Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer

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EGFR is frequently mutated and amplified in lung adenocarcinomas sensitive to EGFR inhibitors gefitinib and erlotinib. A secondary mutation, T790M, has been associated with acquired resistance but has not been shown to be sufficient to render EGFR mutant/amplified lung cancers resistant to EGFR inhibitors. We created a model for studying acquired resistance to gefitinib by prolonged exposure of a gefitinib-sensitive lung carcinoma cell line (H3255; EGFR mutated and amplified) to gefitinib in vitro. The resulting resistant cell line acquired a T790M mutation in a small fraction of the amplified alleles that was undetected by direct sequencing and identified only by a highly sensitive HPLC-based technique. In gefitinib-sensitive lung cancer cells with EGFR mutations and amplifications, exogenous introduction of EGFR T790M effectively conferred resistance to gefitinib and continued ErbB-3/P13K/Akt signaling when in cis to an activating mutation. Moreover, continued activation of P13K signaling by the PIK3CA oncogenic mutant, p110ε E545K, was sufficient to abrogate gefitinib-induced apoptosis. These findings suggest that allelic dilution of biologically significant resistance mutations may go undetected by direct sequencing in cancers with amplified oncogenes and that restoration of P13K activation via either a T790M mutation or other mechanisms can provide resistance to gefitinib.

Introduction

The EGFR is a member of a family of closely related growth factor receptor tyrosine kinases that includes EGFR (ErbB-1), HER2/neu (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4). As EGFR is expressed in a majority of non–small cell lung carcinomas (NSCLCs), it has been an attractive target for the development of therapeutic agents (1–3). The small-molecule EGFR tyrosine kinase inhibitors (TKIs), including gefitinib (Iressa; AstraZeneca) and erlotinib (Tarceva; OSI Pharmaceuticals), have been evaluated in clinical trials for patients with NSCLC. Both agents cause partial responses in 10%–20% of all NSCLC patients (4–7). Tumors that possess activating mutations and/or amplification of the EGFR locus appear to be particularly sensitive to EGFR TKIs (8–14). In fact, lung cancers with EGFR mutations often harbor concurrent EGFR amplifications (13, 14). NSCLC cell lines in which EGFR is mutated and amplified, including HCC827 and H3255, are expressly sensitive in vitro to EGFR TKIs (8, 15, 16). Although other cell lines (e.g., breast cancer cell lines) have been used as model systems to investigate sensitivity to gefitinib, the EGFR mutated and amplified lung cancer cell lines used in this study are greater than 10- to 100-fold more sensitive to gefitinib (IC50 ~10–100 nM) than other cell lines and serve as faithful in vitro models for the lung cancers with the most dramatic clinical responses to EGFR inhibitors (8, 15–19).

Acquired resistance to gefitinib occurs in NSCLC patients with somatic activating mutations in EGFR, who initially respond clinically to treatment with gefitinib but then develop progressive cancer. This acquired resistance has been associated with the development of a secondary mutation in EGFR analogous to those observed in BCR-ABL and KIT in imatinib-resistant chronic myelogenous leukemia and gastrointestinal stromal cell tumors, respectively (20, 21). Initial studies have identified a secondary EGFR mutation, T790M, in NSCLC tumor biopsies from 4 of 8 individuals who developed disease progression while receiving EGFR TKI treatment (22–24). The T790M mutation is believed to abrogate gefitinib’s ability to bind and inhibit the EGFR. When EGFR T790M alone or cis to an activating EGFR mutation is transfected into Cos-7 or Ba/F3 cells, the EGFR autophosphorylation is resistant to inhibition by gefitinib (24, 25). However, it remains unknown whether acquisition of T790M alone is sufficient to make a gefitinib-sensitive EGFR mutant NSCLC resistant to gefitinib-induced cell death. Additionally, the importance of whether T790M occurs cis or trans to the somatic activating EGFR mutation in gefitinib-resistant tumors remains to be determined.

Nonstandard abbreviations used: ca-Akt, constitutively active Akt; Del/T790M, EGFR with a T790M mutation cis to an exon 19 deletion mutation; DHPLC, denatur ing HPLC; GR, gefitinib resistant; L858R/T790M, EGFR with a T790M mutation cis to an L858R mutation; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenox- y)-2-(4-sulfophenyl)-2H-tetrazolium; NSCLC, non–small lung carcinoma (cancer); p-, phosphorylated; PARP, poly ADP-ribose polymerase; PTEIN, phosphatase and tumor homolog; shRNA, short hairpin RNA; TKI, tyrosine kinase inhibitor; WT, T790M, WT EGFR with a T790M mutation.

Conflict of interest: Pasi A. Jänne and Bruce E. Johnson are part of a pending patent application on EGFR mutations.


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be determined. Moreover, some acquired-resistance tumors have been shown to harbor a very low percentage of T790M-containing sequences (22, 23). The mechanism by which a small proportion of T790M sequences confers resistance remains undefined. Further-

more, the clinical significance, if any, of rare T790M sequences is not known.

The in vitro sensitivity of NSCLC cell lines to EGFR TKI treatment is closely correlated with downregulation of the PI3K/Akt pathway (17, 26). Moreover, in a previous study we demonstrated that NSCLC cell lines sensitive to gefitinib are distinct in that they use ErbB-3 to activate the PI3K/Akt pathway (26). In fact, preliminary studies demonstrate that ErbB-3 protein expression is associated with efficacy of EGFR TKI therapy in patients with NSCLC (27). However, it is unknown whether downregulation of ErbB-3/PI3K/Akt signaling correlates with sensitivity to gefitinib or if it is necessary for gefitinib to promote cell death.

