DANIEL BOTELHO COSTA

DETERMINANTES MOLECULARES DE RESPOSTA E RESISTÊNCIA AOS INIBIDORES DA TIROSINA QUINASE (TKI) EM PACIENTES COM CARCINOMA DE PULMÃO NÃO PEQUENAS CÉLULAS (CPNPC) COM MUTAÇÕES NO GENE DO RECEPTOR DO FATOR DE CRESCIMENTO EPIDÉRMICO (*EGFR*)

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Orientador: Prof. Dr. Lair Zambon

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LISTA DE ABREVIATURAS

AKT	Família de oncogenes genes AKT (AKT1, AKT2, AKT3).
ASCO	Sociedade Americana de Oncologia Clínica (American Society of Clinical Oncology)
ATP	Adenosina trifosfato
BIDMC	Centro Médico Beth Israel Deaconess (Beth Israel Deaconess Medical Center)
BIM	BCL2-like 11, é uma proteína pró-apoptótica da família antiapoptótica BCL2
BRC-ABL	Gene híbrido BCR/ABL, responsável pela leucemia mielóide crônica (LMC)
CP	Carcinoma de pulmão
CPNPC	Carcinoma de pulmão não pequenas células (non-small cell lung cancer)
SCLC	Carcinoma de pulmão de pequenas células (small cell lung cancer)
DFHCC	Dana Farber/Harvard Cancer Center
DNA	Ácido desoxirribonucléico
EGFR	Receptor do fator de crescimento epidérmico
ERK	Quinases reguladas por sinal extracelular
FDA	Administração de alimentos e drogas dos EUA (Food and Drug Administration)
GIST	Tumores estromais gastrointestinais
KIT	Oncogene c-KIT. Família KIT quinase

LMC	Leucemia mielóide crônica
LREA	Região conservada no exon 19 do gene *EGFR*. A mutação mais comum do *EGFR* consiste em uma pequena deleção ao redor do principal local conservado LREA no exon 19 (resíduos 747-750), seguido por uma mutação no exon 21 (L858R, que é a substituição da leucina por arginina)
MAPK	Mitogen Actived Protein Kinase (Proteína quinase ativada por mitógeno)
MET	Oncogene c-MET. Família MET quinase
PDGFRA	Platelet derived growth factor receptor, alpha polypeptide (Receptor do fator de crescimento derivado de plaquetas, polipeptídeo alfa). Oncogene PDGFR
RNA	Ácido ribonucléico
si RNA	Ácido ribonucléico de interferência
SMG	Sobrevida média global
SLP	Sobrevivência livre de progressão. O tempo entre o começo de uma terapia antineoplásica e a progressão radiológica ou morte do paciente
SRC	Família SRC quinase. Oncogene SRC
TKI	Inibidores da atividade tirosina quinase
TNM	Tumor, nódulo, metástase.
TR	Taxa de resposta

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RESUMO

A maioria dos carcinomas de pulmão não pequenas células (CPNPC) em estádios avançados com mutações ativadoras (deleções do exon 19 ou a mutação L858R do exon 21) do receptor do fator de crescimento epidérmico (*EGFR*) respondem inicialmente, aos medicamentos gefitinib e erlotinib, que são inibidores da tirosina quinase (TKIs) do EGFR. Porém em uma média de 6-12, meses esses tumores desenvolvem resistência adquirida aos TKIs do EGFR. Dois mecanismos de resistência ao gefitinib/erlotinib explicam porque os CPNPC com mutações do *EGFR* se tornam resistentes aos TKIs: mutações de resistência secundária e um sistema de "troca de oncogenes". A mutação T790M-*EGFR* secundária ocorre em 50% dos pacientes com mutação no *EGFR* com resistência adquirida aos TKIs do EGFR, e em *in vitro* esta mutação T790M-*EGFR* inativa a hipersensitividade das mutações ativadoras do *EGFR* ao gefitnib ou erlotinib. Outras mutações de resistência secundárias (D761Y, L747S, A854T) são raras. Um outro mecanismo de resistência é a amplificação adquirida do oncogene *MET*, que ocorre em mais ou menos 20% do pacientes resistentes ao gefitinib/erlotinib e, em metade destes casos, em conjunção com T790M. O MET ativa sinais de sinalização que contornam o EGFR inibido, gerando um sistema de "troca de oncogenes" nesses tumores. Esses dados pré-clinicos relevantes aos CPNPCs com o *EGFR* mutado e resistência ao gefitinib ou erlotinib levaram ao desenvolvimento de experimentos clínicos com novos inibidores do EGFR que inibem "in vitro" a mutação T790M-*EGFR* (HKI-272, XL-647, BIBW-2992 e PF00299804), e inibidores de MET mais TKIs do EGFR em combinação. Neste trabalho: 1) Agrupamos e resumimos os dados dos experimentos clínicos prospectivos com o gefinitib em pacientes com o *EGFR* mutado. Mais de 80% dos pacientes com deleções do exon 19 ou a mutação L858R do *EGFR* tiveram resposta radiográfica, com sobrevivência livre de progressão de 7,7 a 12,9 meses nos estudos identificados, e sobrevivência geral acima de 15 meses; 2) Usamos células CPNPC com mutações do *EGFR* para identificarmos a molécula pró-apoptótica BIM como o efetor principal da apoptose induzida pelos TKIs do EGFR; 3) Caracterizamos a mutação resistente ao gefinitib *EGFR*-L858R-L747S, e determinamos que L858R-L747S apresenta um padrão de resistência menos acentuado ao gefitinib do que o observado com

L858R-T790M; e 4) Avaliamos os efeitos do erlotinib em pacientes com CPNPC *EGFR* mutado e resistência ao gefitinib, caracterizando a correlação da resposta radiográfica e clínica com os mecanismos conhecidos de resistência ao TKIs do EGFR (as mutações de resistência secundárias T790M e L747S, e a amplificação do *MET*). A maioria (mais de 83%) dos pacientes resistentes ao gefitinib tiveram progressões radiográficas nos primeiros 2 a 4 meses de exposição ao erlotinib 150 mg/dia. Isto é consistente com nossas observações pré-clínicas, indicativas de que a maioria dos tumores resistentes ao gefitinib possui predominantemente T790M e/ou amplificações do *MET*, que são resistentes tanto ao gefitinib quanto erlotinib. Pesquisas pré-clínicas e experimentos clínicos futuros do CPNPC com *EGFR* mutado têm o potencial de melhorar os resultados do tratamento clínico de pacientes com essas mutações somáticas.

ABSTRACT

Most advanced non-small cell lung cancers (NSCLCs) with activating epidermal growth factor receptor (*EGFR*) mutations (exon 19 deletions or L858R) initially respond to the EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib. However, over time (median of 6-12 months) most tumors develop acquired resistance to EGFR TKIs. Intense research in these NSCLCs has identified two major mechanisms of resistance to gefitinib/erlotinib: secondary resistance mutations and "oncogene kinase switch" systems. The secondary T790M mutation occurs in 50% of *EGFR* mutated patients with TKI resistance, and *in vitro* this mutation negates the hypersensitivity of activating *EGFR* mutations. Other secondary resistance mutations (D761Y, L747S, A854T) seem to be rare. The amplification of the *MET* oncogene is present in 20% of TKI-resistant tumors; however in half of the cases with this "oncogene kinase switch" mechanism the T790M is co-existent. The growing pre-clinical data in *EGFR* mutated NSCLCs with acquired resistance to gefitinib or erlotinib has spawned the initiation or conception of clinical trials testing novel EGFR inhibitors that *in vitro* inhibit T790M (HKI-272, XL-647, BIBW-2992 and PF00299804), and MET inhibitors in combination with EGFR TKIs. In this work we: 1) Pooled and summarized data from prospective clinical trials of gefitinib for *EGFR* mutated patients. More than 80% of patients with exon 19 deletions or the L858R *EGFR* mutation attained a radiographic response with progression-free survival of 7.7 to 12.9 months in the identified studies, and overall survival exceeding 15 months; 2) Identified the pro-apoptotic molecule BIM as the main effector of EGFR TKI-induced apoptosis using NSCLC cell lines with *EGFR* mutations; 3) Characterized the L858R-L747S gefitinib-resistant mutation, and demonstrated that L858R-L747S has a partial resistance pattern when compared to L858R-T790M; and 4) Evaluated the effects of erlotinib in *EGFR* mutated NSCLC with resistance to gefitinib while characterizing the correlation of response and resistance to this approach to the known mechanisms of resistance to EGFR TKIs (the secondary mutations T790M and L747S, and the amplification of MET). Our clinical observation was that the majority (over 83%) of the gefitinib-resistant patients given erlotinib 150 mg/day had radiographic progression within the first 2 to 4 months of exposure. This is consistent with our pre-clinical

observations, since we expected gefitinib-resistant tumors to predominantly harbor T790M and/or *MET* amplification, which are cross-resistant to both gefitinib and erlotinib. Ongoing pre-clinical and clinical research in *EGFR* mutated NSCLC has the potential to significantly improve the outcomes of patients with these somatic mutations.

1- INTRODUÇÃO

1.1- Considerações gerais

Em 2008, o câncer de pulmão (CP) continua liderando as mortes relacionadas ao câncer nos Estados Unidos, tanto para homens quanto para mulheres (Jemal et al., 2008), e a tendência é similar em outros países, incluindo o Brasil. O CP é dividido em dois subgrupos: carcinoma de pulmão de pequenas células (CPPC) e carcinoma de pulmão não pequenas células (CPNPC). Mais de 80% dos cânceres de pulmão são CPNPC e o prognóstico dos pacientes diagnosticados com CPNPC avançado continua a ser extremamente desfavorável (Ardizzoni et al., 2007). Apesar da conhecida associação entre fumo e os efeitos carcinogênicos do cigarro (Alberg e Samet, 2003), aproximadamente 20% dos casos de CPNPC ocorrem em indivíduos que nunca fumaram (Sun et al., 2007). Cigarros a base de tabaco contêm muitos carcinógenos, como os hidrocarbonetos policíclicos aromáticos, sendo o antraceno e, principalmente, o benzopireno o principal indutor da carcinogênese (Pfeifer et al., 2002).

A interação entre o tempo de exposição a estes carcinógenos, associado às variações individuais genéticas (como dos genes da glutathione-S-transferases e p53), determinam a capacidade de levar danos ao DNA no epitélio das vias aéreas e as subseqüentes transformações malignas (Vineis e Caporaso, 1995). Fatores genéticos tem papel importante no mecanismo de aparecimento desta neoplasia, seja por alterações de genes relacionados com a biometabolização de carcinógenos ou genes relacionados com a diferenciação celular e supressão de tumores (Honma et al., 2008). O aparecimento do CP se deve a múltiplos fatores externos e genéticos e que envolvem múltiplos genes, o que, em parte, pode explicar a dificuldade de desenvolvimento de terapêuticas mais efetivas no CP, quando comparado a outras neoplasias (Zambon et al., 2006).

A base genética do CPNPC não foi completamente elucidada, mas numerosos estudos de associações genômicas começaram a revelar os “loci” que aumentam o risco deste câncer, como a região 15q24 (Thorgeirsson et al., 2008;

Hung et al., 2008). Os fatores de risco para não fumantes com CPNPC são menos claros (Wakelee et al., 2007).

