

DIGITAL ACCESS TO
SCHOLARSHIP AT HARVARD**BIM Mediates EGFR Tyrosine Kinase Inhibitor-Induced
Apoptosis in Lung Cancers with Oncogenic EGFR Mutations**

The Harvard community has made this article openly available.
[Please share](#) how this access benefits you. Your story matters.

| | |
|--------------------------|--|
| Citation | Costa, Daniel B., Balázs Halmos, Amit Kumar, Susan T. Schumer, Mark S. Huberman, Titus J. Boggon, Daniel G. Tenen, and Susumu Kobayashi. 2007. BIM mediates EGFR tyrosine kinase inhibitor-induced apoptosis in lung cancers with oncogenic EGFR mutations. <i>PLoS Medicine</i> 4, no. 10: e315. |
| Published Version | doi://10.1371/journal.pmed.0040315 |
| Accessed | February 19, 2015 4:14:15 AM EST |
| Citable Link | http://nrs.harvard.edu/urn-3:HUL.InstRepos:4817340 |
| Terms of Use | This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA |

(Article begins on next page)

BIM Mediates EGFR Tyrosine Kinase Inhibitor-Induced Apoptosis in Lung Cancers with Oncogenic EGFR Mutations

Daniel B. Costa¹, Balázs Halmos², Amit Kumar³, Susan T. Schumer¹, Mark S. Huberman¹, Titus J. Boggon³, Daniel G. Tenen¹, Susumu Kobayashi^{1*}

1 Division of Hematology/Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, United States of America, **2** University Hospitals of Cleveland and Case Western Reserve University, Cleveland, Ohio, United States of America, **3** Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut, United States of America

Funding: See section at end of manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Academic Editor: Ingo K. Mellinger, University of California Los Angeles, United States of America

Citation: Costa DB, Halmos B, Kumar A, Schumer ST, Huberman MS, et al. (2007) BIM mediates EGFR tyrosine kinase inhibitor-induced apoptosis in lung cancers with oncogenic EGFR mutations. *PLoS Med* 4(10): e315. doi:10.1371/journal.pmed.0040315

Received: May 11, 2007
Accepted: September 14, 2007
Published: October 30, 2007

Copyright: © 2007 Costa et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abbreviations: CML, chronic myelogenous leukemia; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated protein kinase; GIST, gastrointestinal stromal tumors; MAPK, mitogen-activated protein kinase; NSCLC, non-small cell lung cancer; PARP, poly (ADP-ribose) polymerase; SD, standard deviation; siRNA, small interfering RNA; TKI, tyrosine kinase inhibitor; WT, wild type

* To whom correspondence should be addressed. E-mail: skobayas@bidmc.harvard.edu

ABSTRACT

Background

Epidermal growth factor receptor (EGFR) mutations are present in the majority of patients with non-small cell lung cancer (NSCLC) responsive to the EGFR tyrosine kinase inhibitors (TKIs) gefitinib or erlotinib. These EGFR-dependent tumors eventually become TKI resistant, and the common secondary T790M mutation accounts for half the tumors with acquired resistance to gefitinib. However, the key proapoptotic proteins involved in TKI-induced cell death and other secondary mutations involved in resistance remain unclear. The objective of this study was to identify the mechanism of EGFR TKI-induced apoptosis and secondary resistant mutations that affect this process.

Methods and Findings

To study TKI-induced cell death and mechanisms of resistance, we used lung cancer cell lines (with or without *EGFR* mutations), Ba/F3 cells stably transfected with *EGFR* mutation constructs, and tumor samples from a gefitinib-resistant patient. Here we show that up-regulation of the BH3-only polypeptide BIM (also known as BCL2-like 11) correlated with gefitinib-induced apoptosis in gefitinib-sensitive *EGFR*-mutant lung cancer cells. The T790M mutation blocked gefitinib-induced up-regulation of BIM and apoptosis. This blockade was overcome by the irreversible TKI CL-387,785. Knockdown of BIM by small interfering RNA was able to attenuate apoptosis induced by EGFR TKIs. Furthermore, from a gefitinib-resistant patient carrying the activating L858R mutation, we identified a novel secondary resistant mutation, L747S in cis to the activating mutation, which attenuated the up-regulation of BIM and reduced apoptosis.

Conclusions

Our results provide evidence that BIM is involved in TKI-induced apoptosis in sensitive *EGFR*-mutant cells and that both attenuation of the up-regulation of BIM and resistance to gefitinib-induced apoptosis are seen in models that contain the common EGFR T790M and the novel L747S secondary resistance mutations. These findings also suggest that induction of BIM may have a role in the treatment of TKI-resistant tumors.

The Editors' Summary of this article follows the references.



Introduction

Sequencing of the *epidermal growth factor receptor* (*EGFR*) gene in a large number of tumor samples has identified somatic activating mutations in the tyrosine-kinase pocket of *EGFR* [1,2]. These mutations were first described in non-small cell lung cancer (NSCLC) patients treated with specific *EGFR* tyrosine kinase inhibitors (TKIs)—gefitinib and erlotinib—who had radiographic and clinical responses to such agents [3–5]. Two recent transgenic mouse models, in which the overexpression of *EGFR* mutations was targeted in type II pneumocytes, demonstrated that these mutations led to the development of adenocarcinomas and that the tumors responded both to suppression of the *EGFR* driving signal and *EGFR* TKIs [6,7]. As data accumulate, it seems clear that *EGFR*-mutant “oncogene-addicted” cancers represent a distinct form of NSCLC that can be targeted through novel approaches [8]. The tumor cells are dependent on, or addicted to, the *EGFR* mutated oncogene for both maintenance of the malignant phenotype and cell survival. At the time of this writing, phase II trials in which patients with advanced NSCLC are included on the basis of presence of the two most common *EGFR* mutations (either exon 19 deletions or the exon 21 arginine-for-leucine substitution at amino acid 858, or L858R) and are given gefitinib as first-line treatment show radiographic response rates that exceed 75% [9–11]. Mature results of such trials will likely confirm the improved time to progression and survival seen in retrospective studies of patients treated with TKIs in which *EGFR* mutations had been identified [12–15].

Despite the unprecedented responses seen in these specific *EGFR*-mutant tumors, most eventually become resistant to the TKIs and disease progression is noted. Our group and others have identified a second mutation in the *EGFR* kinase domain (the exon 20 methionine to threonine substitution at position 790, or T790M) in repeat tissue samples from patients who initially responded to TKIs but later progressed [16,17]. The two largest cohorts of patients with TKI-resistant NSCLCs, in which a second biopsy was obtained after progression, identified the T790M mutation in around 50% of the samples and one D761 secondary mutation [18,19]. Recently, in four out of 18 (22%) TKI-resistant *EGFR*-mutant tumors, amplification of another oncogene, *MET*, was identified [20]. Other secondary mutations and alternative mechanisms of resistance have not been completely clarified.

One of the major effects of TKIs in sensitive *EGFR*-mutant cell lines is their induction of apoptosis. The exquisite sensitivity of these NSCLCs to gefitinib and erlotinib [3–5] has been supported by the concept of “oncogene addiction” [6,7,21]. A recent report suggested that a common signaling cascade may be involved during apoptosis in cells that depend on oncogenic *SRC*, *BCR-ABL*, and mutant *EGFR* [22]. Interestingly, the BH3-only proapoptotic proteins BIM (also referred to as BCL2-like 11, or BCL2L11), and to a lesser extent BAD (BCL2 antagonist of cell death), mediate imatinib-induced apoptosis of *BCR-ABL* leukemic cells [23]. The key downstream mediators of TKI-induced cell death in *EGFR*-mutant tumors remain unknown. We hypothesized that the BH3-only members might be involved in the apoptotic signal following *EGFR* disruption by TKIs.

In this study we studied BIM's role in TKI-induced apoptosis in *EGFR*-mutant lung cancers. In addition, we

investigated the effect of the resistant mutation T790M and a novel secondary mutation, L747S, on the regulation of BIM and apoptosis.

Methods

Patient Characteristics and Clinical Course after TKI Treatment

Two *EGFR* mutation-positive patients with gefitinib-resistant NSCLCs and secondary *EGFR* mutations were identified from our Thoracic Oncology Clinic database. Their clinical and molecular characteristics, as well as their response to TKI treatment, are detailed in Table S1. Both patients are part of an Institutional Review Board-approved protocol, and written informed consent was obtained for the analysis of their tumors.

Reagents

Gefitinib and erlotinib were purchased from a commercial supplier. CL-387,785 was purchased from Calbiochem (Darmstadt, Germany). Stock solutions for gefitinib, erlotinib, and CL-387,785 were prepared as previously described [16].

Sequencing of the *EGFR* Gene

Both genomic DNA and total RNA were extracted from the tumor cells of a transbronchial biopsy and of pleural fluid in patients 1 and 2 (Table S1), respectively. Genomic DNA was used as a template for sequencing exons 18–21 as previously published [3]. cDNA was transcribed from 1 µg of total RNA with Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). The cDNA was used as a template for subsequent PCR amplifications of *EGFR*. The kinase domain of the *EGFR* coding region was amplified by the use of two sets of oligonucleotides and sequenced: (1) sense primer (5'-GCA CAG GAC GGG GAC CAG ACA ACT-3') and antisense primer (5'-GGA CAT AGT CCA GGA GGC AG-3'); (2) sense primer (5'-GCA CAG GAC GGG GAC CAG ACA ACT-3') and antisense primer (5'-ATG GGT GGC TGA GGG AGG CGT TCT-3'). The PCR products containing exons 19–21 amplified by the use of the latter set of primers were subcloned into the pGEM-T Easy cloning vector (Invitrogen) and sequenced [16].

Cell Culture

Ba/F3 cell lines were maintained in RPMI supplemented with 10% FBS and 5% WEHI conditioned medium as the source of IL3. The human lung cancer-derived cell lines A549, NCI-H460 (H460), NCI-H1975 (H1975), NCI-H3255 (H3255), PC-9, and HCC827 were maintained in RPMI supplemented with 10% FBS.

