RPC-9R, Fig. 1*A*).

Proliferation of RPC-9 cells was slightly faster than parental PC-9 cells (Fig. 1*B*). There was no morphologic difference between the two under gefitinib-free conditions (Fig. 1*C*). Following 72-h culture in the presence of gefitinib, PC-9 cells became rounded and shrunk, and detached from the bottom of the dish. Cell cycle analysis by propidium iodide staining revealed that gefitinib treatment resulted in G_1 cell cycle arrest and an increased fraction of cells with sub-G_1 DNA content in PC-9 cells. However, RPC-9 cells were not affected at all by the same treatment in either morphology or cell cycle analysis (Fig. 1*D*). We also found that RPC-9 cells were

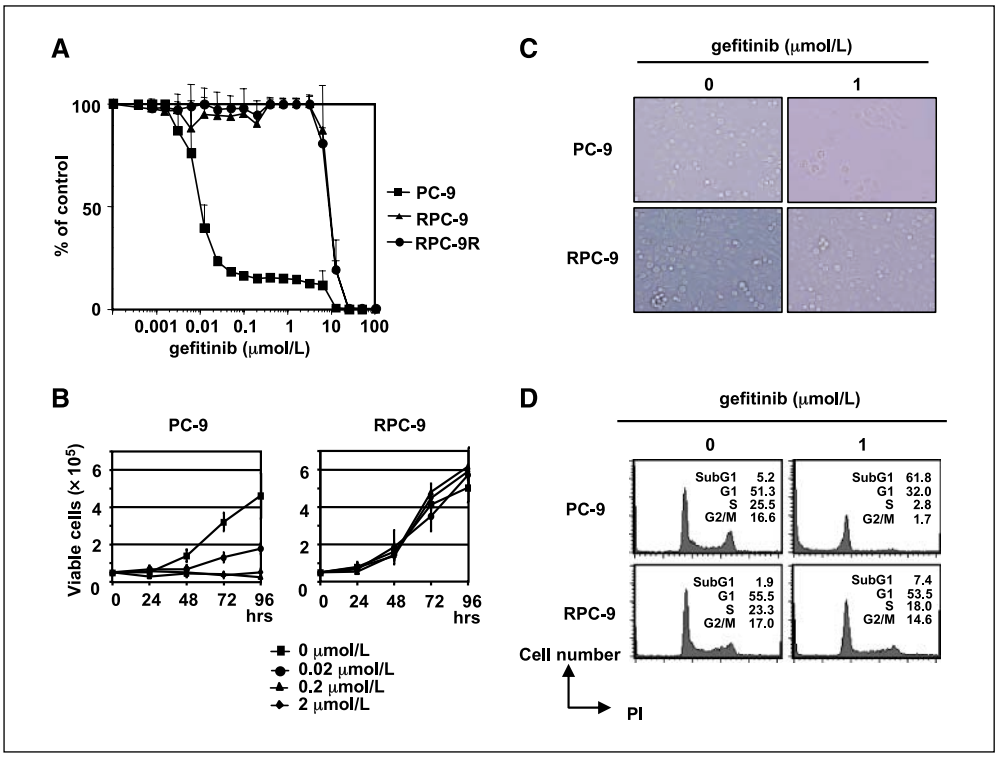

Figure 1. Characterization of gefitinib-resistant cell line RPC-9. *A,* cells (~3,000 per well) were seeded on 96-well plates in quadruplicate, and grown in the absence or presence of the indicated concentration of gefitinib. After 96 h, they were subjected to modified MTT assay. *RPC-9R,* RPC-9 cells maintained in culture for >1 y without gefitinib. *B,* PC-9 and RPC-9 cells were seeded on 24-well plates and allowed to grow in full growth medium with or without serially diluted gefitinib. Cells were trypsinized and counted every 24 h. *Points,* means of the triplicate cultures; *bars,* SD. *C,* light microscopic analysis of PC-9 and RPC-9 cells treated with or without 1 μmol/L of gefitinib for 72 h. *D,* cell cycle profiles of PC-9 and RPC-9 after 72-h treatment with or without gefitinib (1 μmol/L). *PI*, propidium iodide.

strongly resistant to another tyrosine kinase inhibitor, ZD6474 (IC_{50}, 0.1 μmol/L in PC-9 versus >5 μmol/L in RPC-9), which has dual specificity with EGFR and VEGFR, whereas the sensitivity to cisplatin was not significantly altered (IC_{50}, 4.0 μmol/L in PC-9 versus 7.1 μmol/L in RPC-9).

Phosphorylation levels of EGFR in RPC-9 cells do not decrease after gefitinib treatment. We examined the EGFR phosphorylation levels in both PC-9 and RPC-9 cells by Western blotting using an antibody against EGFR Y1068. As previously reported, levels of the phosphorylated Y1068 decreased in PC-9 cells treated with gefitinib (33, 37), whereas RPC-9 cells displayed persistent phosphorylation on Y1068 (Fig. 2*A*). We then asked whether constitutive activation of EGFR in RPC-9 cells was associated with alterations in downstream signaling pathways. As expected, the phosphorylated levels of Akt and Erk1/2 remained high in RPC-9 cells treated with up to 2 μmol/L of gefitinib, whereas both levels were drastically reduced by the same treatment in PC-9 (Fig. 2*A*).