O prognóstico e a estratégia de tratamento para os pacientes com CPNPC dependem do estádio em que o câncer é encontrado. Baseado na classificação do sistema tumor-nódulo-metástase (TNM), a sobrevida de 5 anos para pacientes com estádio IA no CPNPC é de 60%, no IB 40%, II 25-35%, IIIA 15%, IIIB 5% e no estádio IV menos de 1% (Mountain, 1997). O tratamento para CPNPC no estadio I é somente a remoção cirúrgica do tumor. No caso do estádio II para o CPNPC, a remoção cirúrgica é acompanhada de quimioterapia adjuvante. Estes dados são fundamentados nos mais recentes estudos de quimioterapia adjuvante baseada em cisplatina para pacientes com CPNPC após cirurgia (Pisters et al., 2007). Esses estudos mostraram melhora significativa de 5-15% na sobrevida em cinco anos, com a adição da quimioterapia após a remoção cirúrgica para os estádios IB ao IIIA no CPNPC. Em 2007, a Sociedade Americana de Oncologia clínica (ASCO) apresentou recomendações para quimioterapia sistêmica adjuvante para pacientes com CPNPC e concluiu que todos pacientes com CPNPC nos estádios II e IIIA devem receber quimioterapia baseada em cisplatina, após a remoção cirúrgica (Pisters et al., 2007). Pacientes com estádio IIIA irremovível, baseado no envolvimento do linfonodo mediastinal (N2), ou estádio IIIB, são atualmente tratados com quimioterapia e radioterapia, com sobrevida de 5 anos ao redor de 20% (Pisters et al., 2007). Nos casos de estádio IV, o tratamento é paliativo, porque já há metástase e inexiste a possibilidade de cura.

O uso paliativo da quimioterapia com base em cisplatina tem sido a terapia básica para o estádio IV de CPNPC (Schiller et al., 2002). As taxas de respostas (TR) com diferentes combinações de quimioterapia à base de cis ou carboplatina variam ao redor de 20% com uma sobrevida média global (SMG) de 8-10 meses e menos de 15% dos pacientes sobrevivem 2 anos. Mesmo com a adição do anticorpo monoclonal contra o fator de crescimento vascular endotelial (VEGF), chamado “bevacizumab" (Avastin™), a quimioterapia com carboplatina e

paclitaxel, esse tratamento somente consegue melhorar a TR para 30%, a sobrevivência livre de progressão (SLP) para 8 meses e a SMG para 12 meses (Sandler et al., 2006). Mesmo com três terapias de segunda linha aprovadas pela Administração de Alimentos e Drogas dos Estados Unidos (Food and Drug Administration - FDA) para CPNPC platino-progressivo, que incluem docetaxel (Shepherd et al., 2000), pemetrexed (Hanna et al., 2004) e erlotinib (Shepherd et al., 2005), poucos pacientes sobrevivem por mais de 2 anos.

Todavia, existe uma grande heterogeneidade entre pacientes, seus cursos clínicos e respostas a diferentes terapias antineoplásicas.

1.2- Mutações do *EGFR* em CPNPC e "vício oncogênico"

A identificação de mutações no gene do receptor do fator de crescimento epidérmico (*EGFR*) em pacientes com CPNPC proporcionou um dos primeiros exemplos de terapias alvo ("targeted therapies") nesta doença (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004).

Em 2004, três grupos de investigadores (dois do Dana-Farber/Harvard Cancer Center e um do Memorial-Sloan Kettering Cancer Center) identificaram mutações somáticas no domínio da tirosina kinase do *EGFR* em pacientes com CPNPC (Figura 1).

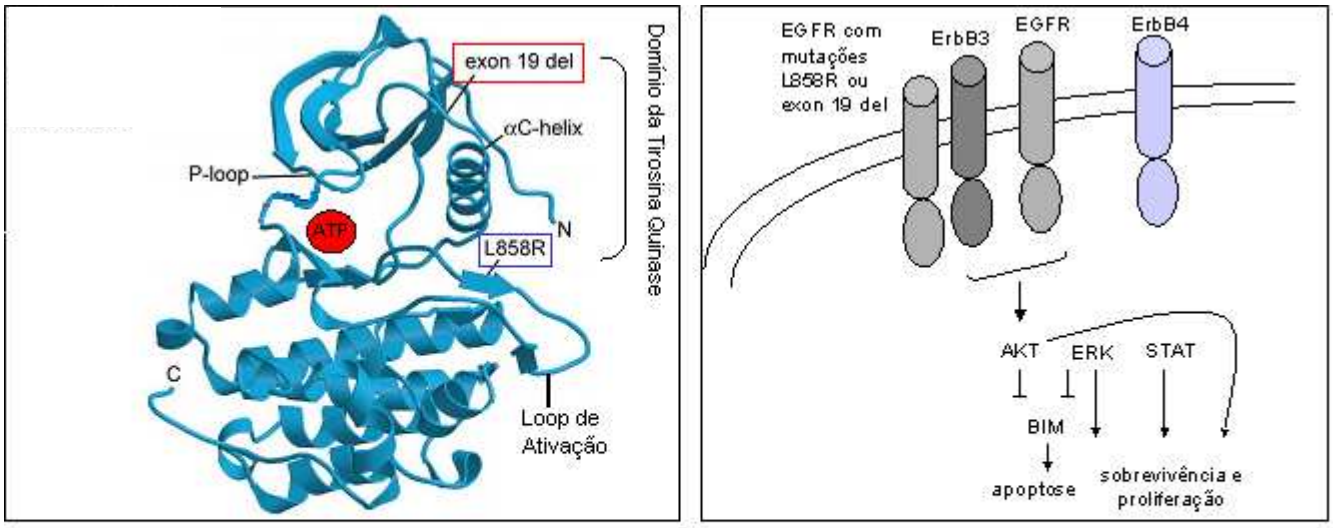

Figura 1. As mutações do *EGFR* com maior prevalência consistem em pequenas deleções em volta do padrão LREA conservado do exon 19 (resíduos 747-750 – deleções no exon 19), seguido de uma mutação (L858R) do exon 21. O EGFR mutado ativa vias intracelulares que inibem apoptose e estimulam sobrevivência e proliferação celular, como a PI3K–Akt, Ras–Raf–MEK–ERK1 e ERK2, e as vias STAT3 e STAT5.

Tentativas de seqüenciamento em grande escala, vêm identificando constantemente mutações no *EGFR* em um subgrupo de pacientes com CPNPC composto de mulheres, não fumantes, com tipo histológico adenocarcinoma e pacientes originários da Ásia (Shigematsu et al., 2005). Mutações no *EGFR* não são encontrados em números significativos em carcinomas de células escamosas do pulmão, CPPC e outras neoplasias epiteliais. Portanto, mutações ativadoras somáticas do *EGFR* representam uma característica própria de uma subclasse do CPNPC.

É estimado que mutações do *EGFR* estejam presentes em 10-15% de todos CPNPC, em populações de descendência européia e por volta de 40% em asiáticos (Kosaka et al., 2004). A porcentagem de casos é muito maior em não fumantes com CPNPC, em que metade dos casos abriga tais mutações ativadoras (Sequist e Lynch, 2008). Os fatores de riscos genéticos e ambientais específicos responsáveis pelas mutações do *EGFR* são ainda desconhecidos

(Sun et al., 2007). Apesar da maior freqüência de casos em não fumantes, um estudo epidemiológico no Japão demonstrou que fumar por si só não protege os indivíduos contra neoplasias com a mutação no *EGFR*, mas pode diluir o número de casos devido ao grande aumento no número de CPNPC por outros fatores genéticos nesta população (Mitsudomi e Yatabe, 2007). Estudos estão sendo realizados para determinar o papel do sexo feminino, níveis de estrógeno, vapores de cozinha e polimorfismos genéticos no gene *EGFR* para tentar identificar os agentes responsáveis pelo CPNPC com mutação no *EGFR* (Fukui e Mitsudomi, 2008).

As mutações do *EGFR* com maior prevalência consistem em pequenas deleções em volta do padrão LREA conservado do exon 19 (resíduos 747-750 – deleções no exon 19), seguido de uma mutação (L858R) no exon 21 (Riely et al., 2006; Sequist et al., 2007b). As deleções no exon 19 e L858R são responsáveis por 90% das mutações do *EGFR* conhecidas (Figura 1).

As mutações do *EGFR* ocorrem no domínio tirosina quinase que abrange os exons 18 a 21 – o domínio inteiro é codificado pelos exons 18 ao 24 – e são agrupados ao redor da bolsa de ATP da enzima (Sharma et al., 2007). Mutações do domínio tirosina quinase são geralmente referidas como mutações ativadoras, pois elas parecem resultar em atividade quinase aumentada do receptor (Sharma et al., 2007). Consistente com o seu papel na etiologia do CPNPC, deleções no exon 19 e L858R do *EGFR* são oncogênicas em cultura celulares e em estudos em ratos transgênicos (Lynch et al., 2004; Paez et al., 2004; Ji et al., 2006; Politi et al., 2006) . Estas mutações do *EGFR* hiperativam a via sinalizadora controlada pelo EGFR e promovem sinais antiapoptóticos e pró-sobrevivência para o tumor. Isto ocorre através de alvos do EGFR, como a PI3K–Akt, Ras–Raf–MEK–ERK1 e ERK2, e as vias STAT3 e STAT5 (Figura 1). Isso faz dessas células com *EGFR* mutado, dependentes do *EGFR* para sua sobrevivência (Sharma et al., 2007).

Ambas as linhagens celulares e modelos animais em ratos com mutações no *EGFR* demonstram que células que possuem estas mutações são extremamente sensíveis à inibição do EGFR (Paez et al., 2004; Politi et al., 2006; Ji et al., 2006). O bloqueio da função do EGFR, nesses sistemas, por meio das pequenas moléculas inibidoras, o RNA de interferência ou a retirada dos indutores nos modelos experimentais leva à inibição das vias Akt e ERK com subseqüente pausa na proliferação celular e apoptose (Paez et al., 2004; Ji et al., 2006; Politi et al., 2006). Entretanto, o sinal pró-apoptótico chave, essencial para a indução da apoptose não havia sido identificado até 2007, quando nosso grupo decidiu investigar este problema.

O CPNPC com o *EGFR* mutado faz parte de uma classe molecular distinta de câncer de pulmão que apresenta o fenômeno de "vício oncogênico" ("oncogene addiction") e portanto, tem um "calcanhar de Aquiles" (seu ponto fraco) na inibição de EGFR por inibidores da tirosina quinase (TKIs) desse receptor. Este fenômeno de "vício oncogênico" gera a oportunidade de uma possível abordagem terapêutica com o objetivo de inibir, com o uso de TKIs, a atividade de tumores com oncogenes tirosina quinases alterados. O sucesso desta abordagem terapêutica foi observado na leucemia mielóide crônica (LMC) e em tumores gastrointestinais estromais (GIST) que dependem dos oncogenes BRC-ABL ou KIT. Esses oncogenes tornam esses tumores extremamente sensíveis à inibição mediada pela droga TKI imatinib que inibe ABL e KIT (Hehlmann et al., 2007; Rubin et al., 2007).

Uma cascata sinalizadora comum está envolvida na apoptose de células com "vício oncogênico" com os oncogenes SRC, BCR-ABL, e EGFR (Sharma et al., 2006). Curiosamente, as proteínas pró-apoptóticas "BH3-only" Bim e Bad mediam a apoptose induzida por "imatinib" em células leucêmicas dependentes no BCR-ABL (Kuroda et al., 2006). Como os mediadores chave para a morte celular induzida por TKIs em tumores *EGFR*-mutante permaneciam desconhecidos, formulamos a hipótese de que membros do "BH3-only" estariam envolvidos no sinal apoptótico após a interrupção da atividade EGFR pelos TKIs.