In this study, we model the development of acquired resistance to gefitinib in patients with NSCLC by generating a gefitinib-resistant H3255 (H3255 GR) cell line in vitro. This cell line acquires a T790M mutation only in a small fraction of the amplified EGFR alleles. The T790M allele is undetectable by conventional sequencing and requires a highly sensitive HPLC-based technique for its detection. We found that exogenous introduction of EGFR T790M conferred resistance to gefitinib-induced cell death in vitro and in vivo when in cis to an activating mutation. We further demonstrated that continued activation of the PI3K/Akt pathway by EGFR-independent mechanisms was sufficient to confer resistance to gefitinib in these EGFR mutant and amplified lung cancers. These observations indicate that restoration of PI3K activation via either a rare T790M mutation or other mechanisms can provide resistance to gefitinib.

**Results**

**Development of H3255 GR, a gefitinib-resistant NSCLC cell line.** Despite the clinical and radiographic benefits of gefitinib and erlotinib treatment in many patients with activating EGFR mutations,
confirmed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-
phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay that this
phosphorylation in the H3255 GR cell line. In light of recent obser-
vation that the presence of an E790M mutation is associated with
acquired resistance to EGFR TKIs in patients (24, 25), the en-
tire EGFR gene from H3255 GR was sequenced, and no changes
in the presence of 1 μM gefitinib (Figure 1D). The H3255 GR cell
line also retained phosphatase and tensin homolog (PTEN) expres-
sion as determined by Western blotting (data not shown).

**Table 1**
Summary of parental and engineered cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EGF R mutation</th>
<th>Expression construct</th>
<th>IC_{50}, gefitinib (μM)</th>
<th>IC_{50}, CL-387.785 (μM)</th>
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<td>Parental</td>
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<tr>
<td>H3255</td>
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<tr>
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<td>ca-Akt</td>
<td>&gt;10</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not done.  

these patients ultimately develop resistance to these agents. In
order to model the mechanisms of acquired resistance to gefitinib
in vitro, we exposed the gefitinib-sensitive H3255 cell line (EGFR
L858R; amplified with >40 copies) to increasing concentrations of
gefitinib using previously established methods (28). H3255 cells
were exposed to serially increasing concentrations of gefitinib for
6 months until the cells were able to proliferate in 100 nM gefitinib
with kinetics similar to those of untreated cells (see Methods).
We confirmed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-
phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay that this
new cell line, H3255 GR, was not growth inhibited by exposure to
different PI3K activity to a greater degree than the parental H3255 cells
lected PI3K signaling by disrupting its association with ErbB-3 (26).
H3255 GR cell lines following treatment with or without 1 μM gefitinib
(Figure 1B). We and others have used 1 μM gefitinib in prior stud-
es, as this is reflective of mean steady-state plasma concentrations
(0.4–1.4 μM) in patients receiving 250 mg gefitinib as determined by
phase I clinical studies, and gefitinib begins to have significant off-
target effects at concentrations greater than 1 μM (16, 26, 29–33).
Although PI3K activity was inhibited in both cell lines following
treatment with gefitinib, the H3255 GR cell line retained approxi-
mately 3-fold greater ErbB-3–associated PI3K activity (35% of con-
trol) compared with the parental H3255 cell line (13% of control).

to determine the mechanism by which H3255 GR cells maintain
ErbB-3/PI3K activity despite treatment with gefitinib, we examined
the effects of gefitinib treatment on phosphorylation of EGFR, ErbB-3, and
downstream signaling molecules (Figure 1C). Unlike the parental H3255 cell line, H3255 GR cells maintained a low level of
EGFR phosphorylation even following treatment with 10 μM
gefitinib (Figure 1C). This subtle but reproducible maintenance of
phosphorylated EGFR (p-EGFR) in H3255 GR was consistently observed in several independent experiments (data not shown).

Similarly, phosphorylation of ErbB-3, Akt, and ERK1/2 was maintained in the H3255 GR cell line (Figure 1C). Upon exposure to 1 μM gefitinib, p-EGFR, p-Akt, and p-Akt persisted at levels approximately 10% of the untreated controls in the H3255 GR cells, but were only approximately 1% of the untreated controls in the parental H3255 cell lines (see Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI28656DS1). The H3255 GR cells, unlike the parental H3255 cells, also maintained low levels of
p-EGFR staining at the membrane