EGFR Mutant Constructs and Transfections

The L747S mutation was introduced into human *EGFR* wild-type (WT) or L858R constructs in the context of the pcDNA3.1 expression vector (Invitrogen) [16] using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The oligonucleotide sequences were as follows: sense primer, 5'-CGT CGC TAT CAA GGA ATC AAG AGA AGC AAC ATC TC-3'; antisense primer, 5'-GAG ATG TTG CTT CTC TTG ATT CCT TGA TAG CGA CG-3'. The resulting constructs were confirmed by sequencing. For transient transfection experiments, COS-7 cells were plated at a concentration of 5×10^4 cells per well in six-well plates. The following day, these cells were transfected with 1 µg of

the expression constructs using Fugene 6 (Roche) and incubated for 12 h when the medium was changed to serum-free. After 12 h of serum starvation, cells were stimulated with 100 ng/ml EGF (Sigma). TKIs were added to the culture medium 3 h prior to the addition of EGF. Cells were exposed to EGF for 15 min. Stable Ba/F3 cell and HCC827 cell lines carrying WT or other mutant EGFR were generated and maintained as previously described [24,25].

Western Blotting and Antibodies

Whole-cell lysates were prepared as previously described [16,26]. The human lung cancer cell lines were treated in RPMI supplemented with 10% FBS in the presence of EGFR inhibitors as indicated. Ba/F3 cells were washed three times with RPMI only and stimulated by EGF as previously described [24]. Gefitinib, erlotinib, or CL-387,785 at increasing concentrations were added to the medium as indicated in the figure legends.

EGFR, caspase-3, BCL-x_L, Bcl2 (mouse specific), and total STAT5 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Total extracellular signal-regulated protein kinase (ERK) antibody was purchased from BD Transduction Laboratories (Lexington, KY). Phospho-EGFR (pTyr1068), phospho-STAT5 (pTyr694), phospho-AKT (pS473), phospho-ERK1/2 (pT202/pY204), BCL2 (human specific), BIM, BAD, phospho-BAD (pS112), poly (ADP-ribose) polymerase (PARP), cleaved-PARP, and AKT antibodies were purchased from Cell Signaling Technology (Beverly, MA). BIM antibody was also purchased from Stressgen (Victoria, Canada). Actin antibody was purchased from Sigma (St. Louis, MO).

Cell Proliferation Assay

Cell counts were performed at daily intervals using Trypan blue dye exclusion. Growth inhibition was assessed by CellTiter 96 Aqueous One solution proliferation kit (Promega, Madison, WI) [24]. Briefly, Ba/F3 stable cells were washed three times with RPMI 1640 only and resuspended in RPMI 1640 supplemented with 10% FBS and 20 ng/ml EGF (Sigma, St. Louis, MO). Then, cells were transferred to triplicate wells at 10,000 cells/well in 96-well flat-bottom plates with various concentrations of inhibitors and the cells were incubated for 48 h.

Flow Cytometric Analysis of Cell-surface Exposure of Phosphatidylserine and Mitochondrial Membrane Potential

For flow cytometric analysis, cells were plated at 1×10^5 /well in six-well plates and treated with DMSO or EGFR inhibitors. Cell-surface exposure of phosphatidylserine was assessed using an Annexin-V-FLUOS staining kit (Roche, Basel, Switzerland) as previously described [24]. For assessment of mitochondrial membrane potential, Ba/F3 cells were incubated with 40 nM DiOC6(3) (Molecular Probes, Eugene, OR) in PBS for 15 min at room temperature as previously described [27].

RNA Interference

BIM-specific and negative control small interfering RNAs (siRNAs) were purchased from Cell Signaling Technology and Dharmacon Research (Lafayette, CO), respectively. Cells were transfected with TransIT-TKO transfection reagent (Mirus,

Madison, WI) according to the manufacturer's protocol in the presence of siRNAs. After 24 h of transfection, cells were washed with RPMI twice and incubated with RPMI containing 10% FBS in the presence of DMSO (control), gefitinib, or CL387,785 for 48 h.

Statistical Analysis

The paired Student t-test was used to determine statistical significance. A *p*-value less than 0.01 was considered significant.

Results

Apoptosis in NSCLCs with and without EGFR Mutations

We selected a set of NSCLC cell lines to identify the differential sensitivity of wild-type (WT) EGFR and mutant tumors to the apoptotic effects of gefitinib. A549 and H460 have WT EGFR and are highly resistant to gefitinib (reported gefitinib sensitivity for proliferation [IC₅₀] are 9.6 and 12.9 μM, respectively) [28], whereas H1650 and HCC827 have a deletion in exon 19 (delE746-A750) with different gefitinib sensitivities (IC₅₀: 1 and 0.005 μM, respectively) [28]. H3255 carries the L858R EGFR exon 21 point mutation and has an IC₅₀ of 0.015 μM to gefitinib [28]. In addition, we also tested PC-9 cells, which have the delE746-A750 EGFR mutation and are sensitive to reversible EGFR tyrosine kinase inhibitors [22].

After 48 hours of 1 μM gefitinib treatment, all the EGFR mutant cell lines showed an increase in the percentage of apoptotic cells when compared to untreated cells (Figure 1). However, the HCC827, H3255, and PC-9 cells had a greater increase in the amount of apoptotic cells after treatment than did the H1650 cells. The non-EGFR mutant A549 and H460 cells displayed almost no changes in the number of apoptotic cells after gefitinib treatment (Figure 1).

Up-Regulation of BIM in EGFR-Mutant NSCLCs Sensitive to Gefitinib

Knowledge of the differential degrees of apoptotic induction by gefitinib in these cell lines prompted us to test our hypothesis that the BH3-only BIM is involved in the cell death execution process mediated by TKIs. The BIM gene encodes three major isoforms: BIM short (BIM_s), BIM long (BIM_L), and BIM extra long (BIM_{EL}). All isoforms contain a BH3 domain that can bind to and inactivate members of the antiapoptotic BCL2 family of proteins [29].

As shown in Figure 2, gefitinib treatment induced rapid and sustained increase in the levels of BIM_{EL} and BIM_L in HCC827, H3255 and PC-9. Two major pathways regulate BIM expression and/or function: the PI3K-AKT-FOXO and the ERK1/2 mitogen-activated protein kinase (MAPK) pathways [29,30]. Consistent with these reports, we detected that the relative electrophoretic migration BIM_{EL} and BIM_L was faster when HCC827, H3255, and PC-9 cells were treated with gefitinib, which suggests that BIM_{EL} and BIM_L were hypophosphorylated due to loss of AKT and/or ERK activity (Figure 2), or other EGFR downstream targets. In contrast, there was no sign of hypophosphorylation or marked up-regulation of BIM in A549 and H460 cells, possibly because of sustained phosphorylation of AKT/ERK (Figure 2). In H1650 cells, there was a slight increase in the level of BIM_{EL} (Figure 2), consistent with the degree of apoptosis seen in these cells (Figure 1). Changes in the proapoptotic BAD and the

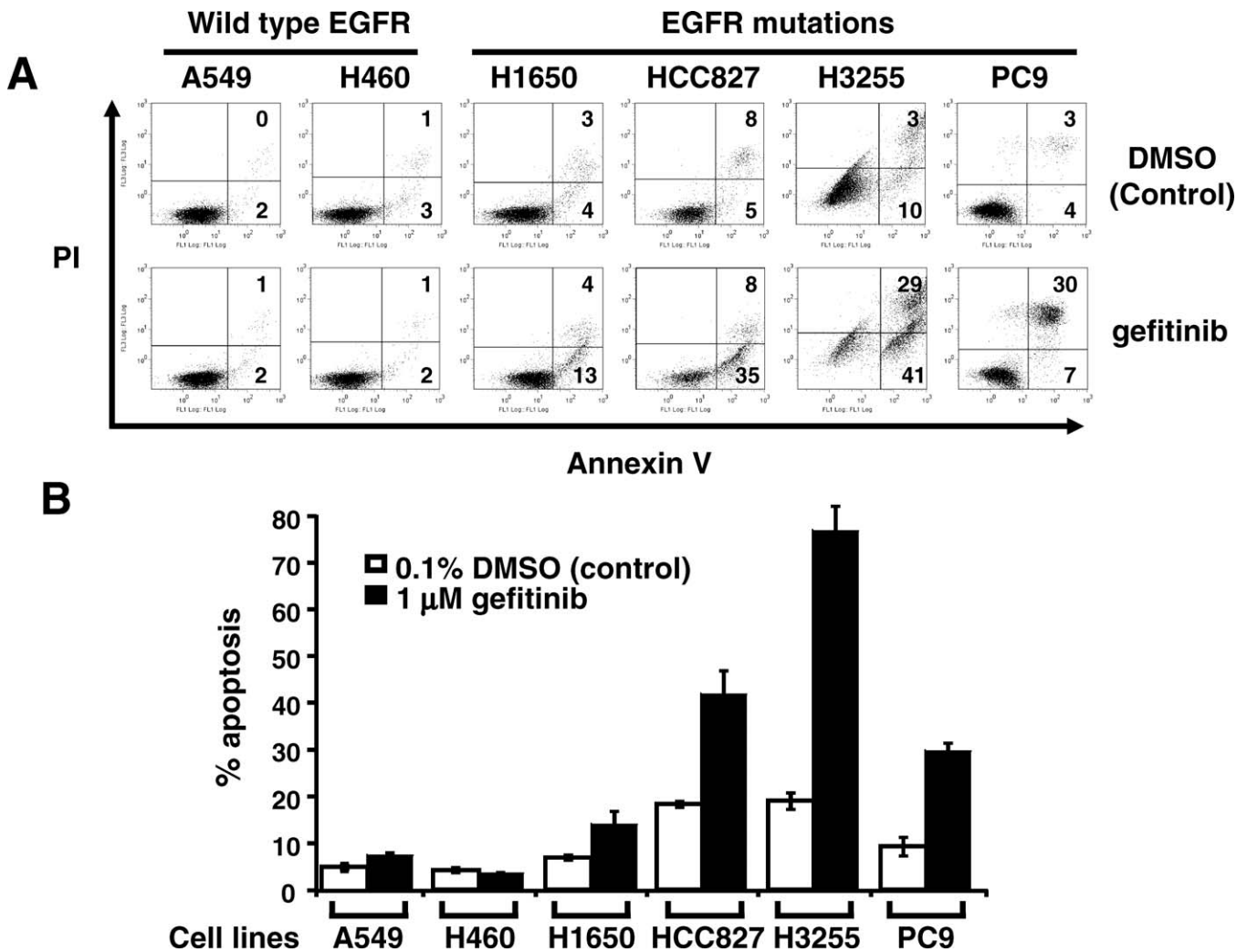


Figure 1. Up-Regulation of BIM Correlates with Gefitinib-Induced Apoptosis in NSCLC Cell Lines

Effect of gefitinib on NSCLC cells. A549, H460, H1650, HCC827, H3255, or PC-9 cells were grown in the presence of 0.1% DMSO (control) or 1 μM gefitinib for 48 h. Apoptosis was assessed using propidium iodide and Annexin-V staining.