1.3- Mutações *EGFR* em CPNPC e as experiências clínicas com os inibidores tirosina quinase do EGFR (EGFR TKIs) gefitinib e erlotinib

O desenvolvimento de inibidores tirosina quinase do EGFR (EGFR TKIs) começou na década de 90 e os primeiros estudos com humanos se iniciaram nos anos subseqüentes (Hidalgo et al., 2001; Baselga et al., 2002). O primeiro composto desenvolvido foi denominado ZD-1839 e depois rebatizado gefitinib. Logo depois, começou o desenvolvimento clínico do composto OSI-774, depois denominado erlotinib.

Os dois compostos receberam aprovação da FDA dos Estados Unidos para uso em pacientes com CPNPC, não-selecionados, como terapia de 2ª e 3ª linha após falha da primeira linha de quimioterapia com drogas baseadas em cis ou carboplatina. Entretanto, nesta população de pacientes não-selecionados, o benefício clínico foi pequeno. Gefitinib não foi estatisticamente melhor que o placebo para controlar a progressão de estádios avançados em pacientes com CPNPC (Thatcher et al., 2005) e, como conseqüência, o FDA restringiu o seu uso para pacientes que haviam sido beneficiados previamente do tratamento ou estavam participando de ensaios clínicos. Nesse mesmo ensaio de fase III, os grupos de pacientes não fumantes e asiáticos mostraram um benefício clínico evidente com o uso do gefitinib comparados ao placebo (Thatcher et al., 2005). Deste modo, gefitinib continua a ser usado em países do leste asiático e em pacientes com o genótipo *EGFR* mutado (Mitsudomi e Yatabe, 2007; Costa et al., 2007b). Erlotinib foi aprovado para ser utilizado em pacientes não selecionados após falha da quimioterapia (Shepherd et al., 2005). O erlotinib, utilizado como 2ª ou 3ª linha de tratamento para CPNPC, propiciou uma pequena melhora, estatisticamente significativa, na sobrevida, comparada com o placebo, mas o TR foi somente 8,9%, a SLP média de 2,2 meses e SMG de 6,7 meses nos pacientes tratados com a droga.

Estava claro, desde o início do desenvolvimento destes compostos, que somente uma minoria de pacientes poderia obter benefício significativo com o tratamento. As características clínicas que indicariam boa resposta terapêutica

incluíam: sexo feminino, tipo histológico adenocarcinoma, etnia asiática e indivíduos não fumantes (Miller et al., 2004). A identificação de mutações ativadoras do *EGFR,* que ocorrem mais freqüentemente nos grupos citados acima, elucidaram o evento biológico que determina a sensibilidade observada com o tratamento com os EGFR TKIs no CPNPC (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004). Estas descobertas também reforçaram a noção de que CPNPCs com *EGFR* mutado são tumores com "vício oncogênico".

Estudos retrospectivos de milhares de pacientes tratados com os dois EGFR TKIs disponíveis, gefitinib e erlotinib, como terapias de 2ª ou 3ª linha em CPNPC (Mitsudomi et al., 2005; Sequist et al., 2007a) demonstraram que a maioria (cerca de 80%) dos pacientes com tumores com mutações clássicas do *EGFR* obtiveram respostas radiográficas e clínicas, após tratamento com esses agentes orais. Em algumas séries, tanto a SLP como a sobrevida global foi significantemente melhor em pacientes tratados com EGFR TKI e portadores de mutações *EGFR,* quando comparados com casos sem mutações (Sequist et al., 2007a). A avaliação da mutação *EGFR* como um marcador prognóstico e preditivo em CPNPC está sendo explorada em múltiplos ensaios clínicos de fases II e III.

Nove ensaios clínicos prospectivos de fase II avaliaram gefitinib e erlotinib como monoterapia para pacientes selecionados, baseados na presença de mutações do *EGFR* (Asahina et al., 2006; Inoue et al., 2006; Paz-Ares et al., 2006; Sutani et al., 2006; Sunaga et al., 2007; Yoshida et al., 2007; Porta et al., 2008; Sequist et al., 2008; Tamura et al., 2008) (Tabela 1).

Tabela 1- Experimentos clínicos prospectivos de gefitinib ou erlotinib para pacientes com mutações no *EGFR*

Referências	Droga	TR n/total (%)	Média TP/SLP em meses (IC95%)	Sobrevida em 1 ano (%)	SMG em meses	Média em meses
Inoue et al., 2006	gefitinib	12/16 (75%)	9,7 (7,4-9,9)	-	não alcançado	7,6
Asahina et al., 2006	gefitinib	12/16 (75%)	8,9 (6,7-11,1)	83%	não alcançado	12,7
Sutani et al.,2006	gefitinib	21/27 (78%)	9,4 (-)	-	15,4	-
Yoshida et al.,2007	gefitinib	19/21 (90.5%)	7,7 (-)	80%	não alcançado	-
Sunaga et al.,2007	gefitinib	16/19 (84%)	12,9 (-)	85%	não alcançado	12,6
Tamura et al.,2008	gefitinib	21/28 (75%)	11,5 (7,3-não alcançado)	79%	não alcançado	18,6
Sequist et al.,2008	gefitinib	17/31 (55%) *	9,2 (6,2-11,8)	73%	17,5	12,3
Paz-Ares et al., 2006 #	erlotinib	31/38 (82%)	13,3 (-)	82%	não alcançado	7
Porta et al., 2008 #	erlotinib	111/152 (73%)	12 (-)	-	24	10

TR,taxa de resposta; TP,tempo para progressão SLP, sobrevida livre de progressão; SMG, sobrevida média global; -, não relatado no material publicado; * este teste incluiu pacientes com outras mutações no EGFR como G719A, L861Q, T790M/L858R e a inserção no exon 20. Nenhum desses pacientes tiveram resposta. A TR para L858R foi de 78% e para a deleção do exon 19 foi de 59%; # dados atualizados apresentados na conferência ASCO.

Esses experimentos confirmaram que por volta de 75% dos pacientes com mutações do tipo L858R ou deleções do exon 19 atingem respostas (Costa et al., 2007b). Como parte do nosso projeto, consolidamos os dados das cinco primeiras publicações para uma análise global e comparativa dos resultados.

1.4- Mecanismo de resistência adquirida aos inibidores tirosina quinase de EGFR (TKIs) em CPNPC com *EGFR* apresentando mutações

Apesar da eficácia do gefitinib e do erlotinib como monoterapia para CPNCP com mutações do *EGFR*, a resistência adquirida à terapia com TKI do EGFR ocorre na maioria dos pacientes. Em grande parte dos testes prospectivos, a SLP não passou de 12 meses (Costa et al., 2007b).

O primeiro mecanismo de resistência adquirida a esses medicamentos relatado na literatura foi a aquisição da mutação EGFR T790M (Kobayashi et al., 2005a; Pao et al., 2005). Este trabalho foi conduzido na instituição Beth Israel Deaconess Medical Center (BIDMC) e no Dana-Farber/Harvard Cancer Center (DFHCC) em 2005 (Kobayashi et al., 2005a). O resíduo de metionina na posição 790 gera uma corrente lateral mais robusta que afeta a ligação de gefitinib/erlotinib ou aumenta a afinidade da bolsa EGFR tirosina quinase ao ATP, diminuindo a ligação efetiva de gefitinib e erlotinib à bolsa de tirosina quinase do EGFR (Kobayashi et al., 2005a; Yun et al., 2008). Existe uma grande similaridade entre as estruturas de receptores tirosina quinase e algumas das mutações de resistência adquiridas. Este é o caso das mutações T315I, T670I, e T790M em ABL1, KIT e EGFR, respectivamente, em LMC, GIST e CPNPC com mutação do *EGFR* (Carter et al., 2005). Nosso grupo de pesquisadores demonstrou em múltiplos modelos *in vitro* e *in vivo* que T790M em *cis* há uma mutação ativadora (tanto deleções L858R ou deleções no exon 19) inibem a sensibilidade das doses terapêuticas possíveis de serem alcançadas com gefitinib ou erlotinib (Kobayashi et al., 2005b; Pao et al., 2005). A mutação resistente secundária T790M (Kobayashi et al., 2005a; Pao et al., 2005) acontece em mais ou menos 50% dos pacientes com progressões radiográficas (Balak et al., 2006; Kosaka et al., 2006), após o tratamento com gefitinib ou erlotinib (Figura 2).

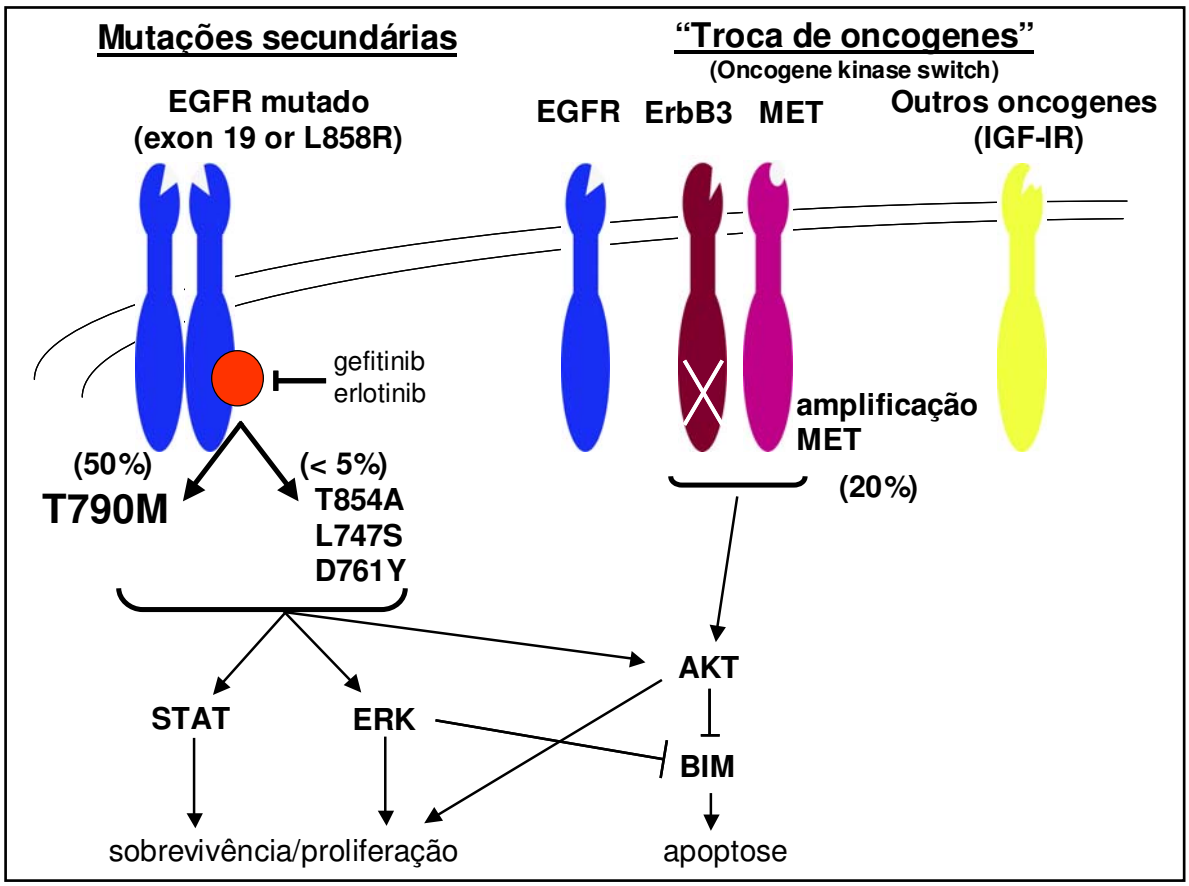

Figura 2- Mecanismos de resistência adquirida aos inibidores tirosina quinase de EGFR (TKIs) em CPNPC com *EGFR* apresentando mutações. Mutações secundárias e "troca de oncogenes" (oncogene kinase switch).