H3255 GR cells acquire an EGF R T790M mutation. The above findings suggested that gefitinib is unable to completely inhibit EGFR phosphorylation in the H3255 GR cell line. In light of recent observations that the presence of an E790M mutation is associated with acquired resistance to EGFR TKIs in patients (24, 25), the entire EGFR gene from H3255 GR was sequenced, and no changes from the parental cell line were identified (data not shown). However, the E790M gene is known to be amplified at least 20-fold (i.e., >40 copies) in H3255 cells, with preferential amplification of the mutant
L858R-containing allele (15, 16). Thus, the H3255 GR could poss-
se a resistant allele only in a fraction of the total E790M alleles that
would be undetected by direct sequencing. Therefore, we employed a more sensitive technique using SURVEYOR, a DNA endonuclease that cleaves DNA heteroduplexes 3′ of a mismatched site. HPLC separation of the DNA fragments was used to screen for low-frequency genetic alterations in H3255 GR. Our previous studies using SUR-
VEYOR demonstrated that this method can detect activating EGF R mutations even if the mutant allele comprises only 1%–5% of the
total alleles (34). We amplified exon 20 from H3255, H1975 (a cell
line with an endogenous T790M mutation; see Table 1), and H3255 GR, treated the PCR products with the SURVEYOR endonuclease,
and separated the digested products using HPLC (Figure 2A). H1975 is heterozygous for a SNP at position 787 (Q787Q) and for T790M,
and thus SURVEYOR digestion produced fragments that migrated as a doublet peak (153 and 154 bps; Figure 2A). These peaks were
not observed in H3255, which is homozygous for the SNP and has
no detectable T790M. However, in H3255 GR, a small peak was
observed at 154 bp following SURVEYOR treatment, suggesting the
presence of a new genetic alteration in exon 20 in this cell line.
Using denaturing HPLC (DHPLC) and fractionation, we enriched for the fractions containing the altered allele and sequenced the
fractions using previously described methods (34). The sequence
tracings from H3255 GR demonstrated the presence of a small
population of T790M containing alleles in this cell line (Figure 2B),
Figure 2
H3255 GR harbors a T790M mutation and is growth-inhibited by an irreversible EGFR inhibitor (CL-387,785) and EGFR-specific shRNA. (A) Exon 20 was amplified by PCR analysis, subjected to SURVEYOR analysis, and analyzed using the WAVE system (see Methods). Shown are HPLC tracings; numbers represent fragment size. Note the presence of a 154-bp fragment in H3255 GR, suggesting a T790M mutation (asterisks), as observed in the H1975 cells (positive control). A549 (dashed line) is shown as a negative control. (B) Exon 20 was amplified from A549 (negative control), H1975 (positive control), H3255, and H3255 GR cells. Products were separated at a partially denaturing temperature using the WAVE system. The indicated fractions (boxed areas) were directly sequenced (see Methods). The T790M mutation was observed in H3255 GR and H1975 cells (asterisks). (C) H3255 and H3255 GR cells were treated with increasing concentrations of CL-387,785 or gefitinib and subjected to an MTS survival assay. (D) Western blot analysis of H3255 and H3255 GR cells treated with increasing concentrations of CL-387,785. Blots of H3255 and H3255 GR extracts for each antibody are the same exposure from a single gel and blot; irrelevant lanes between the H3255 and H3255 GR extracts were omitted. (E) Western blot for PARP with H3255 and H3255 GR cells following treatment with gefitinib or CL-387,785 for 72 hours. (F) Lentiviral constructs containing EGFR shRNAs were infected into H3255 and H3255 GR cells, followed by an MTS assay. Growth was normalized to shRNA construct A (control), which did not cause significant EGFR downregulation (Supplemental Figure 4).
SURVEYOR was also able to detect this mutation in H3255 GR cDNA (Supplemental Figure 2). Using RT-PCR, we amplified exons 20 and 21 from H3255 GR and cloned the PCR products into pCR Blunt (Invitrogen), and 96 individual cDNA clones were sequenced. The T790M allele was the cDNA in 3.3% (3 of 91) of clones analyzed (Supplemental Figure 3). Using an enrichment strategy and screening with SURVEYOR, we verified the T790M mutation in 3 additional cDNA clones from H3255 GR.

Although the methodology used to produce this cell line and the stability of its resistance pattern to gefitinib after more than 15 passages in gefitinib-free media suggests a homogenous population, it remained to be determined whether the mutation was present in all cells in the H3255 GR cell line. Thus we attempted to subclone single cells but were unsuccessful despite culturing in a variety of conditions, including conditioned media. As an alternative approach, we performed PCR on 2 H3255 GR cells followed by SURVEYOR analyses (PCR was unsuccessful from single cells). Analyses of 2 cell PCR products from 8 independent pairs clearly demonstrated the T790M in 7 of 8 PCR products (88%). These findings suggest that in the H3255 GR cell line most, if not all, cells harbor a T790M-containing allele that is present on a small percent of the amplified alleles. This is consistent with the observation that most cells in the H3255 GR population clearly had persistent phosphorylation of the EGFR in the presence of gefitinib (Figure 1D).