Potential mechanisms for gefitinib resistance in RPC-9. The above data raised several possibilities regarding the gefitinib resistance mechanism in RPC-9. First, we considered a decreased intracellular concentration of gefitinib. This was excluded by measuring the uptake of ^{14}C-labeled gefitinib (data not shown). Another possibility was an activating mutation in a gefitinib-insensitive tyrosine kinase other than EGFR, which could trans-phosphorylate EGFR. ErbB2/Her2 was the prime candidate for this because a recent report described that Her2 harboring an insertional mutation in the kinase domain (exon 20) could result in the constitutive phosphorylation and activation of EGFR, which is resistant to gefitinib (38). We thus sequenced exons 18 to 24 of ErbB2, and found no mutation (data not shown). The third possibility was that EGFR is hyperactivated by drastically increased expression of EGFR ligands. Fourth, it was possible that EGFR itself underwent changes that could eliminate inhibition by gefitinib. To exclude these possibilities, we isolated EGFR from PC-9 and RPC-9 by immunoprecipitation, and compared the *in vitro* kinase activity of precipitated EGFR in the presence of gefitinib. Gefitinib clearly suppressed the kinase activity of EGFR isolated from PC-9 cells, but not from RPC-9 cells (data not shown). Collectively, these data indicate that the EGFR in RPC-9 cells was altered such that the EGFR kinase activity was no longer effectively inhibited by gefitinib treatment.

Direct sequencing assay for T790M mutation. Recent observations indicate that an EGFR secondary mutation, T790M, is associated with acquired resistance in patients chronically treated with gefitinib (23–25). To examine whether there were such additional mutations in RPC-9, we did PCR amplification followed by direct sequencing of all of the exons (exons 18–28) encoding the cytoplasmic region of the EGFR. We confirmed the persistence of the original 15 bp deletion. Additionally, a C-to-T base pair change at nucleotide 2369 was identified in exon 20, which leads to a substitution of methionine for threonine at position 790 (T790M; Fig. 3*A*).

To further characterize this additional mutation, we amplified the EGFR exon 19 to 20 from PC-9 or RPC-9 cDNA, and the product was subcloned into plasmid vector, then inserts were isolated and sequenced. Among the 29 sequences from RPC-9, we found that 9 had no mutations (wild-type), 12 had only the 15-bp deletion, and 8 had both the 15-bp deletion and the T790M mutation (Table 1). The T790M mutation was present only on the same clone as the 15-bp deletion. Of 10 clones isolated from PC-9, 6 had no mutation originating from the wild-type allele, whereas the others had the 15-bp deletion. This is at the expected ratio for a heterozygous mutation, and also suggests that the T790M mutation occurred in one or more of the amplified alleles that harbor the 15-bp deletion.

We wished to know how many alleles of EGFR existed in PC-9 or RPC-9 cells. We did CGH analyses using BAC microarray to assess the gain or loss of 530 genes including EGFR (data not shown), and found that the EGFR gene in PC-9 and RPC-9 cells displayed a 1.8- and 1.4-fold signal increase, respectively, compared with the normal human genome. Thus, it is likely that PC-9 or RPC-9 cells had the ~2-fold (four alleles) or ~1.5-fold (three alleles) amplified EGFR gene, respectively. Together with the sequencing data of the subcloned PCR product as described above, these data suggest that parental PC-9 cells had two wild-type alleles and two mutant alleles with the 15 bp deletion. On the other hand, in RPC-9 cells, the ratio between alleles (wild-type versus the allele with 15-bp deletion only versus the alleles with 15-bp deletion and T790M mutation) seemed to be roughly 1:1:1. Thus, it seemed possible that one EGFR allele was lost during the establishment of RPC-9 cells by unknown mechanisms, and that could be the wild-type allele.

Decreased expression levels of ErbB3 in RPC-9 cells. To further characterize any changes in the EGFR signal transduction in RPC-9 cells, we examined PC-9 or RPC-9 cells by 4G10 antiphosphotyrosine Western blot analysis (Fig. 2*B*). In lysates from both cell lines, the band at ~160 kDa in the 4G10 blot seemed to be the phosphorylated EGFR, because of the molecular weight and the fact that treatment with gefitinib resulted in the disappearance of the band in PC-9 lysates. Of note, a prominent phosphotyrosine band at ~200 kDa was observed only in PC-9 but not in RPC-9 cells, which was affected by gefitinib treatment in a similar manner as p-EGFR. The membranes probed with 4G10 were stripped and re-probed with respective ErbB receptor antibodies. Anti-ErbB3, but not anti-ErbB2, detected a band that completely overlapped with the ~200 kDa band in question (Fig. 2*B*; data not shown). ErbB4 was not detectable in either RPC-9 or parental PC-9 cells (data not shown). Interestingly, ErbB3 protein levels were remarkably decreased in RPC-9 cells compared with PC-9 cells as shown in Figs. 2*B* and 5*A*, whereas ErbB2 levels were not appreciably altered (Fig. 2*C*).