(A) Representative flow cytometry data. The numbers represent percentage of cells in the appropriate quadrant. Left lower quadrant, viable cells; right lower quadrant, early apoptotic cells; right upper quadrant, late apoptotic cells.

(B) Quantification of apoptosis. The y-axis plots the sum of early and late apoptotic cells as mean ± standard error of the mean ($n \geq 3$).

doi:10.1371/journal.pmed.0040315.g001

antiapoptotic proteins BCL2 and BCL-x_L did not correlate with cell death upon gefitinib exposure (Figure 2).

These findings suggest that BIM may be a key marker or effector of gefitinib-induced apoptosis in EGFR-mutant lung cells.

The T790M Secondary Resistant Mutation Abrogates the Up-Regulation of BIM by Reversible TKIs

If an increase in BIM expression is important for gefitinib-induced apoptosis, it should be suppressed upon treatment of cell lines expressing the resistant T790M EGFR mutation [16,17] with gefitinib. We tested this hypothesis in gefitinib-sensitive HCC827 stable cell lines expressing activating deletion mutant L747-S752 (HCC/Del) EGFR or in the gefitinib-resistant lines HCC827-delL747-S752-T790M (HCC/Del-TM), harboring both the delL747-S752 and T790M mutations [25], as well as the H1975 cell line harboring L858R-T790M double mutations [24,25].

Gefitinib induced rapid inactivation of EGFR, AKT, and

ERK, and a dramatic increase of BIM in both HCC827 with an empty vector (HCC827/Emp) and HCC/Del cells (Figure 3A). In contrast, the HCC/Del-T790M and H1975 cells had minimal up-regulation of BIM, and the EGFR signaling cascade was less inhibited by gefitinib (Figure 3). Previously, we showed that the irreversible EGFR inhibitor, CL-387,785, can overcome gefitinib resistance and lead to apoptosis in HCC/Del-T790M [25] and H1975 cells [24]. After exposure to CL-387,785 a decrease in phosphorylations of EGFR, AKT, and ERK were observed and accompanied by marked up-regulation of BIM, which correlated with the presence of the cleaved form of PARP (Figure 3).

These results confirm that up-regulation of BIM correlates with effective TKI-induced apoptosis and that T790M suppresses this process in gefitinib-treated cells.

Knockdown of BIM Attenuates TKI-Induced Apoptosis

If BIM functions as a mediator of TKI-induced apoptosis, knockdown of its expression should have detectable effects

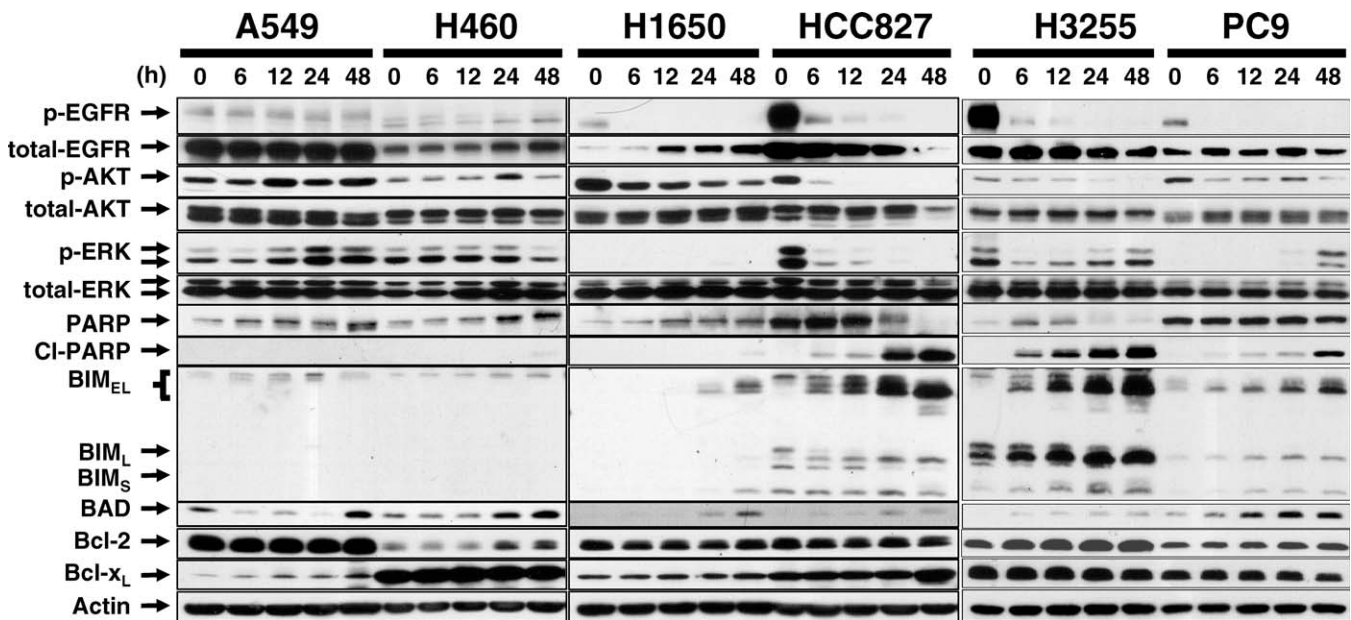


Figure 2. Gefitinib Induces Expression and Dephosphorylation of BIM in Gefitinib-Sensitive *EGFR*-Mutant Cells, but Not in Cells with Wild-Type *EGFR*. Cells were treated with 1 μ M gefitinib for the times (in hours) indicated, and lysates were collected and proteins analyzed by immunoblotting. The three isoforms of BIM, BIM_{EL}, BIM_L, and BIM_S are shown. Hypophosphorylated BIM migrates faster than the other species [23]. doi:10.1371/journal.pmed.0040315.g002

on apoptosis. Therefore, we tested the effects of *BIM* siRNA in HCC827 and H1975 cells treated with gefitinib and CL387,785, respectively. Apoptosis was significantly attenuated by *BIM* siRNA in both cell lines detected by PARP cleavage (Figure 4A) and the Annexin V assay (Figure 4B) after exposure to TKIs that induce dephosphorylation of EGFR, AKT, and ERK (Figure 4A).

These data suggest that BIM plays an important role as a death regulator in TKI-induced apoptosis.

Identification and Characterization of a Novel Secondary Mutation, L747S, in a Gefitinib-Resistant Tumor

Through the sequencing of gefitinib-resistant tumors at our institution we identified a novel second mutation (see Methods). We detected a L858R *EGFR* mutation in the initial biopsies obtained from a 74-year-old white woman affected by an advanced adenocarcinoma with bronchioalveolar and papillary features [31,32]. She maintained a partial response to gefitinib for 40 months without overt clinical or radiographic progression; however, at that point computer tomography scans showed progression of lung lesions, presence of a thickened left pleura with effusion (Figure S1), and bone metastases. Sequencing of the cDNA derived from the pleural fluid of the progressing tumor confirmed the persistence of the initial L858R mutation in addition to a new T-C basepair change in exon 19 (Figure 5A), which results in a predicted amino acid change of leucine (L) to serine (S) at position 747 of EGFR. Most subclones contained the L747S in cis with L858R (Table S1). In the EGFR kinase domain crystal structures [33,34], L747 is located at the start of the loop between strand β 3 and helix α C. This residue is the leucine of the LRE motif that is frequently deleted in exon 19. In crystal structures of both the active and inactive conformations of the EGFR tyrosine kinase domain this residue is oriented toward the back pocket region of the catalytic cleft (Figure

5B). Mutations in the analogous residue of ABL1 (L273M) and ErbB2 (L755S or P) (Figure 5C) have been described in patients with imatinib-resistant chronic myelogenous leukemia (CML) and solid tumors including gastric, breast, and lung cancers, respectively [35–37]. However, functional studies were not performed.

Transient transfection experiments utilizing COS-7 cells demonstrated that auto-phosphorylation of the original L858R EGFR was inhibited by lower concentrations of gefitinib or erlotinib than the L858R-L747S or L858R-T790M constructs (Figure S2A). CL-387,785 partially overcame the observed inhibition (Figure S2B).

To prove the functional significance of the L747S mutation, we generated Ba/F3 cell lines stably expressing mutant EGFR constructs [24]. These cell lines, including L858R-L747S, proliferated in the absence of IL3. However, the proliferation rate of Ba/F3-L858R-L747S cells was not as dramatic as that of Ba/F3-L858R-T790M cells (Figure S3). Ba/F3-L858R cells were extremely sensitive to gefitinib (Figure 5D), whereas Ba/F3-L858R-T790M cells were highly resistant up to 1 μ M gefitinib. All four Ba/F3-L858R-L747S clones demonstrated an intermediate pattern of resistance to the growth inhibition signal induced by gefitinib (Figure 5D). After the cells were exposed to gefitinib for 24 h, Ba/F3-L858R cells were unable to proliferate while Ba/F3-L858R-L747S and Ba/F3-L858R-T790M cells continued to grow even in the presence of 1 μ M gefitinib (Figure 5E).