The irreversible EGFR inhibitor CL-387,785 effectively inhibits the growth of H3255 GR cells. We examined the efficacy of CL-387,785 against H3255 GR cells, as this compound is more effective than gefitinib at inhibiting the expression of HER2 T790M mutant (23, 24). We found that CL-387,785 was more potent than gefitinib at inhibiting the growth of the H3255 GR cell line, while its effect on the parental cell line was similar to that of gefitinib (Figure 2C). Although CL-387,785 is effective against EGFR T790M, it inhibits non–T790M-containing EGFR more potently (24). This is consistent with its greater activity against H3255 compared with H3255 GR. CL-387,785 also inhibited the growth of H1975 cells (Table 1), results consistent with prior studies (25). CL-387,785 had no effect on EGFR T790M confers resistance to gefitinib more effectively when cis to an activating L858R or exon 19 deletion mutation. Both H3255 GR and H1975 cells contained the EGFR T790M mutation and displayed resistance to gefitinib. However, these studies did not address whether EGFR T790M is the sole mechanism of resistance in these cell lines or whether expression of a T790M mutant of EGFR is sufficient to confer a resistant phenotype to a gefitinib-sensitive NSCLC cell line. Thus, we retrovirally infected HCC827 and H3255 cell lines to stably express WT EGFR with a T790M mutation (WT/T790M) or EGFR with a T790M and an activating mutation (i.e., T790M cis to an L858R mutation [L858R/T790M] or an exon 19 deletion mutation [Del/T790M]; see Methods and Table 1). Total EGFR expression, as measured by quantitative RT-PCR, was increased by 1.3- to 2-fold compared with GFP-infected H3255 or HCC827 cells (Supplemental Table 1). Using both direct sequencing and SURVEYOR, we detected EGFR T790M in all of the cell lines. HCC827 cells infected with either GFP control or WT/T790M remained remarkably sensitive to gefitinib (Figure 3A). In contrast, expression of the double mutants L858R/T790M and Del/T790M rendered HCC827 cells resistant to gefitinib (Figure 3A), even though they were expressed at lower levels than WT/T790M (Supplemental Table 1). In fact, direct sequencing revealed that the T790M was a minor but detectable allele in these 2 double-mutant cell lines, consistent with the modest increase in total EGFR expression (Supplemental Table 1 and data not shown). Notably, resistance was promoted by the presence of a different activating EGFR mutation, L858R/T790M, even though the HCC827 cell line contains an EGFR exon 19 deletion (E746_A750; ref. 15). Similarly, stable expression of L858R/T790M or Del/T790M in H3255 rendered this cell line resistant to gefitinib (Figure 3B). Interestingly, although WT/T790M did not cause gefitinib resistance in HCC827 cells, it promoted partial gefitinib resistance—albeit to a lesser extent than when combined with an activating mutation—in H3255 cells. These results were observed in cells derived from 3 independent infections. Gefitinib treatment of HCC827 GFP and HCC827 WT/T790M led to similar inhibition of EGFR, ErbB-3, Akt, and ERK1/2 phosphorylation, with almost complete inhibition observed at concentrations greater than 10 nM (Figure 3C). In contrast, in both HCC827 L858R/T790M and HCC827 Del/T790M, gefitinib (up to 1 μM) was unable to inhibit EGFR phosphorylation and caused only minimal decreases in p-ErbB-3, p-Akt, and p-ERK1/2 (Figure 3C). This is consistent with the notion that the activating mutant EGFRs are more potent signaling proteins than WT EGFR, and thus when resistant to the effects of gefitinib by coexisting T790M mutations, they provide a greater oncogenic stimulus. In H3255 cells, gefitinib was able to partially inhibit p-EGFR, p-ErbB-3, and p-Akt in H3255 WT/T790M cells, while it had no effect on these signaling proteins in H3255 L858R/T790M or H3255 Del/T790M cells (Supplemental Figure 5).

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Figure 3
Expression of EGFR with a L858R/T790M or Del/T790M mutation more effectively promotes gefitinib resistance in HCC827 and H3255 cells. (A) The single-mutant WT/T790M (WT/T), the double-mutant L858R/T790M (L/T) and Del/T790M (Del/T), and GFP (as a control) were introduced into HCC827 cells. The resulting cell lines were subjected to an MTS survival assay in increasing concentrations of gefitinib. (B) The same retroviruses as in A were used to infect H3255 cells, and resulting cell lines were subjected to MTS survival assays in increasing doses of gefitinib. (C) Lysates from HCC827 cells expressing EGFR mutants or GFP control and exposed to increasing concentrations of gefitinib for 12 hours were probed with the indicated antibodies. (D) Xenografts in nude mice were generated from either HCC827 Del/T790M or HCC827 GFP cells. Gefitinib was administered by oral gavage, and tumors were measured 3 times weekly. Mean tumor volumes are shown. (E) The H3255 cell lines were subjected to an MTS survival assay in the presence of increasing concentrations of CL-387,785 and gefitinib. (F) H3255 L858R/T790M and H3255 GFP cells were seeded together at different ratios to determine whether a minor percent of L858R/T790M cells could confer resistance to cells with no T790M mutation. Cells were seeded in the indicated ratios (in parentheses) of H3255 GFP cells to H3255 L858R/T790M cells and subjected to an MTS survival assay.
These observations are consistent with gefitinib's effects on growth in these cell lines (Figure 3B).

Our observations from exogenous introduction of T790M into H3255 and HCC827 cells suggest that EGFR T790M more potently promotes resistance to gefitinib when present cis to the EGFR-activating mutation. In order to determine whether H1975 and H3255 GR also contain the T790M allele in cis to the activating mutation, we performed RT-PCR and sequenced clones from both cell lines. In both cell lines the T790M allele was only found cis to the L858R allele (6 of 6 independent clones in H3255 GR; 21 of 21 independent clones in H1975).

We also generated xenografts in nu/nu mice with HCC827 GFP and HCC827 Del/T790M cell lines and treated these mice with gefitinib (Figure 3D). H3255 did not grow as a xenograft (data not shown). Gefitinib treatment resulted in complete regression (as detected by palpation) of tumors in 5 of 5 HCC827 GFP mice (Figure 3D and Supplemental Figure 6). In contrast, HCC827 Del/T790M tumors were resistant to gefitinib. The comparison between HCC827 GFP and HCC827 Del/T790M growth patterns in mice treated with gefitinib was highly statistically significant (P = 0.0006).

Because the irreversible inhibitor CL-387,785 retains some activity against EGFR T790M, the cell lines stably expressing the T790M-containing constructs were exposed to CL-387,785 and subjected to an MTS survival assay. CL-387,785 treatment inhibited survival of both H3255 L858R/T790M and H3255 Del/T790M cells. Both H3255 GFP and H3255 WT/T790M were growth inhibited at low concentrations of CL-387,785 (Table 1). Similar findings were observed for analogous studies performed on the HCC827 cell lines treated with CL-387,785 (Supplemental Figure 7A and Table 1).