Previous reports showed that PI3K associates with ErbB3 exclusively in gefitinib-sensitive NSCLC cell lines, and treatment with gefitinib dissociates this complex, thereby leading to decreased Akt activity (39). Thus, the EGFR-ErbB3-PI3K-Akt pathway seems to be the key signaling axis, which determines gefitinib sensitivity in NSCLC cells (40). Given the significantly decreased level of ErbB3 expression, this pathway could be dysfunctional in RPC-9 cells. To investigate this possibility, we first looked at the dimerization between ErbB3 and EGFR. Comparison of anti-EGFR immunoprecipitates between PC-9 and RPC-9 cells revealed that EGFR effectively associated with ErbB3 only in PC-9 cells, and that was not affected by gefitinib treatment (Fig. 2*D, top*). Next, we analyzed the interaction between ErbB3 and p85α, which is the regulatory subunit of type 1a PI3K (40). As shown in Fig. 2*D* (*bottom*), ErbB3 coprecipitated with p85α in PC-9 cells, however, the amount of ErbB3 in anti-p85α immunoprecipitates seemed much lower in RPC-9 cells. Western blotting using antiphosphotyrosine 4G10 detected a prominent band in PC-9, which comigrated with ErbB3 (Fig. 2*D, bottom*). Because phospho-Akt levels were as high in RPC-9 as in PC-9 cells (Fig. 2*A*), these data suggest that the pathway which maintained PI3K/Akt activation in RPC-9 was probably altered from that in PC-9 cells, and the roles of ErbB3 could be less prominent.

T790M mutation could be the sole mechanism of RPC-9 resistance. The EGFR phosphorylation in RPC-9 cells was refractory to gefitinib treatment (Fig. 2*A*), and they harbor the T790M mutation (Fig. 3*A*). These facts strongly suggested that T790M is the reason for the resistance to gefitinib in RPC-9 cells. However, to prove that, we need to specifically knock-down the expression of the EGFR harboring T790M. In our hands, the efficiency of small interfering RNA knockdown in PC-9 or RPC-9 cells was far from perfect, and hence, this experiment is not feasible at the moment.

We therefore planned to see whether dilution of the T790M-carrying EGFR by the EGFR without T790M affects the gefitinib

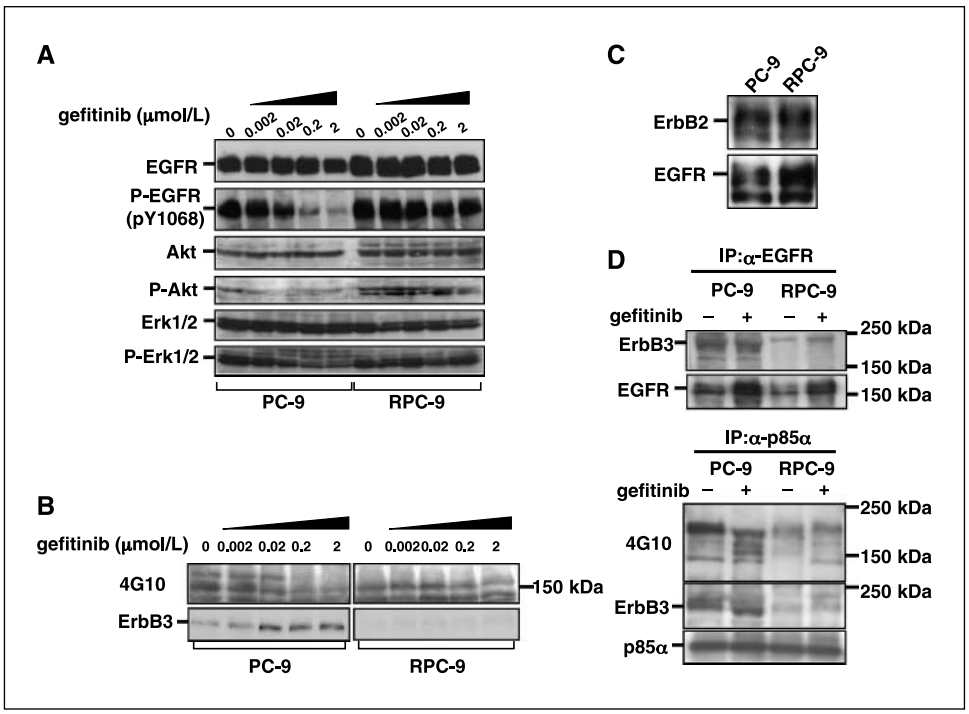

Figure 2. Analysis of signal transduction in PC-9 and RPC-9 cells. *A,* PC-9 and RPC-9 cells were treated with the indicated concentrations of gefitinib for 6 h. Lysates were subjected to Western blotting with the indicated antibodies. *B,* after 8 h of incubation with various concentrations of gefitinib, the extracts from cells were probed with antiphosphotyrosine antibody 4G10. Membranes were then stripped and reprobed with anti-ErbB3 antibody. *C,* Western blot analysis of EGFR and ErbB2 in PC-9 and RPC-9 cells. *D,* cells were grown in the presence or absence of gefitinib (2 μmol/L) for 8 h. Extracts were immunoprecipitated with anti-EGFR (*top*) or anti-p85α antibody (*bottom*), and probed with the indicated antibodies.