These results suggest that execution of apoptosis may be impaired by the presence of T790M and, to a lesser extent, L747S.

Resistance Mutations Attenuate BIM Up-Regulation and the Mitochondrial Apoptosis Pathway

Based on the data obtained from lung cancer cell lines, we further explored the mechanisms by which T790M and L747S

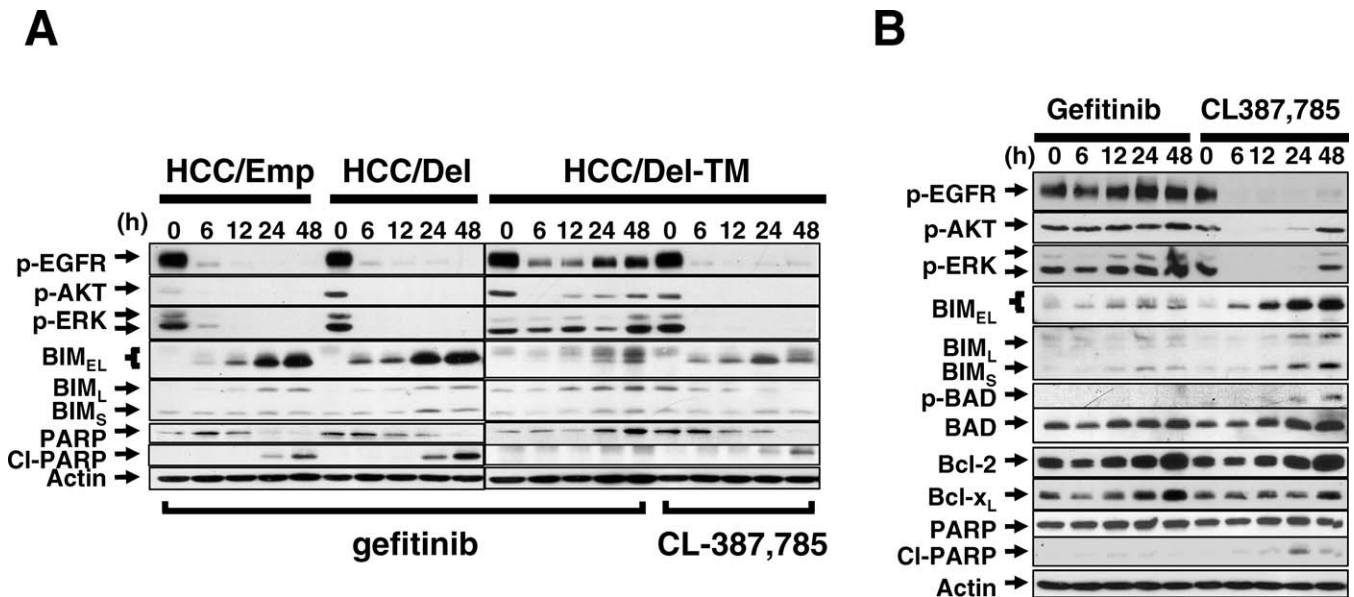


Figure 3. Inhibition of BIM Up-Regulation by the Resistant T790M Mutation

The increase in BIM expression and apoptosis are inhibited by the secondary resistant mutation, T790M.

(A) Modulation of signaling following gefitinib treatment in HCC827 cells expressing EGFR mutants. Time course of gefitinib treatment in HCC827 cells expressing pcDNA3.1 empty vector (HCC/Emp), EGFR-DelL747-S752 (HCC/Del), or EGFR-DelL747-S752+T790M (HCC/Del-TM). Cells were treated with 3 μ M gefitinib or CL-387,785 for indicated times, lysates collected, and proteins analyzed by immunoblotting.

(B) Modulation of signaling following either gefitinib or CL387,785 treatment in H1975 cells. The cells were treated with 1 μ M gefitinib or CL-387,785 for the indicated times and extracts were analyzed by Western blotting.

doi:10.1371/journal.pmed.0040315.g003

resistant mutations affect BIM up-regulation and apoptosis. Gefitinib effectively induced apoptosis in Ba/F3-L858R cells, whereas Ba/F3-L858R-L747S cells were intermediately resistant and Ba/F3-L858R-T790M completely resistant (Figure 6A, top). The up-regulation of BIM was also attenuated in a similar dose-response manner as seen in the apoptosis assay (Figure 6A, bottom), with L858R-T790M completely abrogating the induction of BIM and L858R-L747S attenuating both the up-regulation of BIM and apoptosis.

Phosphorylated EGFR as well as AKT were maintained up to 36 h after gefitinib treatment in Ba/F3-L858R-L747S and L858R-T790M cells. The phosphorylated forms of ERK1/2 were significantly less inhibited in L858R-T790M cells (Figure 6B). Up-regulation of BIM was observed in Ba/F3-L858R cells as early as 3 h after exposure to gefitinib (Figure 6B). Ba/F3-L858R-L747S cells had a delay in the up-regulation of BIM and Ba/F3-L858R-T790M cells had no change in BIM isoforms (Figure 6B). BAD, BCL-x_L, and BCL2 did not change significantly.

In order to explore the consequences of a delay or inhibition in BIM up-regulation on apoptosis, we examined whether gefitinib induced breakdown of the inner mitochondrial membrane potential ($\Delta\Psi_m$), characterized by a sudden increase in permeability of the mitochondrial membrane as a consequence of the charge difference between the mitochondrial matrix and the cytosol [27,38,39].

Treatment with gefitinib led to a significant decrease in $\Delta\Psi_m$ in Ba/F3-L858R cells at 12 h (Figure 6C). The pan-caspase inhibitor z-VAD-fmk did not inhibit the breakdown of $\Delta\Psi_m$ (unpublished data), suggesting that the intrinsic mitochondrial pathway plays a central role during gefitinib-induced apoptosis. It is well known that the BH3-only members are part of the intrinsic apoptotic pathway

[40,41]. Ba/F3-L858R-T790M cells showed no significant change even at 24 h (Figure 6C), and Ba/F3-L858R-L747S cells had no apparent changes at 12 h and only a partial breakdown at 24 h. Activation of caspase-3 and PARP cleavage were consistent with the apoptotic execution process measured by $\Delta\Psi_m$ (Figure 6D).

Taken together, these results suggest that the novel L747S and the T790M mutations attenuate the intrinsic mitochondrial apoptosis pathway by inhibiting the up-regulation of BIM in EGFR-mutant models.

Discussion

We identified BIM as a key apoptotic effector of EGFR TKIs in sensitive cells with the activating L858R or exon 19 deletion EGFR mutations. The common T790M secondary mutation and the novel L747S, in conjunction with an activating mutation, attenuated the up-regulation of BIM and apoptosis.

The discovery that the deregulated tyrosine kinase activity in certain cancers can be targeted has led to major advances in the field of malignant therapeutics [42]. Tyrosine kinases require ATP for their enzymatic activity, and thus small molecules that mimic ATP can bind to mutant or translocated kinases and inactivate them. The most satisfying translational example of this process has been in the case of CML, in which the t(9;22) translocation and the consequent enhanced tyrosine kinase activity of BCR-ABL can be effectively inhibited by imatinib. This oral TKI has revolutionized the treatment of CML and led to sustained responses in a majority of patients [43]. A parallel situation is seen with gastrointestinal stromal tumor (GIST), in which activating mutations of the receptor tyrosine kinases c-KIT or PDGFRA

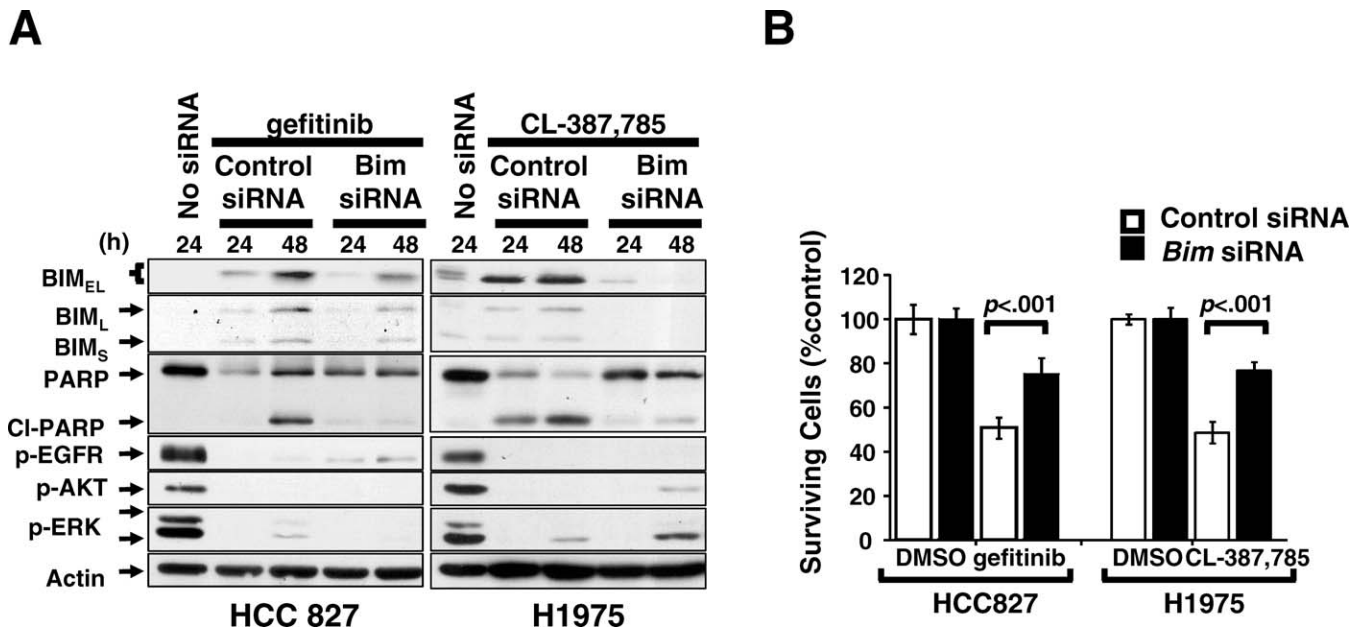


Figure 4. Knockdown of *BIM* Expression Leads to Attenuation of Apoptosis

(A) HCC827 cells (left) or H1975 cells (right) were transfected with *BIM* siRNA oligonucleotides or control oligonucleotides for 24 h prior to 0.5 μ M gefitinib or 1 μ M CL387,785 treatment, respectively. The cells were then treated for 24 and 48 h and lysates were collected and proteins were analyzed by immunoblotting.