EGFR T790M does not confer resistance to surrounding cells via a paracrine effect. Previous studies have suggested that some EGFR TKI-resistant tumors contain a low percentage of T790M-containing cells (23), leading some to speculate that a small percent of resistant cells may confer resistance to the neighboring cells, perhaps via a paracrine effect. To formally test this hypothesis, we sought to determine whether the in vitro resistant H3255 L858R/T790M cells protect sensitive H3255 GFP cells from the effects of gefitinib. We generated mixtures of H3255 GFP with H3255 L858R/T790M in different ratios and examined the effects on growth using the MTS assay. As shown in Figure 3F, the cultures consisting of 10% L858R/T790M cells remained sensitive and those of 100% L858R/T790M cells remained resistant. These results suggest that there is no significant protection of non–T790M-containing cells by cocultured cells harboring a resistant T790M-containing clone. Similar findings were observed for the analogous studies performed on the HCC827 cell lines (Supplemental Figure 7B).

Oncogenic activation of the PI3K/Akt pathway confers resistance to gefitinib. In this and our previous study, we have observed that downregulation of the PI3K/Akt pathway correlates with sensitivity to gefitinib in NSCLC cell lines (26). Although we have demonstrated that an EGFR T790M mutation (when in cis to an EGFR-activating mutation) confers gefitinib resistance and maintains PI3K/Akt signaling, it remains unknown whether continued PI3K/Akt signaling alone is sufficient to make these cancers resistant to gefitinib. Somatic mutations in the PIK3CA gene, which encodes for the p110α catalytic subunit of class IA PI3Ks, have been identified in approximately 5% of NSCLCs (35). We and others have shown that these mutations result in sustained Akt activation under serum-deprived conditions (35–38). To determine whether downregulation of PI3K/Akt signaling is necessary for an EGFR mutant NSCLC cell to be sensitive to gefitinib, we introduced the p110α E545K into HCC827 cells via retroviral infection. HCC827 cell lines harboring activated PI3K (HCC827 p110α E545K) were more resistant to gefitinib in vitro (IC50, 1 μM) compared with those harboring GFP alone (IC50, 0.01 μM; Figure 4A and Table 1). The presence of p110α E545K also protected HCC827 cells from gefitinib-mediated apoptosis, as assessed by either flow cytometry or PARP cleavage (Figure 4, B and C). As shown in Figure 4D, the oncogenic p110α E545K maintained some Akt phosphorylation in the presence of gefitinib (35% of untreated GFP cells and 10% of untreated p110α E545K cells). Additionally, we observed that expression of constitutively active Akt (ca-Akt) also conferred resistance to gefitinib-mediated cell killing (Figure 4, A and E). Therefore, continued activation of PI3K/Akt signaling is sufficient to confer resistance to gefitinib.

Discussion

In the present study, we examined mechanisms of resistance to gefitinib in highly sensitive EGFR mutant and amplified NSCLC cell lines. Prior studies suggest that about 50% of NSCLC patients who initially respond to gefitinib or erlotinib and subsequently develop clinical resistance to EGFR TKIs acquire a detectable secondary T790M mutation in EGFR (22–24). The H3255 GR cell line, a model of acquired resistance to gefitinib, remarkably recapitulated this clinical observation in vitro, as the T790M mutation was detected only in this cell line, not in the parental H3255 cell line. A surprising observation from our studies was the dominant effect of a low fraction of T790M-containing alleles in the H3255 GR cell line. H3255 is amplified more than 20-fold at the EGFR locus. Gefitinib treatment continued to inhibit the majority of EGFR alleles in H3255 GR cells, as demonstrated by the decrease in p-EGFR detected by Western blot analysis and immunofluorescence (Figure 1, C and D). This suggests that the T790M-containing allele(s) can act in a dominant fashion allowing continued maintenance of EGFR, ErbB-3/PI3K/Akt, and ERK1/2 phosphorylation in the H3255 GR cell line and inhibition of apoptosis (Figure 1, B and C, and Figure 2E). Although T790M EGFR is resistant to gefitinib’s inhibitor effects, the T790M mutant may also make EGFR a more potent signaling protein. It has previously been demonstrated that there is greater constitutive EGFR autophosphorylation of a T790M mutant EGFR compared with WT EGFR — more specifically, by EGFR L858R/T790M compared with EGFR L858R when expressed in Ba/F3 cells (25, 39). Additionally, the recent report that germline EGF T790M mutations are linked with familial lung cancers underscores the increased activity of the T790M mutant (40). Furthermore, cell lines (such as H1975) and patients with L858R/T790M who never received gefitinib treatment have been described, suggesting an independent, oncogenic role for the T790M mutation (41). These observations may account for the resistance promoted by only a small fraction of EGFR L858R/T790M alleles in H3255 GR cells. Furthermore, these findings are consistent with the observed differences in gefitinib resistance when T790M was introduced in cis compared with trans with an activating EGFR mutation into HCC827 and H3255 cell lines.