Um outro mecanismo de resistência é a amplificação adquirida do oncogene *MET*, que ocorre em mais ou menos 20% do pacientes resistentes ao gefitinib/erlotinib e em metade destes casos em conjunção com T790M (Bean et al., 2007; Engelman et al., 2007b). Os mecanismos de resistência nos tumores restantes não foram completamente esclarecidos e apenas uma quantidade pequena de outras mutações secundárias como L858R-D761Y, e L858R-T854A (Balak et al., 2006) foram identificados. Como parte do nosso projeto, identificamos e caracterizamos uma mutação nova do *EGFR, a* L858R-L747S. A Figura 2 mostra os mecanismos de resistência descritos acima.

1.5- Testes clínicos para pacientes com resistência adquirida ao EGFR TKIs e CPNPC com mutações no *EGFR*

O melhor tratamento desta crescente população de CPNPC resistente aos EGFR TKIs não foi ainda estabelecido, mas o sucesso de qualquer terapêutica será dependente do mecanismo específico de resistência adquirida do tumor. Várias medidas foram iniciadas com o objetivo de utilizar dados pré-clínicos no planejamento racional de testes clínicos em pacientes com resistência adquirida ao gefitinib ou erlotinib. Nosso grupo mostrou que alguns inibidores irreversíveis do EGFR de segunda geração podem, *in vitro,* superar parcialmente a resistência proporcionada pela mutação T790M (Kobayashi et al., 2005a; Kobayashi et al., 2005b; Carter et al., 2005; Kwak et al., 2005). Este conhecimento gerou os ensaios clínicos de fase II dos compostos HKI-272, BIBW-2992, XL-647, e PF00299804 (Engelman et al., 2007a) nesta população de pacientes selecionados.

Em outros tumores com "vício oncogênico", como LMC e GIST, em que a translocação *BCR-ABL* ou mutações *KIT*, respectivamente, fazem com que estas neoplasias sejam sensíveis ao imatinib, parece que a dose do TKI faz diferença (Rubin et al., 2007). Nessas duas doenças, uma medida clínica a ser tomada, quando a resistência aparece, é aumentar a dose de imatinib de 400 mg a 600 mg/dia ou até doses superiores (Hehlmann et al., 2007; Kantarjian et al., 2007a; Blanke et al., 2008). Esta estratégia de incremento gradual de dose é somente efetiva em alguns pacientes, possivelmente inibindo mutações secundárias com resistência "borderline" ao imatinib ou afetando mecanismos não dependentes de mutação, com curtos períodos de controle da doença (Hehlmann et al., 2007; Blanke et al., 2008). Inibidores de ABL e KIT de segunda geração, recentemente receberam aprovação do FDA como terapias alternativas para LMC e GIST (Kantarjian et al., 2007b; Goodman et al., 2007).

Em tumores com *EGFR* mutado, é desconhecido se aumentos na dose de EGFR TKI, na presença de resistências adquiridas ou *de novo*, alteram seu curso clínico. Para avaliar a eficácia dessa medida, estudamos,

retrospectivamente, a evolução clínica de pacientes com mutação no *EGFR* que receberam primeiramente gefitinib 250 mg/dia e após apresentarem resistência ao gefitinib foram expostos ao erlotinib 150 mg/dia. Esta troca do gefitinib para o erlotinib tem como conseqüência que os pacientes recebem quase o dobro da dose biologicamente ativa desses EGFR TKIs (Hidalgo et al., 2001; Baselga et al., 2002). Como EGFR-T790M e a amplificação do *MET* geram níveis altos de resistência *in vitro* ao gefitinib e erlotinib (Kobayashi et al., 2005a; Engelman et al., 2007b), é nossa hipótese que o erlotinib não beneficiaria pacientes com resistência ao gefitinib, somente alterando a resposta em casos de resistência adquirida em clones portadores de mutações raras gefitinib-resistentes de menor resistência como L858R-D761Y ou L858R-L747S.

2- OBJETIVOS

Como parte de nosso trabalho para entender os determinantes moleculares de resposta e resistência ao EGFR TKIs em pacientes com CPNPC portadores de mutações ativadoras do *EGFR*, os objetivos propostos de cada artigo foram:

1) Coletar e analisar os dados clínicos de cinco ensaios clínicos prospectivos de gefitinib para pacientes com mutações do *EGFR*;

2) Identificar o peptídeo pró-apoptótico chave requerido para os efeitos apoptóticos do gefitinib, erlotinib e outros inibidores EGFR TKI em células com CPNPC com *EGFR* mutado;

3) Identificar e caracterizar novas mutações secundárias (L747S) que têm como conseqüência resistência adquirida à terapia EGFR TKI em CPNPC com *EGFR* mutado;

4) Analisar os efeitos clínicos e radiográficos do erlotinib 150 mg/dia em CPNPC com *EGFR* mutado e com resistência *de novo* ou adquirida ao gefitinib 250 mg/dia, e correlacionar as respostas radiográficas com os mecanismos moleculares de resistência.

3- PUBLICAÇÃO DOS RESULTADOS OBTIDOS

1. **Costa DB**, Kobayashi S, Tenen DG, Huberman MS. Pooled analysis of the prospective trials of gefitinib monotherapy for *EGFR*-mutant non-small cell lung cancers. Lung Cancer 2007b; 58(1):95-103.

2. **Costa DB**, Halmos B, Kumar A, Schumer ST, Huberman MS, Boggon TJ, Tenen DG, Kobayashi S. BIM mediates EGFR tyrosine kinase inhibitor-induced apoptosis in lung cancers with oncogenic *EGFR* mutations. PLoS Med 2007a; 4(10):1669-1679.

3. **Costa DB**, Schumer ST, Tenen DG, Kobayashi S. Differential responses to erlotinib in *EGFR* mutated lung cancers with acquired resistance to gefitinib carrying the L747S or T790M secondary mutations. J Clin Oncol 2008b; 26(7):1182-1184.

4. **Costa DB**, Nguyen KH, Cho BC, Sequist LV, Jackman DM, Riely GJ, Yeap BY, Halmos B, Kim J, Janne PA, Huberman MS, Pao W, Tenen DG, Kobayashi S. Effects of erlotinib in *EGFR* mutated non-small cell lung cancers with resistance to gefitinib. Clin Cancer Res 2008a;14(21): 7060-7067.

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Pooled analysis of the prospective trials of gefitinib monotherapy for EGFR-mutant non-small cell lung cancers

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Prospective;
Lung cancer;
Non-small cell lung cancer

Summary

Purpose: Epidermal growth factor receptor (EGFR) mutations have been found in the majority of gefitinib-responsive non-small cell lung cancer (NSCLC) patients from retrospective studies. We sought to compile the available phase II and prospective trials of this EGFR tyrosine kinase inhibitor (TKI) to better understand the efficacy and safety of selecting patients to receive gefitinib based on their genotype.

Design: We searched published trials involving EGFR-mutant patients and gefitinib. Five reports were identified (published between June 2006 and April 2007) in which gefitinib was given in a prospective manner to EGFR mutation positive patients at a dose of 250 mg/day. Responses were determined by RECIST and toxicities by NCI-CTC.

Results: A total of 101 patients were pooled from these studies. Fifty-nine received gefitinib as their first line of therapy and 42 after having received chemotherapy. The combined rate of complete and partial response (CR + PR) in the 99 measured patients was 80.8% (80/99) and only 7.1% (7/99) had progressive disease as best response. The response rate (CR + PR) for exon 19 deletion and L858R patients were 80.3% (53/66) and 81.8% (27/33), respectively. The median progression-free survival ranged from 7.7 to 12.9 months. Overall survival had not been reached in 4/5 reports and was 15.4 months in one of them. Gefitinib administration was safe (<50% of patients developed grades 1–2 skin rash or diarrhea) and interstitial lung disease was only reported in two patients (2%), without deaths.

Conclusions: Gefitinib monotherapy leads to objective responses in most patients with EGFR mutations. Both L858R and deletion 19 mutations derived similar clinical benefits. Small molecule TKIs are the new treatment paradigm for EGFR-mutant NSCLC.

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1. Introduction

Lung cancer is the leading cause of malignancy-related death in the United States [1] and the world. Patients diagnosed with metastatic and advanced non-small cell lung cancer (NSCLC) have a dismal prognosis that seldom reaches over 1–2 years. The use of combination platinum-based chemotherapeutics has been the cornerstone of palliative treatment of advanced NSCLC [2]. However, even the addition of the vascular endothelial growth factor monoclonal antibody bevacizumab to a platinum-based doublet was only able to achieve response rates (RR) of around 30% in the phase II [3] and III trials [4] and the progression-free survival (PFS) did not exceed 7.5 months. Median survival was barely above 12 months [4]. It is increasingly necessary, in this heterogeneous disease, to identify sub-sets of NSCLC patients who can receive tailored therapies to improve outcomes.

In 2004, three groups of investigators identified somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) in patients with NSCLC [5–7]. The most exciting translational finding of these initial studies was the almost ubiquitous presence of these mutations in patients who had radiographic and clinical responses the specific EGFR tyrosine kinase inhibitor (TKI), gefitinib [5,6]. Subsequent population based efforts to sequence EGFR in NSCLC have consistently identified EGFR mutations in an enriched cohort of women, non-smokers, adenocarcinomas and East Asians [8,9]. The most prevalent EGFR mutations consist of small inframe deletions around the conserved LREA motif of exon 19 (residues 747–750), followed by a single point mutation in exon 21–L858R. Both of these mutations compromise around 90% of known EGFR mutations [5,8,9].

Both cell line and mouse models of EGFR mutations demonstrate that tumor cells that harbor such mutations are exquisitely sensitive both to suppression of the EGFR driving signal or EGFR TKIs [6,10,11]. As data grows, it seems clear that EGFR-mutant NSCLCs compromise a distinct molecular class of lung cancers that display the phenomenon of ''oncogene addiction'' [12] and therefore have an ''Achilles' heel'' in the inhibition of EGFR by small molecule tyrosine kinase inhibitors. The success of such an oncogene-targeted approach for cancer therapy has been fulfilled in chronic myeloid leukemia (CML) and gastrointestinal stromal tumor (GIST) [13,14].

Recent reviews on EGFR mutations and lung cancer have been published, both exploring the biology of this disease [15] and also the retrospective patient data gathered over the last years [16–18]. Over 268 EGFR-mutant patients have been identified in retrospective case series in which gefitinib or erlotinib (another anilinoquinazoline EGFR inhibitor) were given to patients with metastatic NSCLCs, mostly as second or third line therapies [6–8,16,19–37]. The overwhelming observation was that the objective response rate of these EGFR mutant tumors reached 78%. In the same studies, the EGFR mutation negative tumors only showed a response rate of 10% [16]. In contrast to the astonishing responses seen in these molecularly classified tumors, the phase III trials of the anilinoquinazoline EGFR inhibitors in unselected NSCLCs who had failed chemotherapy demonstrated that the benefits of TKI therapy in lung cancer are not applicable to all patients. Erlotinib, in the second or third line setting, derived a small, statistically significant, improvement in survival compared to placebo [38], but RR was only 8.9%, median PFS 2.2 months and overall survival 6.7 months in the treatment arm. In the other hand, gefitinib, versus placebo in a similar group of patients, only achieved a statistically significant survival improvement in a planned sub-group analysis of non-smoking and Asians patients and not in the complete population studied [39]. The later results led to the Food and Drug Administration (FDA)'s limitation in the use of gefitinib in unselected patients with lung cancer in the United States. Patients in the United States can only receive gefitinib now as part of investigational protocols.