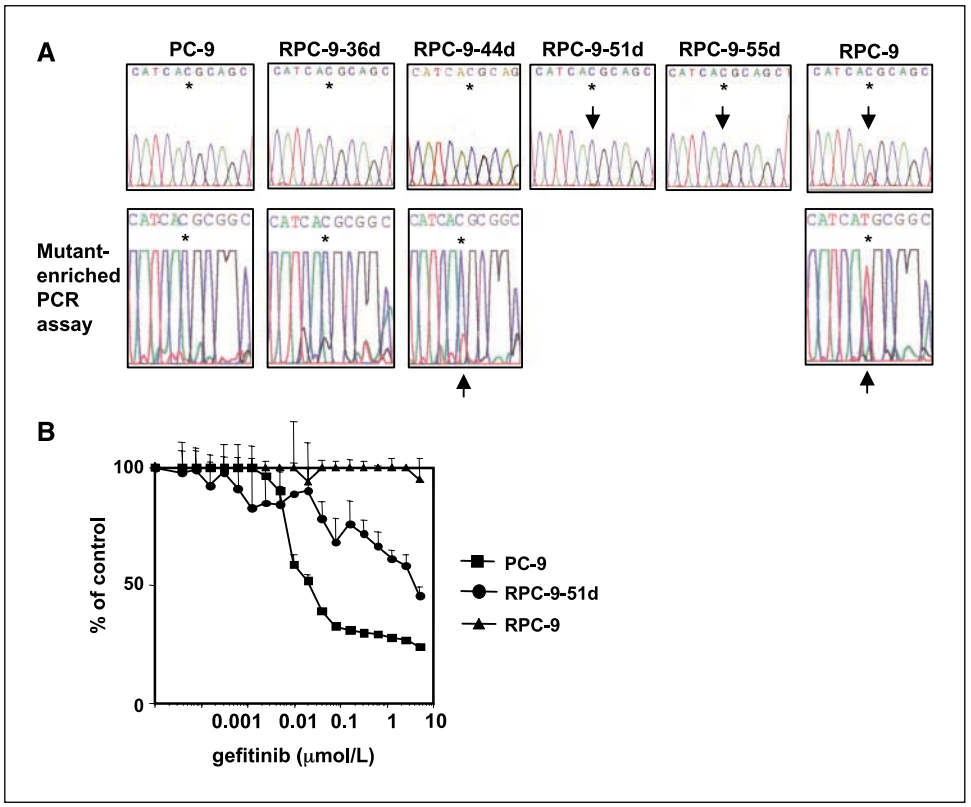

Figure 3. T790M mutation was detected in RPC-9 cells. *A,* sequencing of EGFR RT-PCR products (*top*) from late passage cells (RPC-9-51d, 55d, RPC-9) revealed a C-to-T base pair change (*arrows*) that corresponds to the T790M. Although this mutation was not detectable by direct sequencing of RT-PCR products from PC-9, RPC-9-36d, or -44d cells, mutant-enriched PCR assay (*bottom*) confirmed the presence of T790M mutation in RPC-9-44d (*arrows*). *B,* the sensitivity of the indicated cells was determined in the absence or presence of different doses of gefitinib for 96 h using modified MTT assay.

resistance of RPC-9. To this end, RPC-9 cells were retrovirally transduced with the FLAG-tagged EGFR carrying only the 15-bp deletion. Western blotting with anti-FLAG antibody confirmed the expression of the transduced EGFR, and surface expression of EGFR was modestly increased as revealed by fluorescence-activated cell sorting analysis (Fig. 4*A*). Perhaps surprisingly, the EGFR-infected cells displayed a significant reversal of the gefitinib resistance (Fig. 4*B*). We also analyzed lysates from transduced cells that were preincubated with increasing concentrations of gefitinib by anti–phospho-EGFR (Y1068) immunoblotting. Consistent with the reversal of the resistance, the phosphorylation of EGFR was significantly inhibited in EGFR-transduced RPC-9 cells but not in control cells (Fig. 4*C*).

To test whether the gefitinib resistance of RPC-9 is a consequence of ErbB3 down-regulation, RPC-9 cells were transduced with retrovirus encoding ErbB3 tagged with enhanced green fluorescent protein (Fig. 5*A*). Expression of ErbB3-GFP has previously been used for examining signal propagation in the plasma membrane (41). We found that forced ErbB3 expression in RPC-9 cells had no effect on the resistance to gefitinib (Fig. 5*B*). In addition, the levels of ErbB3 were further decreased in RPC-9 cells in which gefitinib tolerance was reversed by ectopic expression of EGFR with the 15-bp deletion (Fig. 4*A*).

A small fraction of T790M-positive cells arise during gefitinib exposure. PC-9 cells were stocked periodically during the course of gefitinib exposure on days 36, 44, 51, and 55 before establishment of RPC-9 (designated RPC-9-36d, RPC-9-44d, RPC-9-51d, and RPC-9-55d, respectively). The sequencing analysis of EGFR T790 from these cells is summarized in Fig. 3*A*. The late passage cells (RPC-9-51d and 55d) clearly contained a T790M mutation, and the C-to-T peak became more prominent at 55 days, which suggested a subclone of cells harboring the secondary mutation emerged during gefitinib treatment and became dominant under chronic selection by gefitinib. We compared the gefitinib sensitivity of PC-9, RPC-9, and RPC-9-55d cells by using MTT assay (Fig. 3*B*). The level of resistance in RPC-9-55d was milder than that of RPC-9. This could be explained by the smaller fraction of T790M-containing cells existing in RPC-9-55d cultures compared with RPC-9.