(B) Annexin V apoptosis assay. The cells were treated as above and analyzed after 48 h. The percentage of surviving cells—both Annexin V⁻ and propidium iodide⁻ cells—was compared with 0.1% DMSO control. The data are reported as mean \pm standard deviation (SD) ($n \geq 3$). doi:10.1371/journal.pmed.0040315.g004

(platelet-derived growth factor receptor, alpha polypeptide) contribute to the disease, and again imatinib is an effective treatment option improving patient survival [44]. A similar clinical benefit is observed in patients with lung cancers harboring the two most common *EGFR* activating mutations, L858R and exon 19 deletions [3–5,9].

The striking initial response to small molecule tyrosine kinase inhibitors is thought to stem from the phenomenon of “oncogene addiction” [21], and a common signaling cascade may be involved in the apoptosis induced by multiple TKIs [22]. Rapid inactivation of phosphorylated ERK, AKT, and STAT3/5, and the delayed accumulation of phosphorylated p38 are commonly observed in SRC-, BCR-ABL-, and EGFR-dependent cells after exposure to the specific inhibitors SU6655, imatinib, and gefitinib, respectively [22].

Recently, it has been shown that BIM is one of the main effectors of imatinib-mediated apoptosis in BCR-ABL-positive leukemia cells [23,45,46]. BIM belongs to the BH3-only group of proteins that bind and neutralize various anti-apoptotic BCL2 family members so that they cannot inhibit the proapoptotic effects of BAX and BAK in the intermembrane mitochondrial space [40,41]. BIM is regulated by multiple stimuli, including the PI3K-AKT-FOXO and the ERK1/2 MAPK pathways [29,30]. Cytokine withdrawal or inhibition of PI3K-AKT leads to dephosphorylation and nuclear entry of the forkhead transcription factor FOXO-3A, which induces *BIM* mRNA expression [29]. Inhibition of ERK1/2 also induces *BIM* mRNA expression by unknown mechanisms [29]. In addition, ERK1/2 regulates the function of BIM_{EL} by post-translational modifications. ERK1/2-dependent phosphorylation antagonizes BIM_{EL} by proteasomal degradation or disruption of BIM_{EL}-BAX interactions [29].

Of relevance to our studies is that the ErbB family receptors activate the PI3K-AKT and the ERK1/2-MAPK pathways, both of which mediate regulation of BIM. Overexpression of EGFR inhibits anoikis—apoptosis induced by lack of correct cell and extracellular matrix attachment [47]—in mammary epithelial cells by restoring ERK activation and blocking BIM up-regulation, which is reversed by either EGF withdrawal or EGFR inhibition [48]. It has also been shown that EGFR affects the post-translational control of BIM expression through a pathway requiring PKC δ and MEK/MAPK activation [49]. Although the role of p38 as an apoptosis mediator has not been completely characterized [22], p38 activity is required for *EGFR* down-regulation resulting in attenuation of downstream signaling [50]. In addition, p38 activation leads to BIM induction during glucocorticoid-induced apoptosis in lymphoblastic leukemia cells [51].

These observations and the concept of “oncogenic shock,” which postulates that prosurvival signals are shut down quickly, whereas proapoptotic signals remain active enough to induce apoptosis upon oncoprotein inactivation [22], prompted us to hypothesize that BIM plays an important role in apoptosis induced by gefitinib and other EGFR TKIs. Our data indicate that the intrinsic mitochondrial pathway is involved in gefitinib-induced cell death and that the up-regulation of the proapoptotic polypeptide BIM was consistently seen during TKI-induced apoptosis. In addition, sustained activation of EGFR, AKT, and/or ERK caused by the T790M and L747S resistant mutations delayed BIM up-regulation and apoptosis. We also showed, in two distinct cell lines, that knockdown of BIM led to significant reduction in the amount of cell death. These observations suggest that

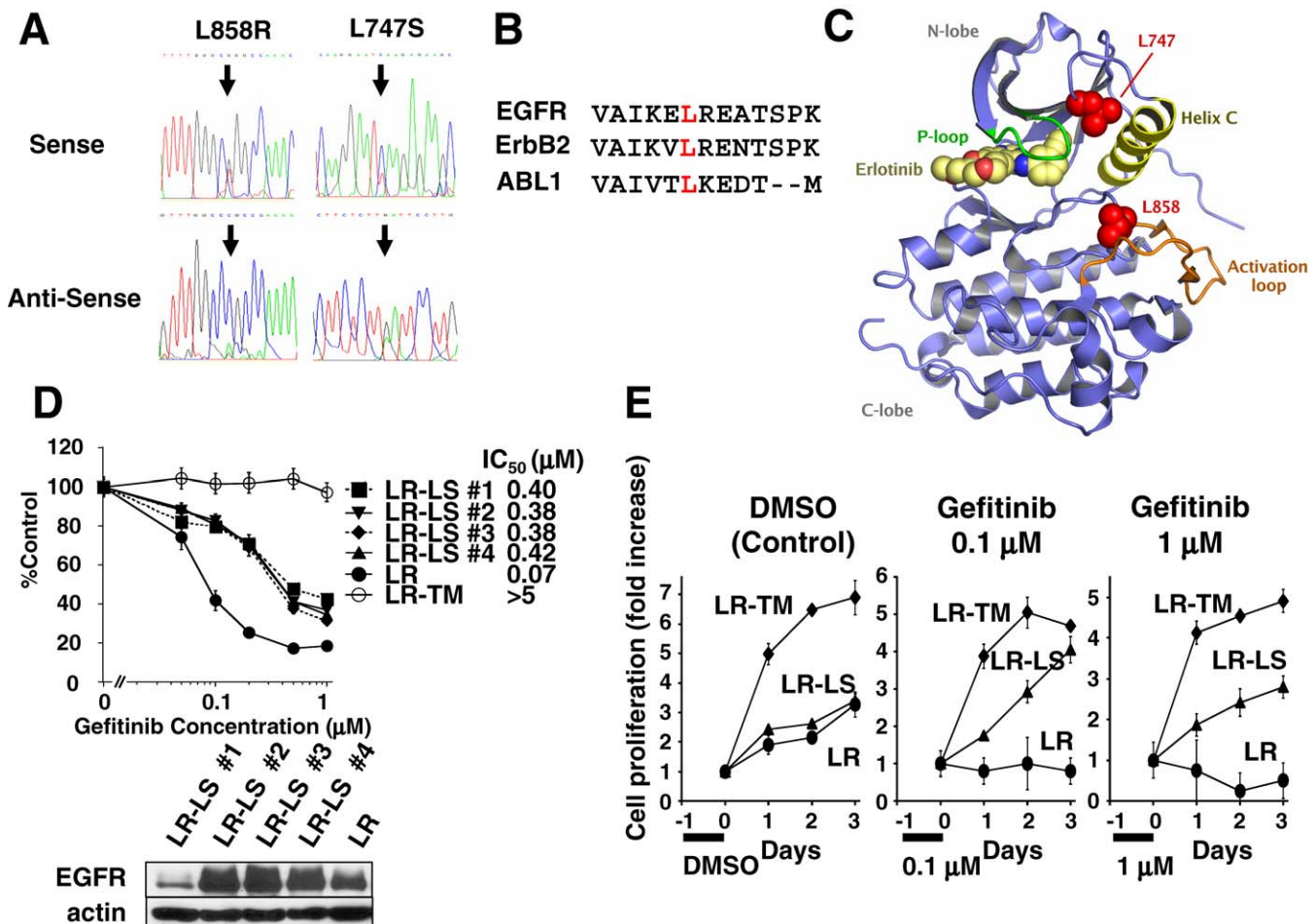


Figure 5. Identification of a Novel Secondary Resistant EGFR Mutation, L747S

(A) Sequencing chromatograms with the EGFR L747S exon 19 and L858R exon 21 by RT-PCR.

(B) Amino acid alignments of the tyrosine kinase domain in EGFR, ErbB2, and ABL1.

(C) Active conformation crystal structure of the kinase domain of EGFR in complex with erlotinib [34]. The graphic shows the spatial locations of residues L747 and L858 as red spheres. The activation loop is orange, helix α C yellow, the glycine-rich P-loop green, and the inhibitor erlotinib yellow. The figure was made using the program PYMOL (<http://pymol.sourceforge.net/>). (D) Top: Dose-dependent growth inhibition of Ba/F3 cells expressing EGFR L858R (LR), L858R-T790M (LR-TM), or L858R-L747S (LR-LS) detected by the MTS assay. Error bars indicate standard deviation ($n = 4$). Four LR-LS clones (#1–4) were obtained from G418 selection. Bottom: Expression of EGFR in Ba/F3 cells expressing L858R, L858R-L747S #1–4, or L858R-T790M.