Our goal in this report is to compile the current phase II and prospective trials of gefitinib monotherapy for EGFR-mutant patients.

2. Material and methods

2.1. Literature search

We performed a systematic search of the MEDLINE and PUBMED databases to identify all recent clinical trials and prospective patient cohorts that contained EGFR mutant NSCLC patients that were treated with gefitinib. The search strategy included articles from 2004 through April of 2007 indexed under the subject headings *epidermal growth factor receptor mutation*, *EGFR*, *gefitinib*, *Iressa*, *tyrosine kinase inhibitor*, *prospective*, *phase II and trial*. We chose to start with the year 2004 because this marked the identification of EGFR mutations. The search did not restrict the type of publication or periodical. We did not include preliminary sets published as abstracts or meeting's proceedings. We selected all prospective trials that included patient selection based on EGFR genotyping and that clearly described that gefitinib was offered to patients based on this genotype identification. The search was also restricted to published manuscripts in the English language.

2.2. Patient selection and EGFR genotype in the identified trials

All the identified trials had to include an institutional approved protocol for human studies and genomic analysis of stored/collected tumor tissue. The inclusion criteria for administration of gefitinib had to include the identification of EGFR mutations in exons 18–21 of EGFR. However, we did not exclude any of the trials based on the method of DNA isolation (if from fresh tissue or paraffin-embedded tissue) or the strategy used to enhance tumor-derived DNA (either micro-dissection or use of more sensitive polymerase chain reaction (PCR) amplification techniques). The trials identified included only adult patients, most with advanced stage IIIB or IV NSCLC. Two trials only included chemotherapy naive patients who were treated in the first line setting, while the others contained a mixed population of patients who had received prior chemotherapy for their NSCLC or were receiving gefitinib as first line therapy.

2.3. Treatment schedules, response, survival assessment and statistical analysis in the identified prospective studies

All the identified trials had the same treatment schedule for gefitinib. This medication was given orally at a dose of 250 mg/day. The primary end point of most of these studies was tumor response rate and feasibility. Progression-free survival (PFS) and overall survival (OS) were also measured, but they were not primary end points in any of the studies identified. Toxicities, as defined by the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTC, either version 2 or 3), were monitored in five trials identified.

The objective tumor response was determined by response evaluation criteria in solid tumors (RECIST) [40]. A complete response (CR) was defined as complete disappearance of all clinically detectable tumors for at least 4 weeks, a partial response (PR) as a 30% or more decrease in the sum of the longest diameter of the target lesions for a minimum of 4 weeks with no new areas of tumors, and progressive disease (PD) defined as at least an increase in 20% in the sum of the longest diameter of the target lesions or a new tumor lesion. Stable disease (SD) was defined as any measurement in between a PR and PD for at least 8 weeks. Response rates (RR) were defined as CR + PR. The trials that monitored for PFS and OS used the date of enrollment as the starting point of measurement. Median PFS and OS were calculated using the Kaplan–Meier method [41] and differences based on type of mutation compared by the log-rank test [42]. Fisher's exact test was used to compare response rates in the different types of mutations and the two-tailed probability reported.

We used direct data as extracted from the author's publications for response rate, PFS and OS. However, for 1-year survival rates, when not available, we extrapolated the results based on the available Kaplan–Meier survival curves provided.

3. Results

3.1. Identification of five prospective trials of patients with EGFR mutations treated with gefitinib

Based on our search criteria, we identified six reports that prospectively evaluated the effects of gefitinib monotherapy for advanced NSCLC based on the presence of an EGFR tyrosine kinase domain mutation in the patient's tumor specimens [43–48]. All trials were published between 2006 and 2007. Five of these stemmed from Japanese and one from European groups. Since the European trial [48] only had three patients in which gefitinib was given prospectively and no data on survival or toxicity, we decided to exclude this trial from further analyses. Table 1 summarizes the five identified Japanese-based studies. The characteristics of these EGFR-mutant patients in these publications [43–47] were similar among the different studies. Most patients were women, non-smokers and had adenocarcinomas (Table 2).

Table 1 Characteristics of the prospective trials of gefitinib monotherapy for advanced EGFR mutation positive non-small cell lung cancers (NSCLC)

Publication source (reference)	Gefitinib 250 mg/day treatment	Number of patients (1st line/2nd line)	Country of origin	EGFR mutation analysis
Inoue et al. [43]	1st line therapy (chemotherapy naive patients)	16 (16/0)	Japan	PCR amplification and sequence of exons 18–23
Asahina et al. [44]		16 (16/0)	Japan	PCR amplification and sequence of exons 18–21
Sutani et al. [45]	Mixed 1st and subsequent lines of therapy (both patients who had received or not prior chemotherapy)	27 (4/23)	Japan	Peptide nucleic acid-locked nucleic acid (PNA-LNA) clamp of exons 18–21
Yoshida et al. [46]		21 (12/9)	Japan	Common fragment analysis of PCR amplification for exon 19 deletions and Cycleave real-time PCR for the L858R mutation
Sunaga et al. [47]		21 (11/10)	Japan	PCR amplification and sequence of exons 19–21
Total**	–	101 (59/42)	Japan	–

EGFR, epidermal growth factor receptor; PCR, polymerase chain reaction; (**) another prospective study by van Zandwijk et al. [48] was exluded from this analyses due to the small number of patients that received gefitinib ($n = 3$) and lack of survival data.

Table 2 Clinical characteristics of the EGFR-mutant patients (also includes EGFR genotyped patients that never received gefitinib as part of the prospective treatment)

Source (reference)	Gender (female/male)	Histology (adenocarcinoma/other)	Smoking status (non-smoker/smoker)[a]
Inoue et al. [43]	21/4	24/1	22/3
Asahina et al. [44]	15/5	19/1	15/5
Sutani et al. [45]	23/15	33/5	25/13
Yoshida et al. [46]	17/10	27/0	17/10
Sunaga et al. [47]	17/4	21/0	19/2
Total (n = 131)	93/38	124/7	98/33

EGFR, epidermal growth factor receptor.
[a] Not all trials defined either lifetime non-smokers vs. former smokers vs. smokers, however most detailed non-smokers vs. ever-smokers. This separation was used for this table.

Overall 101 patients with EGFR mutations received gefitinib. Two of the reports were phase II trials of first line gefitinib therapy for chemotherapy naïve EGFR-mutant stage IIIB or IV NSCLCs [43,44], while the remaining four contained a mixed population of patients, which were either receiving gefitinib as first line therapy or after tumor exposure to chemotherapy (second and subsequent lines of therapy). Overall, 59 patients received gefitinib as first line therapy and 42 after having received chemotherapy (Table 1).

The method of identification of EGFR genotypes is listed in Table 1. Most of the trials isolated tumor DNA from either diagnostic paraffin-embedded or fresh tissue (pleural fluid). Most of the reports identified the need to use micro-dissection techniques; however the details of the percentage of tumors where this was necessary are not disclosed. Four of the trials used direct polymerase chain reaction (PCR) amplification of DNA using EGFR primers that spanned between exons 19 and 21. One of the studies [45], used a more sensitive method for mutation identification, called peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp that has been reported to identify EGFR mutations in the presence of 100-fold background levels of wild-type EGFR from normal cells [45]. And the remaining trial only analyzed for the presence of exon 19 deletions or L858R [46].

Almost all of the patients available for analysis contained either exon 19 in frame deletions or the L858R mutation. In none of the patients was the TKI-resistant T790M mutation [49] identified or reported during the initial sequencing of the tumors.

3.2. Response rates in the total population and by different EGFR mutations

Of the 99 patients that were evaluated for a response in the five identified trials, 80 (80.8%) achieved either a complete or partial response (CR + PR). Another 12 (12.1%) had SD and only 7 (7.1%) displayed PD as best response (Table 3). In the phase II trials that evaluated only chemotherapy naïve patients, the combined CR + PR rates were 75% [43,44].

All of the trials identified objective response rates in the different types of EGFR mutations. As indicated in Table 4, the total number of patients with exon 19 deletions was 66 (around 67% of the patients evaluated for a response) and the other 33 patients had the L858R mutation (around 33% of total). This distribution of EGFR mutations had been expected by the retrospective studies of EGFR sequencing in non-treated patients [8,9].

The percentage of patients achieving either a CR or PR for exon 19 deletions and L858R mutations were 80.3% and 81.8%, respectively (Table 4). In only one of the trials did the exon 19 deletion cohort have a significantly higher response rate than the L858R patients, however even in that trial the investigators noted that the L858R patients with SD by RECIST had measurable tumor responses that did not qualify

Table 3 Overall responses in the 99 EGFR-mutant evaluated patients

Patient population	Source (reference)	CR + PR (%)	SD (%)	PD (%)	Total
Chemotherapy naïve (1st line therapy)	Inoue et al. [43]	12 (75%)	2 (12.5%)	2 (12.5%)	16
	Asahina et al. [44]	12 (75%)	1 (6%)	3 (19%)	16
Mixed 1st and subsequent lines of therapy	Sutani et al. [45]	21 (78%)	5 (18.5%)	1 (3.5%)	27
	Yoshida et al. [46]	19 (90.5%)	1 (4.8%)	1 (4.8%)	21
	Sunaga et al. [47]	16 (84%)	3 (15%)	0 (0%)	19[a]
Total		80 (80.8%)	12 (12.1%)	7 (7.1%)	99

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.
[a] In the prospective trial by Sunaga et al. [47], 21 patients were entered, however 2 discontinued treatment and only 19 were available for analysis of overall response to gefitinib therapy.

Table 4 Responses rates (combined CR + PR) by type of EGFR mutation

	Source (reference)	EGFR Mutation		p-Value[a]
		Deletion 19	L858R	
CR + PR/total (%) in 1st line therapy	Inoue et al. [43]	6/9 (67%)	6/7 (86%)	0.585
	Asahina et al. [44]	10/13 (77%)	2/3 (67%)	1
CR + PR/total (%) in mixed 1st subsequent lines of therapy	Sutani et al. [45]	15/20 (75%)	6/7 (86%)	1
	Yoshida et al. [46]	7/8 (87.5%)	12/13 (92.3%)	1
	Sunaga et al. [47]	15/16 (94%)	1/3 (33%)	0.050
CR + PR/total (%) in the combined group	Total (99 patients)	53/66 (80.3%)	27/33 (81.8%)	1

EGFR, epidermal growth factor receptor; CR, complete response; PR, partial response; SD, stable disease; PD, progressive.
[a] Two-tailed probability by Fisher's exact test.

Table 5 Progression-free survival (PFS) and overall survival (OS)

	Source (reference)	Median PFS in months (95% CI)	1-Year survival (%)	OS in months	Median follow-up (months)
1st line therapy	Inoue et al. [43]	9.7 (7.4–9.9)	–	Not reached	7.6
	Asahina et al. [44]	8.9 (6.7–11.1)	83	Not reached	12.7
Mixed 1st and subsequent lines of therapy	Sutani et al. [45]	9.4 (–)	–	15.4	–
	Yoshida et al. [46]	7.7 (–)	80	Not reached	–
	Sunaga et al. [47]	12.9 (–)	85	Not reached	12.6

PFS, progression-free survival; OS, overall survival; –, not reported in the published material.

as PR [47]. Overall, in these prospectively collected patients the L858R patients were as likely as the exon 19 deletion ones to derived responses from gefitinib monotherapy.

3.3. Progression-free and overall survival in EGFR-mutant patients treated with gefitinib

The follow-up of these patients was short. In the trials that reported median follow-up, it ranged from 7.6 to 12.7 months (Table 5). In only one of the reports did the authors obtain enough number of deaths to calculate median overall survival [45]. In all the other trials, median overall survival had not been reached at time of publication.