To detect T790M mutation in a more sensitive manner, we did a modified mutant-enriched PCR assay (30). This assay was originally reported to detect one copy of the mutant T790M allele in the presence of 1,000 copies of wild-type alleles (30). To check the

Table 1. Fraction of cloned PCR products with indicated mutations

Cells	No. of clones sequenced (%)			
	Wild-type*	15-bp deletion*	15-bp del+ T790M*	T790M†
PC-9	6/10 (60%)	4/10 (40%)	0/10 (0%)	
RPC-9	9/29 (31%)	12/29 (41%)	8/29 (27%)	
RPC-9-36d				0/20 (0%)
RPC-9-44d				0/20 (0%)
RPC-9-55d				1/40 (2.5%)

*PCR was carried out from cDNA, and products were subcloned and sequenced.
† EGFR exon 20 was amplified from genomic DNA, and products were subcloned and sequenced.

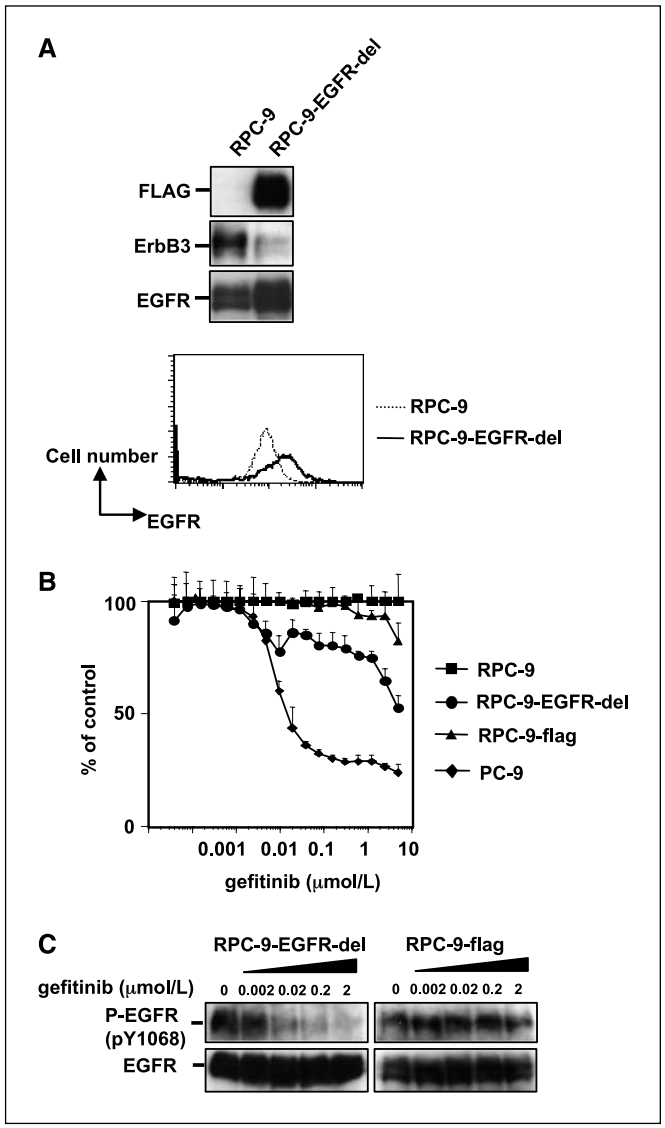

Figure 4. Effects of ectopic expression of EGFR on resistance to gefitinib in RPC-9. *A,* expression of FLAG-tagged EGFR carrying the 15-bp deletion without T790M in RPC-9 (RPC-9-EGFR-del). Whole cell lysates were prepared from the indicated cells, and blotted with anti-FLAG, anti-ErbB3, or anti-EGFR antibody (*top*). Cells were stained with PE-conjugated anti-EGFR antibody, and subjected to fluorescence-activated cell sorting analysis (*bottom*). *B,* cells were plated on 96-well plates and exposed to gefitinib for 96 h, then subjected to modified MTT assay. RPC-9 cells transduced with EGFR carrying the 15-bp deletion (RPC-9-EGFR-del) or control retrovirus encoding only FLAG epitope (RPC-9-flag) were analyzed in parallel with PC-9 and RPC-9 cells. *C,* RPC-9 cells transduced with EGFR carrying the 15-bp deletion (RPC-9-EGFR-del) or control retrovirus (RPC-9-flag) were treated for 6 h with gefitinib at the indicated concentrations. Cell lysates were prepared and subjected to immunoblots with anti-p-EGFR (Y1068) and anti-EGFR antibodies.

sensitivity of our modified version of the assay, PC-9 and RPC-9 cells were mixed at a ratio of 1:1, 10:1, 100:1, and 1,000:1, and genomic DNA was extracted and analyzed. We were able to detect the presence of the T790M allele in 1:1, 10:1, and 100:1 cell mixtures but not in the 1,000:1 mixture (data not shown). Given the number of EGFR alleles as described above, the detection limit of the assay lies somewhere between ~1:400 and ~1:4,000 alleles. We found that T790M-positive cells were already detectable in RPC-9-44d but not in RPC-9-36d (Fig. 3*A*). Consistently, RPC-9-44d displayed weak gefitinib resistance in the MTT assay (data not shown).