(E) Gefitinib release assay. Ba/F3 cells expressing L858R (LR), L858R-L747S #4 (LR-LS), or L858R-T790M (LR-TM) were plated in 24-well plates at a density of 1×10^5 /well and treated with 0.1% DMSO (control) or gefitinib for 24 h. Then the cells were washed three times with RPMI and cultured in medium containing 20 ng/ml EGF. The cells were stained with Trypan blue and counted daily. The data are reported as mean \pm SD ($n = 3$).

doi:10.1371/journal.pmed.0040315.g005

BIM is a key effector of TKI-induced apoptosis in EGFR-driven tumors and that the up-regulation of BIM may be one of the common mechanisms by which tumor cells driven by “oncogenic addiction” undergo apoptosis and “oncogenic shock” [22]. However, knockdown of BIM did not completely inhibit the TKI-induced apoptosis in our cell lines, which may be explained either by the residual level of BIM protein in siRNA transfected cells (Figure 4A), or involvement of other proapoptotic regulators, such as other BH3-only members. One of the possible candidates is BAD, another BH3-only proapoptotic regulator, which was shown previously to play a role in imatinib-induced apoptosis in CML [23]. However, the role of BAD remains unclear so far, since inhibition of BAD phosphorylation, which is believed to be important for preventing apoptosis, was not detected in the NSCLC cells we tested (Figure 3B and unpublished data). In addition, double knockdown of BIM and BAD did not show significant

increase in the survival of gefitinib-treated HCC827 cells compared to single BIM knockdown (unpublished data). Further studies are required to define other relevant apoptotic pathways involved in gefitinib-induced apoptosis.

Furthermore, our data indicate that the degree of BIM up-regulation was directly proportional to the amount of apoptosis and that the up-regulation of BIM determines the sensitivity of lung cancer cells to the apoptotic effects of the TKIs. This effect became evident when we examined three cell lines carrying the same EGFR mutation, HCC827, PC-9, and H1650 (all have the delE746-A750 mutation), and noted that the degrees of gefitinib-induced apoptosis were strikingly different, with the former two being more sensitive than the later. The up-regulation of BIM was minimal in H1650 cells, which lack PTEN [52], compared to HCC827 and PC-9.

Acquired resistance to imatinib commonly occurs in CML and often in GIST [53,54]. In both diseases, secondary

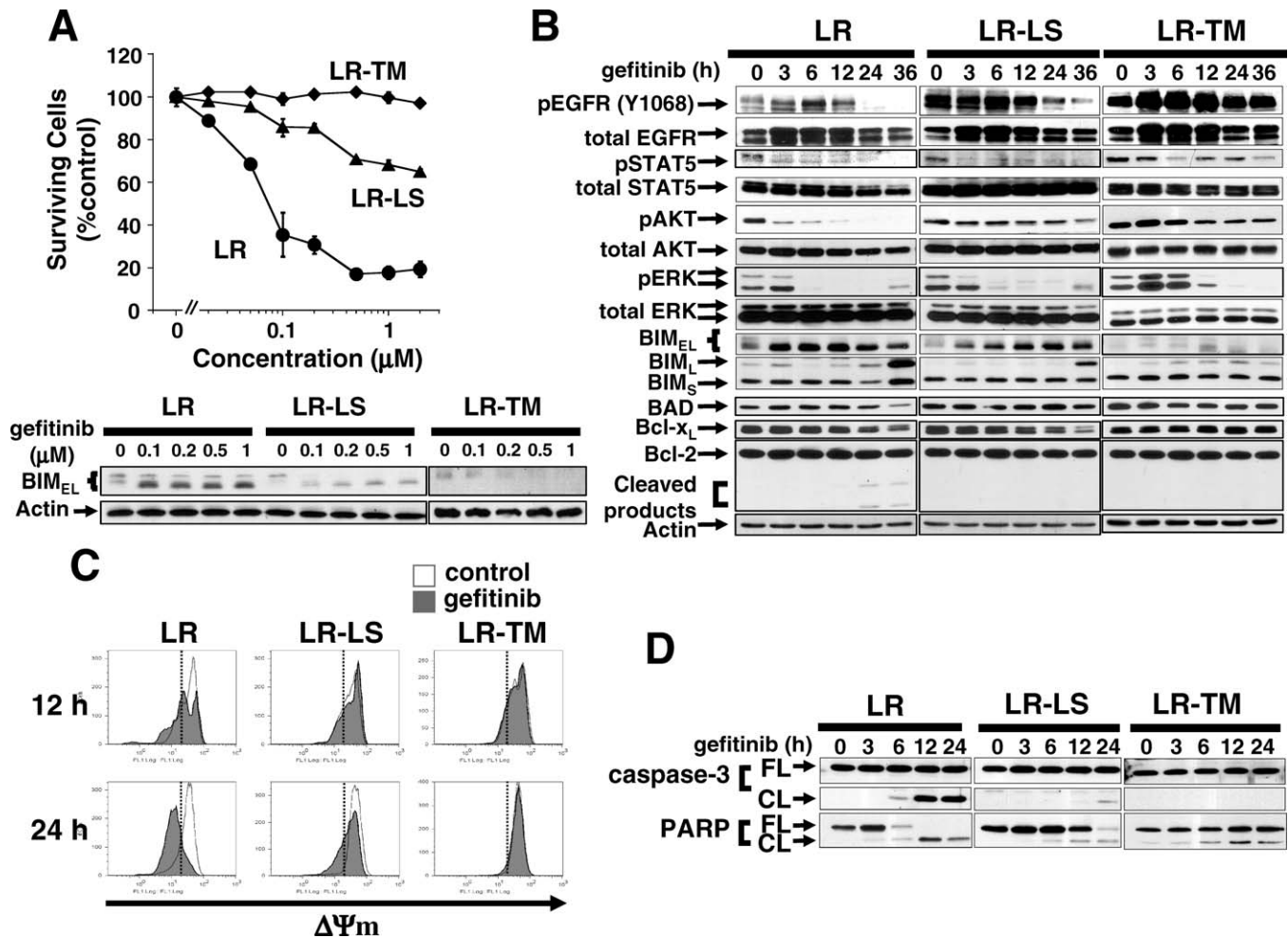


Figure 6. The Secondary Resistant Mutations L747S and T790M Affect Gefitinib-Induced Apoptosis and Inhibit BIM Up-Regulation (A) Top: Annexin V apoptosis assay: Ba/F3 cells expressing L858R (LR), L858R-L747S #4 (LR-LS), or L858R-T790M (LR-TM) cells were grown in the absence or presence of gefitinib for 24 h. The data are reported as mean ± SD (n ≥ 3). Bottom: After the cells were treated for 3 h with increasing concentrations of gefitinib, lysates were collected and proteins analyzed by immunoblotting. BIM expression correlated with the amount of apoptosis in L858R, L858R-L747S, and L858R-T790M cells. (B) Modulation of signaling following gefitinib treatment in Ba/F3 cells expressing EGFR mutants. Time course of gefitinib treatment in L858R (LR), L858R-L747S #4 (LR-LS), or L858R-T790M (LR-TM) cells. Cells were treated with 0.2 μM gefitinib for indicated times in the absence of IL3 and in the presence of EGF 20 ng/ml. Note that increase in BIM expression is delayed in LR-LS cells or minimal in LR-TM cells. (C) Flow cytometric analysis of the inner mitochondrial membrane potential ($\Delta\Psi_m$) breakdown. Cells were treated in the presence or absence of 0.2 μM gefitinib for 12 or 24 h, and stained with DiOC6(3). (D) Activation of caspase-3 and cleavage of PARP are attenuated in L858R-L747S #4 (LR-LS) and L858R-T790M (LR-TM) cells. Cell extracts described in (B) were analyzed by Western blotting. doi:10.1371/journal.pmed.0040315.g006

mutations in either *ABL1* or *KIT* and *PDGFRA* have been identified as main mechanistic factors that re-establish the oncogene signaling in these tumors. In the case of CML, more than 35 mutations have been described in the ABL kinase domain from patients with imatinib resistance [55], and in GIST a similar pattern of multiple sites of secondary kinase domain mutations has been seen [54]. There are many similarities among structures of tyrosine kinases, and some of the secondary mutations fall at exactly the same amino acid residue. This is the case of the T315I, T670I, and T790M mutations in *ABL1*, *KIT*, and *EGFR*, respectively [56]. Mutation of these key gatekeeper residues can prevent inhibitor access to the kinase back pocket region and can disrupt hydrogen bonds or other interactions between the inhibitor and the kinase [16,56]. Despite the similarity between CML, GIST, and *EGFR*-mutated NSCLC in the

patterns of secondary resistance, in NSCLC only the T790M [16,17] and D761Y [19] secondary mutations have been described so far.

In this report, we identify and characterize a novel *EGFR* secondary mutation, L747S. Our in vitro studies demonstrated that both of the secondary mutations initially identified in our lab (T790M and L747S) confer varying degrees of resistance to the apoptotic signals initiated by gefitinib. These changes may reflect the progressing radiological and clinical pictures of our patients while on gefitinib monotherapy for their metastatic NSCLC. T790M results in ineffective TKI inhibition and sustained down-stream signaling from the mutant *EGFR* [16,17], as was seen in all cell lines carrying T790M in the current report. *EGFR*, *AKT*, and *ERK1/2* were not inhibited in our T790M gefitinib-treated models. The L747 residue is oriented toward the back pocket region of the

catalytic cleft both in the active and inactive conformation of the EGFR tyrosine kinase domain [33,34]. It is unclear from the current crystallographic data and from our molecular dynamics simulations how acquisition of this mutation results in resistance to ATP-competitive EGFR inhibitors; there may be differential effects on ATP and small molecule binding, as seen for L858R and G719S mutations [57] or a shift in the conformational equilibrium of the kinase between the active and inactive states. Further crystallographic studies are required to more fully understand the structural basis for the effects of this mutation.

Our data show that L858R-L747S demonstrated a pattern of resistance that was less pronounced than that observed with L858R-T790M with increasing doses of gefitinib (Figures 5D and 6A). These results are similar to the ones observed in the previously reported secondary L858R-D761Y mutation [19]. It is possible to conceive that an increase in the clinical doses of gefitinib or switching to erlotinib, which is given at its maximal tolerated dose [58], may lead to beneficial clinical effects, possibly by increasing BIM expression, in patients with *EGFR* mutations who acquired L747S after exposure to gefitinib.