Since EGFR is amplified in the H3255 cell line, the presence of the secondary mutation in a minority of EGFR alleles in H3255 GR was initially not detected using conventional direct sequencing and was only detectable using a more sensitive enzymatic technique. Our findings suggest that the prevalence of the T790M mutation as a mechanism of resistance to gefitinib and erlotinib may be
more common than previously described. To this end, we have now identified 3 patients who did not have a T790M mutation detected by direct sequencing in whom SURVEYOR detected the presence of a T790M mutation (Table 2). Two of the patients were treated with gefitinib and had T790M present only in the relapsed tumor specimen, while in the remaining patient, T790M was present in a pretreatment tumor specimen. In patient 3, the T790M mutation was detected only in 2 of 5 independent direct sequencing reactions from the relapsed specimen (Table 2). Therefore, direct sequencing alone is not a sensitive enough method to detect the T790M mutation in patients with NSCLC. The findings in this study suggest that the presence of rare T790M-containing sequences in some gefitinib-resistant NSCLC tumors are due to rare T790M-containing alleles in most tumor cells, not to rare T790M-containing cells within the tumor (23). We demonstrated that these T790M alleles have clinical significance and are sufficient to confer resistance to gefitinib. To date, the finding of a limited number of resistant alleles in the amplified locus appears unique to EGFR in lung cancers. The majority of EGFR mutant NSCLC tumors and cell lines contain concurrent EGFR amplifications (13–16). In contrast, amplification of KIT or PDGFRα in gastrointestinal stromal cell tumors or BCR-Abl in chronic myelogenous leukemia occurs only in the minority of imatinib-resistant patients (20, 21, 42–44). These findings have potential implications for detecting resistance mutations in other oncogenes associated with amplification, such as ErbB-2 in breast cancer and EGFR in glioblastoma multiforme (45, 46). Recent studies have used genome-wide sequencing efforts to identify mutations in the entire family of kinases in human cancers (8, 47). Our findings suggest that sequencing alone may be insufficient to identify all functionally and clinically significant mutations, such as T790M, and should be combined with more sensitive techniques of mutation detection.

The irreversible EGFR inhibitor CL-387,785, which binds covalently to cys-773 of EGFR, retains some ability to inhibit EGFR containing the T790M mutation, causes growth inhibition and downregulation of the ErbB-3/PI3K/Akt axis, and induces apoptosis in T790M-containing H3255 GR. Of note, the H3255 GR cells retained PTEN expression, had not acquired a PIK3CA gene mutation, and demonstrated decreased p-Akt levels and loss of PI3K–ErbB-3 interaction upon exposure to CL-387,785 (Figure 2D and data not shown). These findings are consistent with — but do not absolutely prove — the notion that PI3K/Akt signaling remains under the control of EGFR (via ErbB-3) as in the parental H3255 cells. The acquisition of the T790M allele is likely what confers continued signaling in the presence of gefitinib. We further demonstrated, using oncogenic p110α E545K and ca-Akt, that downregulation of the PI3K/Akt signaling pathway not only correlated with sensitivity to gefitinib, but was required for gefitinib to promote its proapoptotic effect. Thus, continued activation of the PI3K/Akt pathway either through EGFR-dependent (T790M) or independent mechanisms is sufficient to render an EGFR mutant NSCLC resistant to gefitinib. This distinction has clinical relevance and may assist in guiding therapeutic decisions for lung cancer patients developing acquired resistance to gefitinib. Patients with EGFR-dependent mechanisms of resistance to gefitinib may still clinically benefit from a newer generation of EGFR inhibitors, which are presently undergoing clinical evaluation (24, 25). However, those with EGFR-independent mechanisms of resistance may need alternative forms of treatment.

Other recent studies have observed that persistent PI3K signaling confers resistance to ErbB inhibitors in other cellular contexts. For example, the efficacy of the anti–ErbB-2 monoclonal antibody trastuzumab is significantly reduced in patients with ErbB-2–amplified breast cancer that lack PTEN (48). Similarly, EGFR TKIs are ineffective in glioblastoma tumors and cell lines as well as in breast cancer.
cer cell lines that lack PTEN expression (18, 45). Restoration of PTEN expression into PTEN mutant MDA-468 breast cancer cells can sensitize them to gefitinib, and downregulation of PTEN using shRNA in the EGFR TKI–sensitive A431 cells (amplified WT EGFR) reduces the apoptosis induced by either erlotinib or the EGFR/ErbB-2 TKI PKI-166 (45). Our observations that continued PI3K signaling conferred resistance to gefitinib even in these EGFR mutant and amplified lung cancers further strengthens the concept that downregulation of PI3K signaling is necessary for ErbB inhibitors to be effective. Furthermore, this is the first study to our knowledge to observe that the recently identified PIK3CA oncogenes are sufficient to promote continued PI3K signaling in gefitinib-sensitive cancer cells treated with gefitinib. Thus, these studies predict that cancers with PIK3CA oncogenic mutations are unlikely to respond to EGFR inhibitors.

Our experimental findings that in vitro and in vivo resistance was generated by introducing EGFR-activating mutations in cis with the T790M mutation provide several important observations regarding the biology of EGFR mutations and EGFR inhibitors. They confirm that EGFR is the bona fide target of gefitinib in these cell lines, as introduction of a mutant EGFR conferred resistance to gefitinib. Interestingly, WT/T790M was able to exert partial resistance to gefitinib in H3255 cells (which naturally contains a L858R mutation), while HCC827 cells (which naturally harbor an exon 19 deletion) remained sensitive to gefitinib (Figure 3, A and B). Recent clinical studies have also demonstrated that EGFR inhibitors are more effective in patients with exon 19 deletion mutations than in those with L858R mutations (49, 50). Patients with exon 19 deletion mutations have significantly slower disease progression and longer overall survival when treated with gefitinib or erlotinib compared with those with L858R mutations. Accordingly, both H3255 and HCC827 are gefitinib-sensitive cell lines, the HCC827 cells are substantially more sensitive to gefitinib and CL-387,785 (Table 1). Thus it is tempting to speculate that L858R-containing lung cancer cells and tumors are relatively more resistant to gefitinib and that introduction of WT/T790M is a sufficient boost to render them partially resistant to gefitinib. In contrast, the modest amount of resistance promoted by a WT/ T790M allele was inconsequential in the highly sensitive exon 19 deletion cancer cells.