Discussion

In this study, we have established a gefitinib-resistant NSCLC cell line, RPC-9, from highly sensitive PC-9 cells that carry a 15-bp deletion in EGFR gene exon 19 (36). We found that in RPC-9 cells, the phosphorylation level of EGFR did not respond to gefitinib treatment. Consistently, the secondary mutation, T790M, was detected in RPC-9, and this was likely to be a major reason for the resistance. Several lines of evidence supported this conclusion. First, the T790M mutation occurred in cis to the 15-bp deletion; therefore, the T790M mutation directly affected the activated EGFRs that were promoting the growth of the cell line. Second, in cell specimens periodically banked during the 2-month course of gefitinib exposure, there was an apparent correlation between the gradually increased fraction of cells carrying the T790M mutation and the increase in resistance. Third, introduction of the EGFR carrying the activating 15-bp deletion into RPC-9 cells reversed gefitinib resistance, suggesting that the ratio between gefitinib-sensitive and gefitinib-resistant alleles could be an important factor in determining gefitinib sensitivity. This observation also suggested that the growth and survival of RPC-9 cells was probably still heavily dependent on EGFR signaling. Fourth, we have excluded a number of potential contributing factors, such as intracellular concentration of gefitinib, as described in Results.

It is currently unclear whether the T790M mutation occurred *de novo* during the gefitinib exposure or if we simply picked out preexisting RPC-9 type cells from PC-9 culture. Consistent with the latter possibility, accumulating evidence now indicates that T790M mutation emerges without gefitinib treatment as a minor clone in some patients (29, 30). Because the mutant-enriched PCR assay

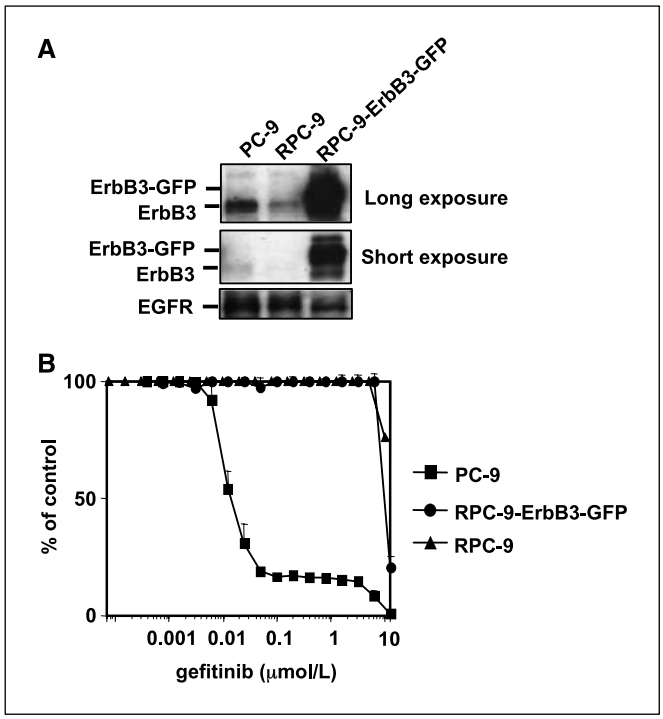

Figure 5. Overexpression of ErbB3-GFP in RPC-9 cells could not restore gefitinib sensitivity. *A,* RPC-9 cells stably transfected with ErbB3-GFP were lysed and subjected to Western blotting using anti-ErbB3 or anti-EGFR antibodies. In the case of anti-ErbB3 Western blotting, results with both long and short exposures are shown. *B,* cells were treated with or without gefitinib for 96 h on 96-well plates, and subjected to modified MTT assay.

could not detect T790M in PC-9 cells, the fraction of such clones in the PC-9 population, if present, should be <1:100.

We estimate that T790M exists in one of the two mutant EGFR alleles, and possibly one of the two wild-type alleles may have been lost in RPC-9. This may have provided growth advantage by increasing the ratio of active EGFR in the pool of total EGFR molecules, and this could be an additional, albeit minor, mechanism of gefitinib resistance in RPC-9. Alternatively, it is also possible that loss of the wild-type allele was a neutral event that occurred by chance in this setting. However, consistent with the former possibility, we detected mild reversal of the resistance in RPC-9 transfected with wild-type EGFR (data not shown).

Of note, we also found a decreased association of EGFR with ErbB3 in RPC-9, accompanied by a change in the signal transduction pathway. This could be due to the decreased expression levels of ErbB3 in RPC-9, with activation of Akt kinase by EGFR possibly being mediated through another adapter molecule. For example, Grb2-associated binding protein 1 (Gab1) can bind directly to EGFR, and it facilitates PI3K signaling through its tyrosine phosphorylation sites in cells not expressing ErbB3 (42, 43). To exclude the contribution of decreased ErbB3 to the resistance, we introduced ErbB3-GFP into RPC-9, but this could not restore the sensitivity.

Besides our study, there has been only one published report that established a gefitinib-resistant NSCLC cell line that has a T790M mutation (28). This study used a H3255 cell line carrying a EGFR L858R mutation, in which the EGFR gene had undergone extensive amplification (>40 alleles). The resistant subline, H3255 GR, harbors only a very limited number of EGFR alleles with a T790M mutation because 3 out of 91 (3.3%) cloned reverse transcription-PCR products carry T790M. In our study, nearly half of the transcripts from the activated EGFR allele also had a T790M mutation, and a modest amount of ectopically expressed EGFR restored sensitivity. This discrepancy could be related to the use of different cell lines, or the presence of different mutations (L858R versus 15-bp deletion). In any case, our study predicts that a very small number of EGFR carrying T790M in a given NSCLC cell does not necessarily confer high levels of gefitinib resistance. The resistance effects of T790M could be diluted in cells with a large number of active EGFR alleles until the allele with T790M itself is amplified to certain levels. Therefore, it is conceivable that NSCLC patients with EGFR amplification could sustain response to gefitinib for longer durations than NSCLC without amplification, as indicated by a recent clinical study (44, 45).