The five publications reported PFS (Table 5). In the phase II first line therapy trials, the PFS were 9.7 and 8.9 months [43,44]. In the prospective cohorts with a mixed treatment population, the PFS ranged from 7.7 to 12.9 months [45–47]. The 1-year survival, as derived from the investigators or extrapolated by the Kaplan–Meier curves, ranged from 80 to 85% (Table 5).

It was not possible to extract PFS and OS for the individual types of EGFR mutations from three out of the five trials. In the phase II trial by Asahina et al. [44], the median PFS for exon 19 deletion patients was 8.3 months [44,50] and not reached for the L858R patients (of the three L858R patients, two were alive and progression-free after more than 11.7 months of therapy). The other only trial that reported PFS by mutational status was that of Yoshida et al. [46]. They noted a median PFS of 7.8 months (95% CI, 7.6—not reached) for exon 19 deletions ($n = 8$) and 6.0 months (95% CI, 2.6–7.7 months) for the L858R group ($n = 13$)

Table 6 Gefitinib-related adverse events

Source (reference)	Skin		Diarrhea		ILD
	Grades 1–2/no. pts (%)	Grades 3–4/no. pts (%)	Grades 1–2/no. pts (%)	Grades 3–4/no. pts (%)	Grades 1–4/no. pts (%)
Inoue et al. [43]	12/16 (75%)	0/16 (0%)	3/16 (19%)	0/16 (0%)	0/16 (0%)
Asahina et al. [44]	7/16 (44%)	1/16 (6%)	7/16 (44%)	0/16 (6%)	1/16 (6%)
Sutani et al. [45]	9/27 (78%)	1/27 (18.5%)	5/27 (3.5%)	0/27	0/27 (0%)
Yoshida et al. [46]	6/21 (28.5%)	0/21 (0%)	6/21 (28.5%)	2/21 (9.5%)	0/21 (0%)
Sunaga et al. [47]	13/21 (62%)	1/21 (4.7%)	2/21 (9.5%)	0/21 (0%)	1/21 (4.7%)
Total	47/101 (46.5%)	3/101 (3%)	23/101 (23%)	2/101 (2%)	2/101 (2%)

No. pts, number of patients; ILD, interstitial lung disease.

(log-rank test, $p = 0.04$). No other results can be extracted from the other trials regarding differential PFS by mutation type.

3.4. Gefitinib-related adverse events reported in these prospective trials

Adverse events were reported based on the NCI-CTC guidelines. A total of 101 patients had their common grades 1–4 toxicities reported (Table 6). The most common toxicities were those related to the skin. The trials reported them as rash, skin toxicity, skin pruritus or dry skin. 46.5% of the patients developed grade 1 or 2 skin side-effects, however only 3% were reported as having serious grade 3 or 4 skin toxicities. The second most common adverse event was diarrhea, with 23% of patients reporting grades 1–2 and 2% grades 3–4, respectively. Other less common effects reported, but not displayed in Table 6, included elevations in aspartate and alanine aminotransferases, fatigue and anorexia [43–48].

The most dreaded adverse effect associated with TKI exposure in NSCLC, interstitial lung disease (ILD), was noted in only 2% of the treated patients. In one patient this was classified as grade 3 toxicity and the patient recovered after steroids [47]. The other ILD patient (grade 1) recovered without need of steroids [44]. No deaths were attributed to ILD or gefitinib-treatment in these 101 patients.

4. Discussion

The discovery of EGFR mutations has sparked an input of renewed interest in lung cancer translational research. Our current report pooled from five prospective cohorts, of Japanese patients, who were genotyped for EGFR mutations and selected to receive gefitinib based on the presence of an activating somatic mutation. We believe that our pooled analysis, combining 101 patients (99 of which had their tumor's radiographic response measured by RECIST), is able to strengthen the individual observations of each of these small prospective and phase II studies alone. The primary outcome measured in all trials was objective tumor response rate and therefore they have not been powered or followed long enough to generate detailed information on patient survival.

A great majority, over 80%, of the 99 patients displayed either a CR or PR as best response to 250 mg/day of gefitinib. These unheard of results in NSCLC confirm the retrospective RR of 78% previously reported in 268 EGFR-mutant patients [16]. Gefitinib monotherapy in EGFR-mutated patients has been described as a ''weapon of mass destruction'' [51] and our compilation reaffirms this concept.

Due to the large number of homogenous patients in the group described here we were also able to evaluate the predictive role of the two most common EGFR mutations. Two well conducted retrospective reports from the Dana-Farber/Harvard Cancer Center (DF/HCC) [52] and the Memorial Sloan-Kettering Cancer Center [53] combining 32 (22 exon 19 deletions, and 10 L858R) and 34 patients (23 exon 19 deletions, and 11 L858R), respectively, indicated that the response rates, progression-free and overall survival of gefitinib or erlotinib treated individuals was superior in exon 19 deletions than the L858R mutation patients [52,53]. Two retrospective East Asian cohorts did not show significant differences in survival between the gefitinib-treated L858R and exon 19 deletions [25,28,50], and a recent Japanese retrospective report actually described an improved survival for L858R-bearing patients after gefitinib therapy [54].

From our data, it is clear that, in the 99 patients reviewed here, the response rates were almost identical for patients with either exon 19 deletions or L858R mutations—80.3% and 81.8%, respectively ($p = 1$). Of note, all of the patients described in our report were Japanese. The Massachusetts General Hospital and DF/HCC Thoracic Oncology groups have completed a phase II trial of gefitinib as first line therapy for EGFR-mutant tumors (iTARGET). The results of these patients have not been reported yet, however we believe that this North American trial will help both confirm the remarkable response rates of gefitinib in a western population and also determined if L858R and exon 19 deletion mutations confer different prognostic degrees in these non-Asian ethnical groups. Despite the current controversy regarding possible clinical differences between the exon 19 and 21 mutations, we view our report as reassurance that both of these classes of mutations are highly sensitive to TKIs initially in a predominantly Asian population. The mature follow-up of the 99 patients compiled here will, in the future, permit a better understanding of patterns of survival in the different mutations.

The time to progression in the five trials reported here are also impressive. In the two prospective phase II studies of gefitinib for chemotherapy naïve patients the median PFS exceeded 8.9 months [43,44]. And even more striking was the result of the three other trials with a mixed population of previously treated and untreated advanced NSCLC. In two of those trials, the median PFS exceeded 9.4 months [45,47]. The overall survivals have not been reached yet, however we speculate that longer follow-up will confirm the improved survival seen in retrospective studies of patients treated with TKIs in which EGFR-mutations had been identified [19,23,52,53].

Another observation made possible by our pooled analysis was that of the safety of gefitinib administration to EGFR-mutant NSCLC patients. The most common toxicities were those of skin rash and diarrhea, which had been expected from the large phase III trial of gefitinib [39]. Almost all were reported as mild toxicities and few patients required a dose reduction or discontinuation. Most notably, the rate of ILD was only 2% and no deaths occurred in the patients in which this developed. Previous case series had identified this as a feared complication of TKI therapy. In over 1900 gefitinib-treated patients, around 3.5% developed ILD and over 1.5% died [55]; with male sex, smoking history and interstitial pneumonia identified as risk factors. Of interest, the only patient that developed a grade 3 ILD from our pooled analysis was a male smoker with an EGFR mutation [47].

Despite the incredible response rates and efficacy of gefitinib monotherapy for EGFR-mutant NSCLC, our results confirm that acquired resistance to EGFR TKI therapy is seen in most of the patients. As described above, in almost all trials the PFS did not exceed 12 months. This is a reminder that, different than CML where most patients retain a response

even after 5 years of imatinib [56], EGFR-mutant tumors have a high propensity to loose their sensitivity to continuous TKI therapy. Two cohorts of patients with TKI-resistant NSCLCs, in which a second biopsy was obtained after progression, identified the secondary resistant T790M mutation [49] in around 50% of the samples [57,58]. The acquired amplification of the MET oncogene was recently reported as occurring in around 20% of TKI-resistant patients [59]. The mechanisms of resistance in the remaining tumors have not been completely clarified and very few other secondary mutations identified [57]. T790M, by introducing a bulkier amino acid side chain at this position, may affect the binding of gefitinib/erlotinib to the EGFR tyrosine kinase domain [49] and results in ineffective TKI function and sustained down-stream signaling from the mutant EGFR [60]. *In vitro*, T790M causes significant resistance to the effects of gefitinib but this can be partially overcome by irreversible EGFR inhibitors [60]. These findings have prompted ongoing trials of second-generation irreversible EGFR inhibitors in this patient population, such as an ongoing trial of the HKI-272 compound [61]. The clinical efficacy of this approach is still unknown. The re-administration of gefitinib in previously responsive patients that show radiographic progression has also been reported to improve symptoms and the clinical course of patients [62], suggesting a role for continued TKI use to control the non-TKI resistant clones of these ''oncogene addicted'' cancers. The optimal management of EGFR-mutant tumors that progress on gefitinib or erlotinib has not been established.

Our compilation of the first 101 published patients selected by their EGFR-mutant genotype to receive gefitinib monotherapy for advanced NSCLC confirms the high response rate (over 80%) of this agent in this molecularly classified population. Longer follow-up and phase III trials comparing gefitinib to conventional platinum-based chemotherapy are necessary to define the extent of improvement in survival for these patients. We believe that gefitinib will be a most valuable ''weapon'' in the fight against EGFR-mutant NSCLC.

Conflict of interest statement

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

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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

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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 cells 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 oligonucleotides 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 cells 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 [IC_{50}] 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 (IC_{50}: 1 and 0.005 μM, respectively) [28]. H3255 carries the L858R *EGFR* exon 21 point mutation and has an IC_{50} 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

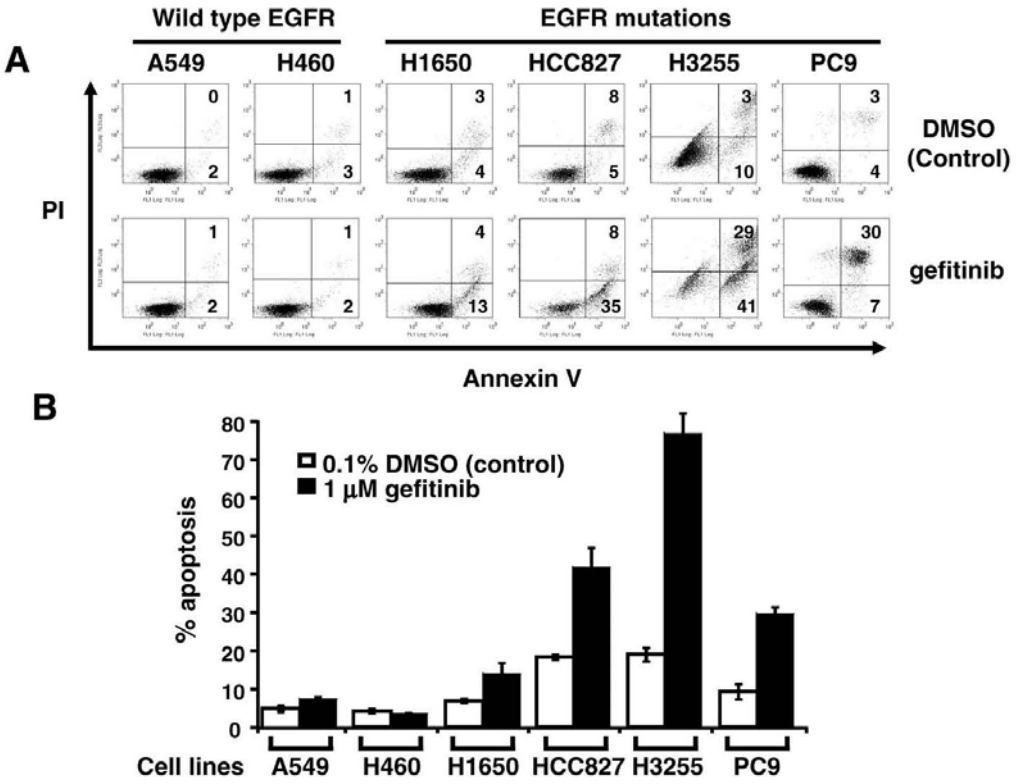

Figure 1. Up-Regulation of BIM Correlates with Gefitinib-Induced Apoptosis in NSCLC Cell Lines

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 ($n \geq 3$).