The data presented here indicate that BIM is both a marker and an effector of TKI-induced apoptosis in *EGFR*-mutant NSCLC cells. Furthermore, we identified a novel acquired *EGFR* secondary mutation, L747S, and showed that both the L747S and the common T790M in cis to an activating *EGFR* mutation (either L858R or an exon 19 deletion) cause resistance to EGFR TKI-induced apoptosis and attenuate the up-regulation of BIM. In this context, it is possible that enhancement of BIM expression or activation of its downstream targets may be a promising strategy for the treatment of *EGFR*-mutant NSCLC, particularly in the context of mutations conferring secondary resistance to TKI inhibitors.

Supporting Information

Figure S1. Chest CT Scans Before (Left) and After (Right) Progression of Lung Tumors

Note that pleural effusion was detected in left cavity.

Found at doi:10.1371/journal.pmed.0040315.sg001 (330 KB PPT).

Figure S2. The EGFR-L747S-L858R Double Mutant Is Less Sensitive to Inhibition by Gefitinib and Erlotinib

(A) Autophosphorylation of EGFR tyrosine 1068 is detected by immunoblots of whole-cell extracts isolated from transfected COS-7 cells after a 3-h incubation with different concentrations of gefitinib. Total EGFR expression is shown as loading control.

(B) The inhibition of EGFR autophosphorylation by CL-387,785. Blots were probed with EGFR tyrosine 1068 (left) and total EGFR antibody (right).

Found at doi:10.1371/journal.pmed.0040315.sg002 (398 KB PPT).

Figure S3. Functional Analyses of Ba/F3 Cells Expressing *EGFR* Mutants

Top: Expression of EGFR in Ba/F3 cells expressing L858R (LR), L858R-L747S (LR-LS#4), wild-type EGFR (WT), L747S (LS), or L858R-T790M (LR-TM). Bottom: IL3-independent growth of Ba/F3 cells expressing the EGFR mutants. Cells were seeded at a density of 1×10^4 /ml and counted daily.

Found at doi:10.1371/journal.pmed.0040315.sg003 (4.4 MB PPT).

Table S1. Characteristics and Clinical Course of Patients with Gefitinib-Resistant *EGFR*-Mutant NSCLCs and Secondary *EGFR* Mutations from the Thoracic Oncology Clinic at Beth Israel Deaconess Medical Center

Found at doi:10.1371/journal.pmed.0040315.st001 (28 KB DOC).

Accession Numbers

The NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>) accession numbers for the genes discussed in this paper are *BAD* (NM_032989), *BIM* (AF032458), and *EGFR* (NM_005228). The Protein Data Bank (<http://www.pdb.org/>) accession number for the EGFR tyrosine kinase domain with the 4-anilinoquinazoline inhibitor erlotinib is 1M17.

Acknowledgments

We thank Geoffrey Shapiro for PC-9 cells. We are indebted to Alexander Ebraldize and Ulrich Steidl for scientific and technical advice. We would also like to thank members of the Tenen laboratory for their helpful comments and suggestions, and William Pao, Mark Cragg, and Andreas Strasser for sharing unpublished results.

Author contributions. DBC conducted the experiments and analyzed the clinical and experimental data. AK and TJB generated the structural model of EGFR with secondary mutations and edited the manuscript. STS and MSH contributed to the clinical care, sample collection, and data acquisition of the gefitinib-resistant patients. BH and DGT supervised all aspects of this study including study design, execution, and interpretation. SK designed and conducted the experiments and analyzed the clinical and experimental data. DBC, BH, DGT and SK wrote the final manuscript.

Funding. This work was supported by US National Institution of Health grant (K99CA126026 to SK) and Specialized Program of Research Excellence (SPORE) in Lung Cancer (CA090578 to SK and DGT); an American Association for Cancer Research (AACR)–AstraZeneca–Cancer Research and Prevention Foundation Fellowship in Translational Lung Cancer Research and a Clinical Investigator Training Program–Beth Israel Deaconess Medical Center, Harvard Medical School/MIT (to DBC); an American Society of Hematology Junior Faculty Scholar Award (to TJB); and a Flight Attendant Medical Research Institute Young Clinical Scientist Award (to BH). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

- Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, et al. (2005) Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst* 97: 339–346.
- Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, et al. (2004) Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res* 64: 8919–8923.
- Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, et al. (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 350: 2129–2139.
- Pao W, Miller V, Zakowski M, Doherty J, Politi K, et al. (2004) 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* 101: 13306–13311.
- Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, et al. (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 304: 1497–1500.
- Ji H, Li D, Chen L, Shimamura T, Kobayashi S, et al. (2006) The impact of human EGFR kinase domain mutations on lung tumorigenesis and in vivo sensitivity to EGFR-targeted therapies. *Cancer Cell* 9: 485–495.
- Politi K, Zakowski MF, Fan PD, Schonfeld EA, Pao W, et al. (2006) 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* 20: 1496–1510.
- Takeuchi T, Tomida S, Yatabe Y, Kosaka T, Osada H, et al. (2006) Expression profile-defined classification of lung adenocarcinoma shows close relationship with underlying major genetic changes and clinicopathologic behaviors. *J Clin Oncol* 24: 1679–1688.
- Inoue A, Suzuki T, Fukuhara T, Maemondo M, Kimura Y, et al. (2006) Prospective phase II study of gefitinib for chemotherapy-naive patients with advanced non-small-cell lung cancer with epidermal growth factor receptor gene mutations. *J Clin Oncol* 24: 3340–3346.
- Asahina H, Yamazaki K, Kinoshita I, Sukoh N, Harada M, et al. (2006) A phase II trial of gefitinib as first-line therapy for advanced non-small cell lung cancer with epidermal growth factor receptor mutations. *Br J Cancer* 95: 998–1004.
- Costa DB, Kobayashi S, Tenen DG, Huberman MS (2007) Pooled analysis of the prospective trials of gefitinib monotherapy for EGFR-mutant non-small cell lung cancers. *Lung Cancer* 58: 95–103.
- Jackman DM, Yeap BY, Sequist LV, Lindeman N, Holmes AJ, et al. (2006) Exon 19 deletion mutations of epidermal growth factor receptor are associated with prolonged survival in non-small cell lung cancer patients treated with gefitinib or erlotinib. *Clin Cancer Res* 12: 3908–3914.
- Mitsudomi T, Kosaka T, Endoh H, Horio Y, Hida T, et al. (2005) Mutations