Koizumi et al. also established a gefitinib-resistant PC-9 subline (termed PC-9/ZD) using a strategy similar to ours but with brief exposure to a mutagenic drug *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (37). However, the T790M mutation was not found in the PC-9/ZD subline. Nonetheless, the overall phosphorylation levels of EGFR are refractory to gefitinib treatment, suggesting the existence of important resistance mechanisms other than T790M mutation. Interestingly, PC-9/ZD has decreased ErbB3 levels similar with RPC-9, raising the possibility that the continuous exposure to gefitinib selects PC-9 cells that have altered EGFR-ErbB3-PI3K signaling.

While this article was under review, the amplification of proto-oncogene MET was reported to cause acquired resistance in *in vitro*–selected resistant sublines of NSCLC cell line HCC827 as well as in patients treated with gefitinib or erlotinib (46). Our BAC microarray analysis showed no MET amplification in RPC-9 (data not shown), excluding this possibility.

In summary, we have isolated a gefitinib-resistant subline, RPC-9, from the highly sensitive NSCLC cell line PC-9. The occurrence of the secondary mutation, T790M, is most likely the predominant mechanism for the resistance, which could be reversed by the expression of gefitinib-sensitive EGFR. We suggest that the balance of expression levels of gefitinib-sensitive or gefitinib-resistant EGFR proteins may play a role in regulating gefitinib sensitivity in patients with NSCLC.

Acknowledgments

Received 2/19/2007; revised 5/19/2007; accepted 6/1/2007.

Grant support: Ministry of Education, Culture, Sports, Science and Technology (K. Kiura).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Masayo Kimura, Emi Uchida, Aiko Kinomura, Hiromi Nakashima, and Yoko Sato for expert technical support; Kyoko Takahashi, Hiroko Shimamoto, and Hisayo Saito for secretarial assistance; AstraZeneca for providing gefitinib, ^{14}C-gefitinib, and ZD6474; Dr. Shigeki Higashiyama (Ehime University, Matsuyama, Japan) for human ErbB3 cDNA; Dr. Toshiyasu Taniguchi (Fred Hutchinson Cancer Center, Seattle, WA) for pMMP-IRES-puro; Dr. Toshio Kitamura (University of Tokyo, Tokyo, Japan) for pMX vector and PlatE cells; Dr. James Cunningham (Harvard Medical School, Boston, MA) for MCAT-1 expression vector; Masahiko Maekawa (GSP Lab, Inc., Kawasaki, Kanagawa, Japan) for CGH analysis using BAC microarray; Dr. Tetsuya Shiraishi (SONY Computer Science Laboratories, Inc., Tokyo, Japan) for advice; Dr. Shinichi Toyooka (Okayama University, Okayama, Japan) for sharing unpublished results.

References

1. Herbst RS, Fukuoka M, Baselga J. Gefitinib—a novel targeted approach to treating cancer. Nat Rev Cancer 2004;4:956–65.
2. Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer 2007;7:169–81.
3. 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.
4. 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.
5. 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.
6. Fujiwara K, Kiura K, Ueoka H, Tabata M, Hamasaki S, Tanimoto M. Dramatic effect of ZD1839 ('Iressa') in a patient with advanced non-small-cell lung cancer and poor performance status. Lung Cancer 2003;40:73–6.
7. Thatcher N, Chang A, Parikh P, et al. Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). Lancet 2005;366:1527–137.
8. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 2005;353:123–32.
9. Tsao MS, Sakurada A, Cutz JC, et al. Erlotinib in lung cancer—molecular and clinical predictors of outcome. N Engl J Med 2005;353:133–44.
10. Bell DW, Lynch TJ, Haserlat SM, et al. Epidermal growth factor receptor mutations and gene amplification in non-small-cell lung cancer: molecular analysis of the IDEAL/INTACT gefitinib trials. J Clin Oncol 2005;23:8081–92.
11. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. Cancer Res 2004;64:8919–23.
12. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst 2005;97:339–46.
13. Calvo E, Baselga J. Ethnic differences in response to epidermal growth factor receptor tyrosine kinase inhibitors. J Clin Oncol 2006;24:2158–63.
14. Politi K, Zakowski MF, Fan PD, Schonfeld EA, Pao W, Varmus HE. Lung adenocarcinomas induced in mice by mutant EGF receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors. Genes Dev 2006;20:1496–510.
15. Ji H, Li D, Chen L, et al. The impact of human EGFR kinase domain mutations on lung tumorigenesis and *in vivo* sensitivity to EGFR-targeted therapies. Cancer Cell 2006;9:485–95.
16. Greulich H, Chen TH, Feng W, et al. Oncogenic