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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

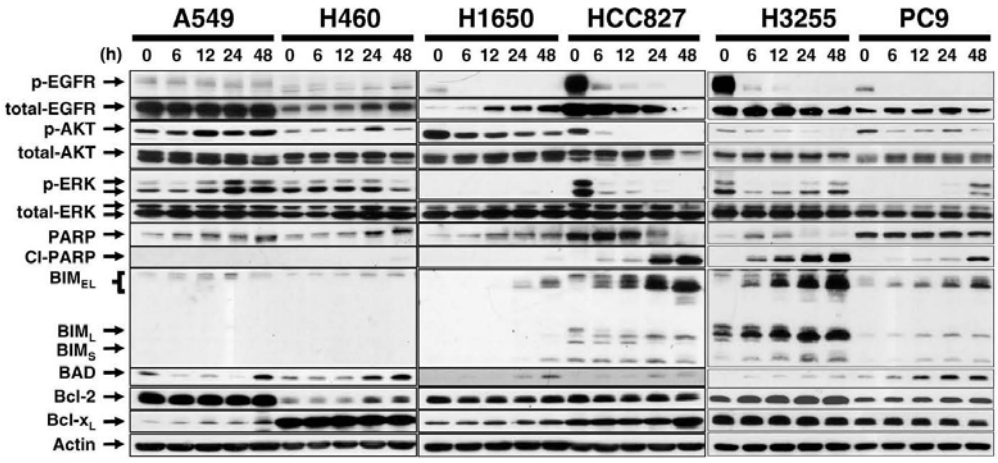

Figure 2. Gefitinib Induces Expression and Dephosphorylation of BIM in Gefitinib-Sensitive *EGFR*-Mutant Cells, but Not in Cells with Wild-Type *EGFR*
Cells were treated with 1 μM gefitinib for the times (in hours) indicated, and lysates were collected and proteins analyzed by immunoblotting. The three isoforms of BIM, BIM_{EL}, BIM_L, and BIM_S are shown. Hypophosphorylated BIM migrates faster than the other species [23].
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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 β3 and helix αC. 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 μM 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 μM 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

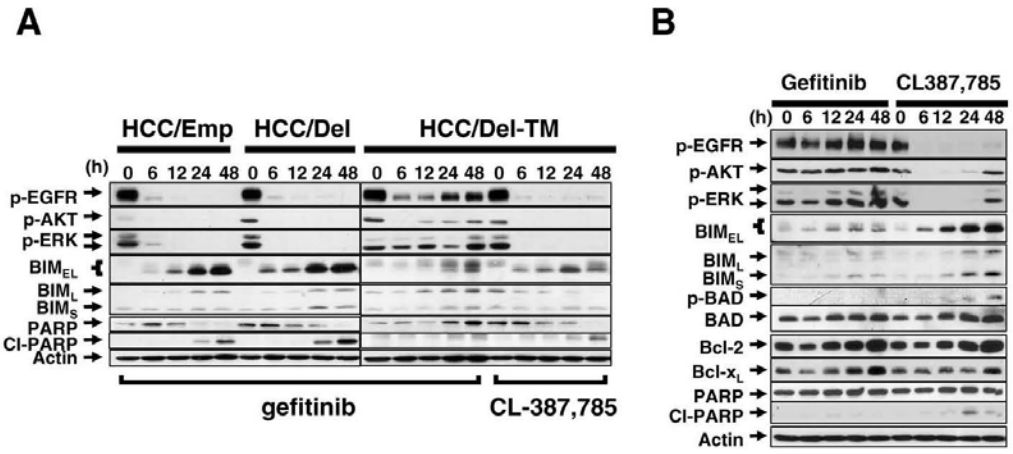

Figure 3. Inhibition of BIM Up-Regulation by the Resistant T790M Mutation

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 μM 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 μM gefitinib or CL-387,785 for the indicated times and extracts were analyzed by Western blotting.
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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

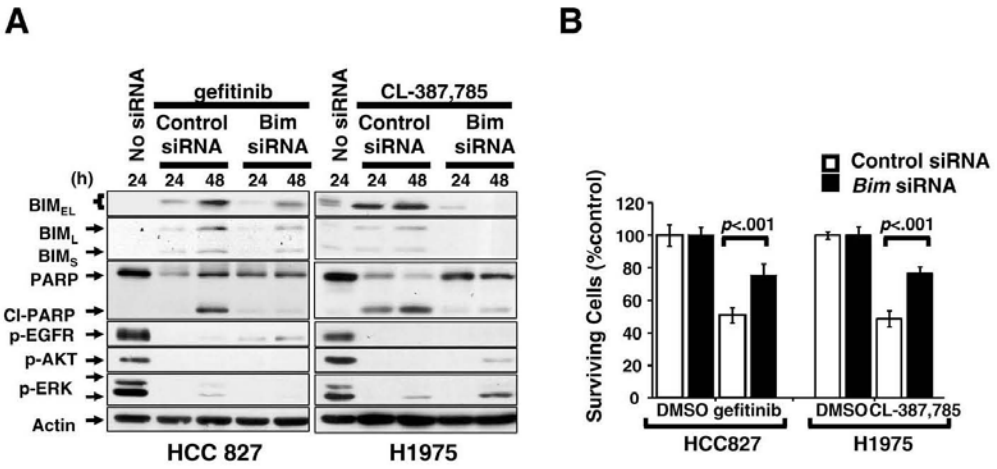

Figure 4. Knockdown of *BIM* Expression Leads to Attenuation of Apoptosis

(A) HCC827 cells (left) or H1975 cells (right) were transfected with *BIM* siRNA oligonucleotides or control oligonucleotides for 24 h prior to 0.5 μM gefitinib or 1 μM 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 ± standard deviation (SD) ($n \geq 3$).

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(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 PKCδ 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

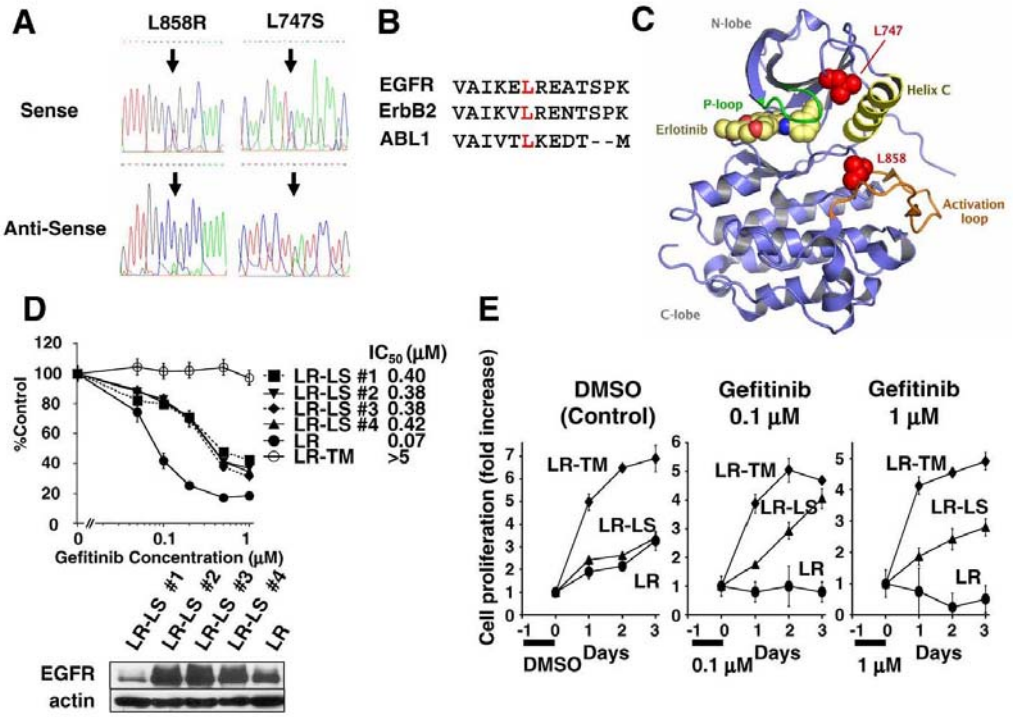

Figure 5. Identification of a Novel Secondary Resistant *EGFR* Mutation, L747S

(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 αC 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 ± SD ($n = 3$).

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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

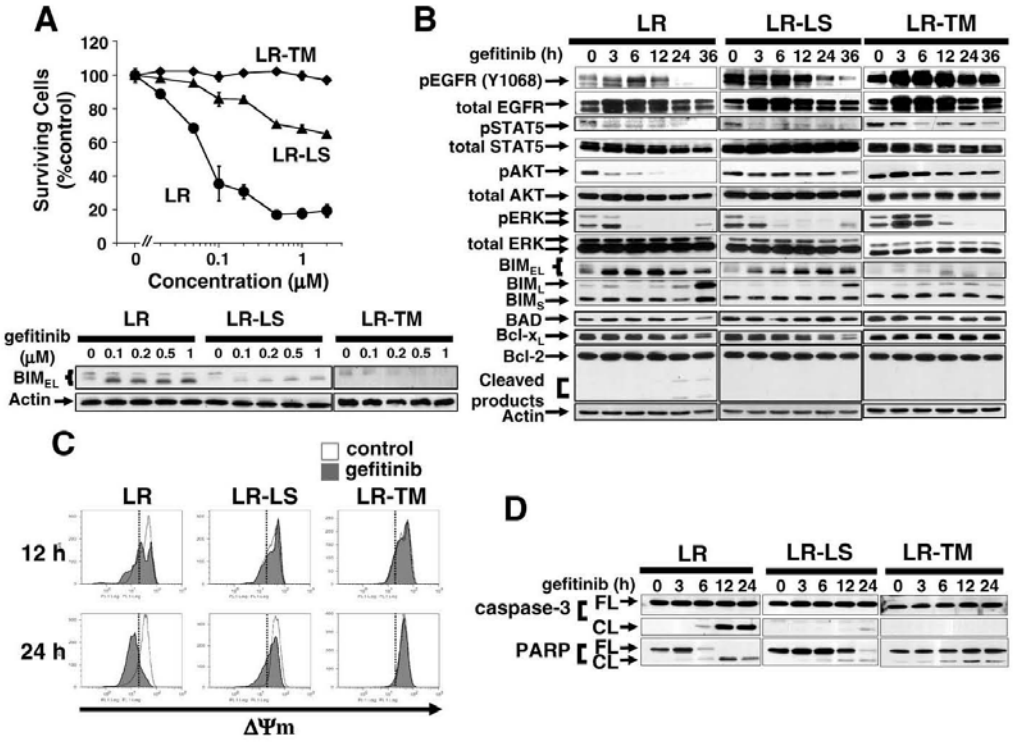

Figure 6. The Secondary Resistant Mutations L747S and T790M Affect Gefitinib-Induced Apoptosis and Inhibit BIM Up-Regulation

(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 ($n \geq 3$). 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.