- of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small-cell lung cancer with postoperative recurrence. *J Clin Oncol* 23: 2513–2520.
14. Riely GJ, Pao W, Pham D, Li AR, Rizvi N, et al. (2006) Clinical course of patients with non-small cell lung cancer and epidermal growth factor receptor exon 19 and exon 21 mutations treated with gefitinib or erlotinib. *Clin Cancer Res* 12: 839–844.
 15. Han SW, Kim TY, Hwang PG, Jeong S, Kim J, et al. (2005) Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small-cell lung cancer patients treated with gefitinib. *J Clin Oncol* 23: 2493–2501.
 16. Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, et al. (2005) EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 352: 786–792.
 17. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, et al. (2005) Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2: e73. doi:10.1371/journal.pmed.0020073
 18. Kosaka T, Yatabe Y, Endoh H, Yoshida K, Hida T, et al. (2006) Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and acquired resistance to gefitinib. *Clin Cancer Res* 12: 5764–5769.
 19. Balak MN, Gong Y, Riely GJ, Somwar R, Li AR, et al. (2006) Novel D761Y and common secondary T790M mutations in epidermal growth factor receptor-mutant lung adenocarcinomas with acquired resistance to kinase inhibitors. *Clin Cancer Res* 12: 6494–6501.
 20. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, et al. (2007) MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 316: 1039–1043.
 21. Weinstein IB (2002) Cancer. Addiction to oncogenes—the Achilles heel of cancer. *Science* 297: 63–64.
 22. Sharma SV, Gajowniczek P, Way IP, Lee DY, Jiang J, et al. (2006) A common signaling cascade may underlie “addiction” to the Src, BCR-ABL, and EGF receptor oncogenes. *Cancer Cell* 10: 425–435.
 23. Kuroda J, Puthalakath H, Cragg MS, Kelly PN, Bouillet P, et al. (2006) Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic. *Proc Natl Acad Sci U S A* 103: 14907–14912.
 24. Kobayashi S, Ji H, Yuza Y, Meyerson M, Wong KK, et al. (2005) An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor. *Cancer Res* 65: 7096–7101.
 25. Kobayashi S, Shimamura T, Monti S, Steidl U, Hetherington CJ, et al. (2006) Transcriptional profiling identifies cyclin D1 as a critical downstream effector of mutant epidermal growth factor receptor signaling. *Cancer Res* 66: 11389–11398.
 26. Kobayashi S, Yamashita K, Takeoka T, Ohtsuki T, Suzuki Y, et al. (2002) Calpain-mediated X-linked inhibitor of apoptosis degradation in neutrophil apoptosis and its impairment in chronic neutrophilic leukemia. *J Biol Chem* 277: 33968–33977.
 27. Hirata H, Takahashi A, Kobayashi S, Yonehara S, Sawai H, et al. (1998) Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. *J Exp Med* 187: 587–600.
 28. Helfrich BA, Raben D, Varella-Garcia M, Gustafson D, Chan DC, et al. (2006) Antitumor activity of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib (ZD1839, Iressa) in non-small cell lung cancer cell lines correlates with gene copy number and EGFR mutations but not EGFR protein levels. *Clin Cancer Res* 12: 7117–7125.
 29. Ley R, Ewings KE, Hadfield K, Cook SJ (2005) Regulatory phosphorylation of Bim: sorting out the ERK from the JNK. *Cell Death Differ* 12: 1008–1014.
 30. Qi XJ, Wildey GM, Howe PH (2006) Evidence that Ser87 of BimEL is phosphorylated by Akt and regulates BimEL apoptotic function. *J Biol Chem* 281: 813–823.
 31. Costa DB, Schurer ST (2006) Three-year survival in metastatic non-small cell lung cancer treated with gefitinib. *Lung Cancer* 53: 123–124.
 32. Janne PA, Borrás AM, Kuang Y, Rogers AM, Joshi VA, et al. (2006) A rapid and sensitive enzymatic method for epidermal growth factor receptor mutation screening. *Clin Cancer Res* 12: 751–758.
 33. Wood ER, Truesdale AT, McDonald OB, Yuan D, Hassell A, et al. (2004) A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. *Cancer Res* 64: 6652–6659.
 34. Stamos J, Sliwkowski MX, Eigenbrot C (2002) Structure of the epidermal growth factor receptor kinase domain alone and in complex with a 4-anilinoquinazoline inhibitor. *J Biol Chem* 277: 46265–46272.
 35. Talpaz M, Shah NP, Kantarjian H, Donato N, Nicoll J, et al. (2006) Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. *N Engl J Med* 354: 2531–2541.
 36. Stephens P, Hunter C, Bignell G, Edkins S, Davies H, et al. (2004) Lung cancer: intragenic ERBB2 kinase mutations in tumours. *Nature* 431: 525–526.
 37. Lee JW, Soung YH, Seo SH, Kim SY, Park CH, et al. (2006) Somatic mutations of ERBB2 kinase domain in gastric, colorectal, and breast carcinomas. *Clin Cancer Res* 12: 57–61.
 38. Verma YK, Gangenahalli GU, Singh VK, Gupta P, Chandra R, et al. (2006) Cell death regulation by B-cell lymphoma protein. *Apoptosis* 11: 459–471.
 39. Ly JD, Grubb DR, Lawen A (2003) The mitochondrial membrane potential ($\Delta\psi$) in apoptosis; an update. *Apoptosis* 8: 115–128.
 40. Strasser A (2005) The role of BH3-only proteins in the immune system. *Nat Rev Immunol* 5: 189–200.
 41. Willis SN, Adams JM (2005) Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol* 17: 617–625.
 42. Sawyers C (2004) Targeted cancer therapy. *Nature* 432: 294–297.
 43. O'Brien SG, Guilhot F, Larson RA, Gathmann I, Baccarani M, et al. (2003) Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med* 348: 994–1004.
 44. Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, et al. (2002) Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med* 347: 472–480.
 45. Kuribara R, Honda H, Matsui H, Shinjyo T, Inukai T, et al. (2004) Roles of Bim in apoptosis of normal and Bcr-Abl-expressing hematopoietic progenitors. *Mol Cell Biol* 24: 6172–6183.
 46. Essafi A, Fernandez de Mattos S, Hassen YA, Soeiro I, Mufti GJ, et al. (2005) Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. *Oncogene* 24: 2317–2329.
 47. Gilmore AP (2005) Anoikis. *Cell Death Differ* 12: 1473–1477.
 48. Reginato MJ, Mills KR, Paulus JK, Lynch DK, Sgroi DC, et al. (2003) Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. *Nat Cell Biol* 5: 733–740.
 49. Quadros MR, Connelly S, Kari C, Abrams MT, Wickstrom E, et al. (2006) EGFR-dependent downregulation of Bim in epithelial cells requires MAPK and PKC-delta activities. *Cancer Biol Ther* 5: 498–504.
 50. Frey MR, Dise RS, Edelblum KL, Polk DB (2006) p38 kinase regulates epidermal growth factor receptor downregulation and cellular migration. *EMBO J* 25: 5683–5692.
 51. Lu J, Quearry B, Harada H (2006) p38-MAP kinase activation followed by BIM induction is essential for glucocorticoid-induced apoptosis in lymphoblastic leukemia cells. *FEBS Lett* 580: 3539–3544.
 52. Janmaat ML, Rodriguez JA, Gallegos-Ruiz M, Kruyt FA, Giaccone G (2006) Enhanced cytotoxicity induced by gefitinib and specific inhibitors of the Ras or phosphatidylinositol-3 kinase pathways in non-small cell lung cancer cells. *Int J Cancer* 118: 209–214.
 53. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, et al. (2001) Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* 293: 876–880.
 54. Heinrich MC, Corless CL, Blanke CD, Demetri GD, Joensuu H, et al. (2006) Molecular correlates of imatinib resistance in gastrointestinal stromal tumors. *J Clin Oncol* 24: 4764–4774.
 55. Jabbour E, Kantarjian H, Jones D, Talpaz M, Bekele N, et al. (2006) Frequency and clinical significance of BCR-ABL mutations in patients with chronic myeloid leukemia treated with imatinib mesylate. *Leukemia* 20: 1767–1773.
 56. Carter TA, Wodicka LM, Shah NP, Velasco AM, Fabian MA, et al. (2005) Inhibition of drug-resistant mutants of ABL, KIT, and EGF receptor kinases. *Proc Natl Acad Sci U S A* 102: 11011–11016.
 57. Yun CH, Boggon TJ, Li Y, Woo MS, Greulich H, et al. (2007) Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity. *Cancer Cell* 11: 217–227.
 58. Cho BC, Im CK, Park MS, Kim SK, Chang J, et al. (2007) Phase II study of erlotinib in advanced non-small-cell lung cancer after failure of gefitinib. *J Clin Oncol* 25: 2528–2533.

Editors' Summary

Background. Most cases of lung cancer—the leading cause of cancer deaths worldwide—are “non-small cell lung cancer” (NSCLC). Many patients with NSCLC die within a year of their diagnosis, but recently, “targeted” therapies have increased the life expectancy of some of them. Like all cancers, NSCLC occurs when cells begin to divide uncontrollably because of changes (mutations) in their genes. Targeted therapies specifically attack these changes and, unlike standard chemotherapy drugs, kill cancer cells without damaging normal cells. The targeted drugs used to treat NSCLC are gefitinib and erlotinib, two epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs). In normal cells, messenger proteins bind to EGFR and activate its tyrosine kinase, an enzyme that sticks phosphate groups on tyrosine (an amino acid) in other proteins. These “phosphorylated” proteins then tell the cell to divide. In some NSCLCs, EGFR drives uncontrolled cell division because its tyrosine kinase is mutated and the cancer becomes dependent on or “addicted” to EGFR signaling for its survival. TKI treatment can dramatically shrink this subset of NSCLCs, most of which lack a specific part of *EGFR* (the gene that encodes EGFR) or have the amino acid leucine instead of arginine at position 858 (an L858R mutation) of EGFR.

Why Was This Study Done? TKI-sensitive NSCLCs eventually become resistant to TKIs because they acquire additional (secondary) mutations. In half of these TKI-resistant tumors, the additional mutation is replacement of threonine by methionine at position 790 (T790M) in EGFR. However, the mutations responsible for the remaining cases of TKI resistance are not known. In addition, little is known about how TKIs induce cell death other than that they induce a type of cell death called apoptosis. A better understanding of how TKIs kill tumor cells and how secondary mutations block their effects could reveal ways to enhance their action and improve the outcome for patients with NSCLC. In this study, the researchers have studied the mechanism of TKI-induced cell death and of resistance to TKIs.

What Did the Researchers Do and Find? The researchers first measured the ability of gefitinib to cause apoptosis (genetically programmed cell death) in NSCLC cell lines (tumor cells adapted to grow indefinitely in dishes) that had the *EGFR* deletion, the L858R mutation, or normal EGFR. Gefitinib caused apoptosis only in cell lines with altered EGFR. Then they asked whether a proapoptotic protein called BIM (a member of the BCL2 family of pro- and antiapoptotic proteins) is involved in TKI-induced cell death—BIM is known to be involved in this process in leukemia (blood cancer) cells. Gefitinib treatment increased the expression of BIM in TKI-

sensitive NSCLC cell lines and reduced the phosphorylation of BIM (which makes BIM more active). By contrast, blocking BIM expression using a technique called RNA interference reduced TKI-induced apoptosis in TKI-sensitive NSCLC cells. Furthermore, introduction of the T790M resistance mutation into these cells blocked gefitinib-induced up-regulation of BIM and apoptosis. Finally, the researchers identified a new TKI resistance mutation (L747S, substitution of serine for leucine at position 747) in a patient whose TKI-sensitive NSCLC had become resistant to gefitinib, and showed that this resistance mutation also reduced TKI-induced apoptosis in cells growing in dishes by interfering with BIM up-regulation.

What Do These Findings Mean? These findings (and those reported by Gong et al. and Cragg et al.) show that BIM is required for TKI-induced apoptosis in *EGFR* mutant NSCLC cells. They also show that mutations that make TKI-sensitive cells resistant to these drugs reduce TKI-induced apoptosis by preventing the upregulation of BIM. These results were obtained by examining the behavior of established cell lines growing in dishes and need to be confirmed in cells freshly isolated from tumors and in tumors themselves. However, they suggest that the efficacy of TKIs could be increased by finding ways to increase BIM expression or to activate other proteins involved in apoptosis. Such approaches might be particularly beneficial for patients with NSCLC whose initially TKI-sensitive tumors have acquired mutations that make them resistant to TKIs.

Additional Information. Please access these Web sites via the online version of this summary at <http://dx.doi.org/10.1371/journal.pmed.0040315>.

- Ingo Mellingerhoff discusses this paper and two related ones in a perspective article
- US National Cancer Institute information for patients and professionals on lung cancer (in English and Spanish)
- Information for patients from Cancer Research UK on lung cancer, including information on treatment with TKIs
- CancerQuest information on all aspects of cancer from Emory University (in several languages)
- Wikipedia pages on apoptosis, epidermal growth factor receptor, and BCL2 proteins (note that Wikipedia is a free online encyclopedia that anyone can edit; available in several languages)
- Information for patients from Cancerbackup on erlotinib and gefitinib