transformation by inhibitor-sensitive and -resistant EGFR mutants. PLoS Med 2005;2:e313.
17. Chen YR, Fu YN, Lin CH, et al. Distinctive activation patterns in constitutively active and gefitinib-sensitive EGFR mutants. Oncogene 2006;25:1205–15.
18. Zhang X, Gureasko J, Shen K, Cole PA, Kuriyan J. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell 2006; 125:1137–49.
19. Choi SH, Mendrola JM, Lemmon MA. EGF-independent activation of cell-surface EGF receptors harboring mutations found in gefitinib-sensitive lung cancer. Oncogene 2006;26:1567–76.
20. Yun CH, Boggon TJ, Li Y, et al. Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity. Cancer Cell 2007;11:217–27.
21. Inoue A, Suzuki T, Fukuhara T, et al. Prospective phase II study of gefitinib for chemotherapy-naïve patients with advanced non-small-cell lung cancer with epidermal growth factor receptor gene mutations. J Clin Oncol 2006;24:3340–6.
22. Sutani A, Nagai Y, Udagawa K, et al. Gefitinib for non-small-cell lung cancer patients with epidermal growth factor receptor gene mutations screened by peptide nucleic acid-locked nucleic acid PCR clamp. Br J Cancer 2006;95:1483–9.
23. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2005;2:e73.
24. Kwak EL, Sordella R, Bell DW, et al. Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. Proc Natl Acad Sci U S A 2005; 102:7665–70.
25. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786–92.
26. Carter TA, Wodicka LM, Shah NP, et al. Inhibition of drug-resistant mutants of ABL, KIT, and EGF receptor kinases. Proc Natl Acad Sci U S A 2005;102:11011–6.
27. Kobayashi S, Ji H, Yuza Y, et al. An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor. Cancer Res 2005; 65:7096–101.
28. Engelman JA, Mukohara T, Zejnullahu K, et al. Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer. J Clin Invest 2006;116:2695–706.
29. Toyooka S, Kiura K, Mitsudomi T. EGFR mutation and response of lung cancer to gefitinib. N Engl J Med 2005;352:2136; author reply.
30. Inukai M, Toyooka S, Ito S, et al. Presence of epidermal growth factor receptor gene T790M mutation as a minor clone in non-small cell lung cancer. Cancer Res 2006;66:7854–8.
31. Bell DW, Gore I, Okimoto RA, et al. Inherited susceptibility to lung cancer may be associated with the T790M drug resistance mutation in EGFR. Nat Genet 2005;37:1315–6.
32. Mellinghoff IK, Wang MY, Vivanco I, et al. Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. N Engl J Med 2005;353:2012–24.
33. Uchida A, Hirano S, Kitao H, et al. Activation of downstream EGFR signaling provides gefitinib-resistance in cells carrying EGFR mutation. Cancer Sci 2007;98:357–63.
34. Lee YC, Saijo N, Sasaki Y, et al. Clonogenic patterns of human pulmonary adenocarcinoma cell lines (PC-9, PC-13 and PC-14) and how they influence the results of test for chemosensitivity to cisplatin in the human tumor clonogenic assay. Jpn J Clin Oncol 1985;15:637–44.
35. Arao T, Fukumoto H, Takeda M, Tamura T, Saijo N, Nishio K. Small in-frame deletion in the epidermal growth factor receptor as a target for ZD6474. Cancer Res 2004;64:9101–4.
36. Ono M, Hirata A, Kometani T, et al. Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. Mol Cancer Ther 2004;3:465–72.
37. Koizumi F, Shimoyama T, Taguchi F, Saijo N, Nishio K. Establishment of a human non-small cell lung cancer cell line resistant to gefitinib. Int J Cancer 2005; 116:36–44.
38. Wang SE, Narasanna A, Perez-Torres M, et al. HER2 kinase domain mutation results in constitutive phosphorylation and activation of HER2 and EGFR and resistance to EGFR tyrosine kinase inhibitors. Cancer Cell 2006;10:25–38.
39. Engelman JA, Janne PA, Mermel C, et al. ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. Proc Natl Acad Sci U S A 2005;102:3788–93.
40. Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature 2006; 441:424–30.
41. Verveer PJ, Wouters FS, Reynolds AR, Bastiaens PI. Quantitative imaging of lateral ErbB1 receptor signal propagation in the plasma membrane. Science 2000;290: 1567–70.
42. Rodrigues GA, Falasca M, Zhang Z, Ong SH, Schlessinger J. A novel positive feedback loop mediated by the docking protein Gab1 and phosphatidylinositol 3-kinase in epidermal growth factor receptor signaling. Mol Cell Biol 2000;20:1448–59.
43. Sithanandam G, Smith GT, Fields JR, Fornwald LW, Anderson LM. Alternate paths from epidermal growth factor receptor to Akt in malignant versus nontransformed lung epithelial cells: ErbB3 versus Gab1. Am J Respir Cell Mol Biol 2005;33:490–9.
44. Cappuzzo F, Hirsch FR, Rossi E, et al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst 2005;97:643–55.
45. Kaye FJ. A curious link between epidermal growth factor receptor amplification and survival: effect of "allele dilution" on gefitinib sensitivity? J Natl Cancer Inst 2005;97:621–3.
46. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007;316:1039–43.

Emergence of Epidermal Growth Factor Receptor T790M Mutation during Chronic Exposure to Gefitinib in a Non–Small Cell Lung Cancer Cell Line

Atsuko Ogino, Hiroyuki Kitao, Seiki Hirano, et al.

Cancer Res 2007;67:7807-7814.