Finally, our studies also provide models in which to test future EGFR inhibitors. Treatment of H3255 Del/T790M, H3255 L858R/T790M, and H3255 GR with CL-387,785 did not restore their sensitivity to that observed with the parental cell line (compare Figure 2C with Figure 3, A and E). Identification of an EGFR inhibitor that is more effective than CL-387,785 in vitro would aid in the prioritization of such an agent for clinical trials for patients who develop T790M-mediated resistance to gefitinib.

Methods

Cell culture and reagents. The EGFR mutant NSCLC cell lines H3255, HCC827, and H1975 (which naturally harbor L858R, del E746_A750, and L858R/T790M mutations, respectively) were used in this study and have been extensively characterized previously (8, 15, 16, 22, 30, 51). H3255 and HCC827 are heterozygous for an EGFR mutation and are amplified at the EGFR locus (15, 16). Gefitinib was a gift of AstraZeneca. Stock solutions of 10 mM gefitinib were prepared in DMSO and stored at −20°C. CL-387,785 was purchased from Calbiochem.

Anti–p-Akt (Ser-473), anti–total Akt, anti-EGFR, and anti–p–ErbB-3 (Tyr-1289) antibodies were obtained from Cell Signaling Technology. Anti-ErbB-3 antibody was obtained from Lab Vision Corp. The p-EGFR (pY1068), total ERK1/2, and p-ERK1/2 (pT185/pY187) antibodies were purchased from BioSource International Inc. Anti-p85 antibody was obtained from Upstate USA Inc.

Cell proliferation and growth assays. Growth and inhibition of growth were assessed by MTS assay. This assay, a colorimetric method for determining the number of viable cells based on the bioreduction of MTS by cells to a formazan product that is soluble in cell culture medium and can be detected spectrophotometrically, was performed according to previously established methods (8, 16, 30). The cells were exposed to treatment for 72 hours, and the number of cells used per experiment was determined empirically and has been as previously established (16, 30). All experimental points were set up in 6–12 wells. Each concentration of drug was performed 6 times, and the mean and SD are shown. Independent experiments were performed at least 3 times, and representative results are shown. The data were graphically displayed using GraphPad Prism version 3.00 for Windows (GraphPad Software). The curves were fitted using a nonlinear regression model with a sigmoidal dose response.

Immunoblotting. Cells grown under the previously specified conditions were lysed in the following lysis buffer: 20 mM Tris, pH 7.4; 150 mM NaCl; 1% Nonidet P-40; 10% glycerol; 1 mM EDTA; 1 mM EGTA; 5 mM sodium pyrophosphate; 50 mM NaF; 10 mM β-glycerophosphate; 1 mM sodium vanadate; 0.5 mM DTT; 4 μg/ml leupeptin; 4 μg/ml pepstatin; 4 μg/ml apro- protein; and 1 mM PMSF. After cell lysis, lysates were centrifuged at 16,000 g for 5 minutes at 4°C. The supernatant was used for subsequent procedures. Western blot analyses were conducted after separation by SDS/PAGE electrophoresis and transfer to nitrocellulose membranes. Immunoblotting was performed according to the antibody manufacturers’ recommendations. Antibody binding was detected using an enhanced chemiluminescence system (New England Nuclear Life Science Products Inc.). Bands were quantified using NIH ImageJ version 1.36b software (http://rsb.info.nih.gov/ij/). Multiple film exposures were used for quantification so that comparisons were made between lanes within the linear range of the film.

Generation of in vitro H3255 GR. In order to generate a resistant cell line, H3255 cells were exposed to increasing concentrations of gefitinib in a manner similar to previously described methods using imatinib (28). The cells

<table>
<thead>
<tr>
<th>Patient</th>
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<th>Treatment duration</th>
<th>Pretreatment EGFR mutation</th>
<th>EGFR mutation at disease progression</th>
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<td>L747,E749</td>
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<td>2</td>
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<td>E746_A750</td>
<td>L746_A750</td>
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<tr>
<td>3</td>
<td>Yes</td>
<td>14 mo</td>
<td>E746_A750</td>
<td>L746_A750</td>
</tr>
</tbody>
</table>

Shown are 3 patients in whom EGFR T790M was detected using the SURVEYOR analyses but not detected by direct sequencing. All specimens in which SURVEYOR indicated a T790M mutation were subjected to DHPLC, fractionation, and sequencing as previously described (32). T790M was detected by direct sequencing in 2 of 5 independent sequence tracings.
were initially treated with 40 nM gefitinib, equivalent to IC_{50} in parental H3255 (Figure 1A). The gefitinib concentrations was increased stepwise to 55 nM, 75 nM, and 100 nM, equivalent to IC_{50}, IC_{90}, and IC_{99} in parental cells, respectively, when the cells reached near-normal growth kinetics. The cells that were able to grow in 100 nM gefitinib were obtained 6 months after initial drug exposure. To confirm the emergence of a resistant clone, MTS assays were performed following growth at each concentration after allowing the cells to grow in drug-free condition for at least 4 days. H3255 cells were maintained concomitantly without gefitinib, and their sensitivity to gefitinib was examined every 5 passages. There was no significant change in the sensitivity to gefitinib in parental cells during the period (data not shown).

SURVEYOR analyses. EGFR exon 20 was amplified using previously described primers and conditions (34). Following PCR amplification, the product was cloned into pCRII-TOPO (Invitrogen) and sequenced with forward and reverse primers. SURVEYOR analyses also confirmed the L858R mutations in H3255 and H1975 DNA (data not shown).

PCR analyses of H3255 GR cells. H3255 GR cells were counted by Coulter counter and seeded in PBS at 20 cells/well in 96-well plates. The number of cells per well was confirmed by counting the number of cells per well in a replica plate seeded in growth media. One-tenth the volume (2 μl) was spiked into PBS (55). The experiment was terminated when the mean tumor volume was 400–500 mm³. Gefitinib was administered at 150 mg/kg/d by daily oral gavage. This dose schedule was previously reported to be highly efficacious in other tumor models, and doses were set at or below the respective maximum well-tolerated doses in nude mice (55). The experiment was terminated when the mean size of the control tumors reached 2,000 mm³.

PI3K assay. The PI3K assays were performed according to previously described methods (37). ErbB-3 was immunoprecipitated overnight at 4°C from either control or gefitinib-treated NSCLC cell lines. The immunoprecipitate was captured on protein G-Sepharose beads. Half of the immunoprecipitate was used for Western blot analysis, and the other half was used for PI3K assays; each was performed in triplicate. The relative amounts of ErbB-3 in the immunoprecipitates were determined by Western blot analyses using the LI-COR Odyssey protein quantification system. The average and standard deviation of the PI3K activity normalized to the amount of ErbB-3 protein is shown in relative units as a percent of control (no gefitinib).

EGFR immunofluorescence. The p-EGFR (pY1068) antibody (Cell Signaling Technology) was used for these studies according to the manufacturer’s recommendations. Cells were plated on coverslips and, following gefitinib and/or EGF treatment (100 ng/ml), fixed using 4% paraformaldehyde. The coverslips were washed, blocked in blocking buffer (5% goat serum, 1% BSA, 0.02% NaN₃, and 1x PBS) for 45 minutes at room temperature, and stained with the p-EGFR antibody overnight at 4°C according to the manufacturer’s recommended conditions. Alexa Fluor 594 goat anti-mouse secondary antibody (Invitrogen) was used at a 1:500 dilution. Images were obtained on a Nikon TE2000 with a PerkinElmer Spinning Disk Confocal Microscope.

Statistics. In order to compare effects of gefitinib in the different xenografts, the data were transformed into the log scale for analysis in order to approximate a linear fit, reflecting the exponential growth of tumors. Some tumors of the gefitinib-treated mice in the HCC827 GFP model had shrunk to the extent that they were no longer measurable. Their tumor volumes were recoded arbitrarily as 0.01 mm³, that is, 2 logs lower than the smallest nonzero tumor volume. As the animals were randomized among treatment groups, an overall mean was assumed for the population of mouse-specific intercepts in each xenograft model. Linear and quadratic terms were fitted as regression effects of treatment in order to model the

expression levels in the transfected cells were analyzed by quantitative real-time PCR using previously established methods (54). EGFR shRNA constructs and lentiviral infection. EGFR shRNA constructs were cloned in plKO.1 puro vector were obtained from Harvard RNAi consortium. Each construct contained a 21-bp sequence targeting different regions of EGFR, a 6-nucleotide hairpin sequence (CTCGAG), and a 21-bp complementary strand sequence. shRNA constructs B and C targeted the 3’ untranslated region of EGFR, while constructs A, D, and E targeted the coding region. The specific shRNA sequences are shown in Supplemental Methods. Lentivirus production and infections were performed as previously described (26). MTS assays were performed 7 days after infection. Results were unchanged when performed under puromycin selection.

 Xenograft studies. Nude mice (nu/nu; 6–8 weeks old; Charles River Laboratories) were used for in vivo studies and were cared for in accordance with the standards of the Institutional Animal Care and Use Committee (IACUC) under a protocol approved by the Animal Care and Use Committee of Children’s Hospital Boston. Mice were anesthetized using a 2% isoflurane (Baxter) inhalation oxygen mixture. A suspension of 5 × 10⁶ HCC827 GFP or HCC827 Del/T790M lung cancer cells (in 0.2 ml PBS) were inoculated subcutaneously into the lower-right quadrant of the flank of each mouse. Five mice were inoculated with either HCC827 GFP or HCC827 Del/T790M cells in the gefitinib treatment group. Tumors were measured twice weekly using calipers, and volume was calculated as length × width² × 0.52. Mice were monitored daily for body weight and general condition. Mice were randomized to treatment when the mean tumor volume was 400–500 mm³. Gefitinib was administered at 150 mg/kg/d by daily oral gavage. This dose schedule was previously reported to be highly efficacious in other tumor models, and doses were set at or below the respective maximum well-tolerated doses in nude mice (55). The experiment was terminated when the mean size of the control tumors reached 2,000 mm³.
initial dip in tumor growth observed in the gefitinib-treated group of the HCC827 Del/T790M model. Growth patterns between groups were compared by the appropriate contrast of fixed regression effects using inference based on the F test. Estimation by restricted maximum likelihood was computed using SAS (version 9.1; SAS Institute Inc.), and model fit was assessed by Akaika information criterion.

The paired Student's t test was used to determine whether growth following shRNA infection of control B was different compared with that following infection of control construct A. Statistical analyses were performed using StatView (version 5.0; SAS Institute Inc.). All statistical tests were 2-sided. A P value less than 0.05 was considered to be statistically significant.

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