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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.

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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.

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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 Mellinghoff 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

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Differential Responses to Erlotinib in Epidermal Growth Factor Receptor (*EGFR*)-Mutated Lung Cancers With Acquired Resistance to Gefitinib Carrying the L747S or T790M Secondary Mutations

To the Editor: Acquired resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), gefitinib, and erlotinib, is emerging as the main obstacle in managing lung cancer patients with activating *EGFR* mutations. The secondary resistant T790M mutation[1] has been identified in around 50% of progressing patients,[2] and ongoing clinical trials of second-generation, irreversible EGFR inhibitors are attempting to overcome the resistance generated by this mutation. The acquired amplification of the *MET* oncogene was recently reported as occurring in around 20% of TKI-resistant patients and sometimes concomitantly with T790M.[3] Few other secondary mutations have been described.[2,4] The optimal management of *EGFR*-mutated tumors that progress on gefitinib or erlotinib has not been established, but likely will depend on the mechanism of acquired resistance. Cho and colleagues published in the *Journal* their phase II trial experience of erlotinib for gefitinib-progressive patients.[5] We propose a molecular explanation for the positive or negative response to a gefitinib to erlotinib switch in gefitinib-resistant *EGFR*-mutated non–small-cell lung cancers by reporting the in vitro and clinical effects of these EGFR inhibitors in two secondary mutations, L747S and T790M.

Our group recently reported a patient with the L858R-*EGFR*–activating mutation that acquired the secondary L747S mutation after a 40-month response to gefitinib.[4] L858R-L747S, in vitro, generated a pattern of partial resistance to both gefitinib and erlotinib (Fig 1A). Increasing doses of these reversible EGFR inhibitors led to enhanced proliferation arrest. However, the L858R mutation alone is more sensitive to the antiproliferative and apoptotic effects of gefitinib and erlotinib at all dose ranges tested.[4] This is similar to the pattern of partial resistance described for the L858R-D761Y gefitinib-resistant mutation.[2] Based on our in vitro data (Fig 1A), we predicted that an increase in the clinical doses of gefitinib or switching to erlotinib, which is given at its maximum tolerated dose,[5] would lead to beneficial clinical effects in L858R-*EGFR*–mutant patients that acquired L747S after exposure to gefitinib. Our in vitro data also predicted that L858R-T790M (Fig 1A) or exon 19 deletion-T790M[1] containing tumors would be resistant to either gefitinib or erlotinib at the currently used clinical doses. The mean steady-state serum concentration of gefitinib following a 225-mg daily dose varies from 0.03 to 0.32 μg/mL, with an average of 0.16 μg/mL[6] or 0.358 μmol/L. The mean concentration increases to 0.24 μg/mL at the 300-mg daily dose and to 1.1 μg/mL at 1,000-mg a day of gefitinib.[6] At the maximum tolerated and currently used dose of erlotinib (150 mg per day), the steady-state through values ranged from 0.33 to 2.64 μg/mL with a median of 1.26 $\pm$ 0.62 μg/mL[7] or 2.9 μmol/L.

After progression on daily 250 mg of gefitinib, the patient with the acquired L858R-L747S-*EGFR* was exposed to an experimental EGFR inhibitor without a measurable response. Due to progressive pulmonary, pleural (Fig 1B), and osseous lesions, erlotinib was started at 150 mg a day. Within 1 week of use, the patient developed a moderate rash involving the face and, to a lesser extent, scalp, accompanied by severe pruritus of the lesions and other skin areas. These toxicities far exceeded her maximum toxicity to gefitinib (Fig 1C). The increase in skin-related side effects likely represents a higher biologic dose of EGFR inhibition conferred by erlotinib 150 mg a day when compared to gefitinib 250 mg a day. The patient's osseous pain and pulmonary symptoms markedly improved, and imaging studies demonstrated a partial response to erlotinib (Fig 1B), which was maintained for 6 months.

Another gefitinib-resistant *EGFR*-mutated patient[1] from our service (carrying the delL747-S752 exon 19 deletion) was also given erlotinib 150 mg a day after progression on gefitinib 250 mg a day; however, progressive symptomatic and radiographic disease was noted within the first weeks of therapy. This patient harbored the T790M mutation[1] and, based on our in vitro models,[1] T790M negates the activating mutation hypersensitivity to EGFR inhibitors (Fig 1A) and generates high-grade resistance to achievable clinical doses of gefitinib and erlotinib.[6,7]

Our clinical and biologic observations may explain the response to a gefitinib to erlotinib switch in the patient with the acquired L858R-L747S-*EGFR* mutation and the lack of response to such an approach in other series of *EGFR*-mutated, gefitinib-responsive patients that eventually progressed,[5] since the T790M mutation might have been the culprit of those cases. These cases also underscore the need for utilizing tumor-derived molecular markers to understand and overcome acquired mechanisms of resistance to TKI therapy in EGFR mutated tumors.

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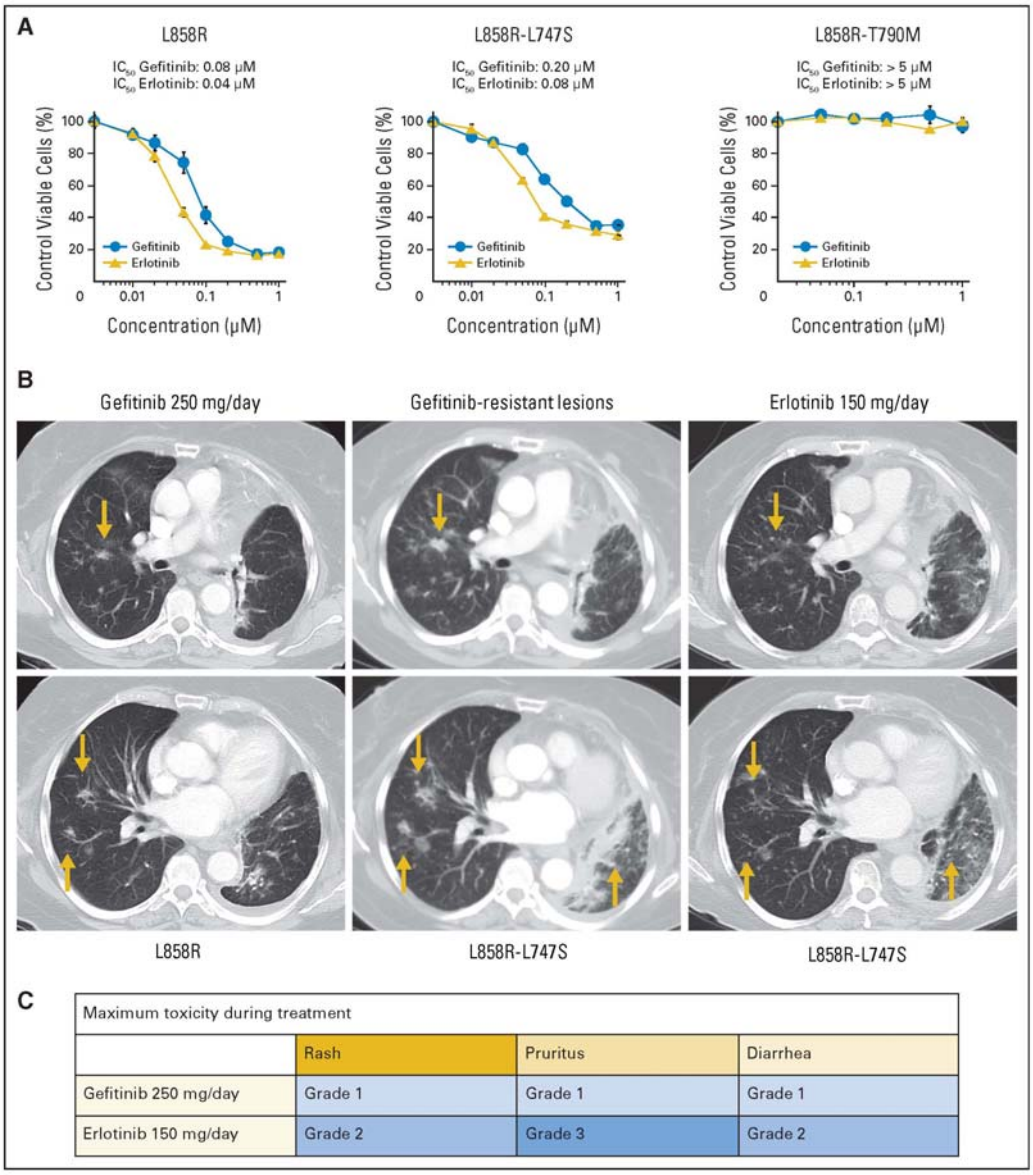

C

Maximum toxicity during treatment			
	Rash	Pruritus	Diarrhea
Gefitinib 250 mg/day	Grade 1	Grade 1	Grade 1
Erlotinib 150 mg/day	Grade 2	Grade 3	Grade 2

Fig 1. Effects of L747S and T790M in the sensitivity of L858R-EGFR to gefitinib and erlotinib. (A) Ba/F3 cells stably containing constructs with the L858R, L858R-L747S or L858R-T790M EGFR, as previously described,[4] were treated with increasing concentrations of gefitinib or erlotinib in an IL-3 free EGF-containing medium,[4] and growth inhibition was assessed by CellTiter 96 AQueous One solution proliferation kit (Promega, Madison, WI) after 24 hours of incubation. Note that L858R-T790M cells were not inhibited, even at 1 μmol/L of gefitinib or erlotinib. However, L858R-L747S cells showed a pattern of partial resistance to both EGFR inhibitors when compared to L858R-only cells. L858R-L747S cells had increased inhibition at higher doses of gefitinib or erlotinib. (B) Computer tomography scans of the patient with the L858R-L747S-EGFR mutation. The first panel shows the maximum response to 250 mg a day of gefitinib, the middle panel progressive pulmonary and pleural lesions of her gefitinib-resistant tumor (arrows) and the last panel the radiographic partial response to erlotinib 150 mg a day. (C) Maximum toxicity, as defined by the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTC, version 3), while on gefitinib or erlotinib for the patient with the acquired L8585-L747S-*EGFR* mutation.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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In Reply: We thank Dr Costa and his collaborators for their interest in our study. In their letter, they proposed a molecular explanation for response to erlotinib in non–small-cell lung cancer with acquired resistance to gefitinib. We do think that their work illustrated why patients in our phase II study and other anecdotal reports derived clinical benefit from erlotinib therapy after failure of gefitinib.

T790M gatekeeper mutations represent approximately 50% of tumors from patients who initially responded and then relapsed.[1] In T790M mutation, the substituted methionine at position 790 makes sterical hindrance. Because of profound gatekeeper effect, T790M mutation can not be overcome by erlotinib. One of the patients in our phase II study revealed T790M mutation in addition to an exon 19 deletion mutation in tumor tissue that became resistant to gefitinib.[2] This patient showed progressive disease on subsequent erlotinib therapy, further supporting this hypothesis. Therefore, strategy for overcoming resistance due to T790M mutation is irreversible inhibitors that covalently bind to epidermal growth factor receptor (EGFR).[3]

Certain second mutations, such as L747S or D761Y, confer much less resistance to gefitinib or erlotinib compared with T790M mutation. Based on crystal structure, D761Y mutation, which occurs in α-helix of EGFR, is not predicted to result in bulky steric clash as with T790M mutation.[4] The importance of presence of T790M mutation as a negative predictor for salvage use of erlotinib after gefitinib therapy was also reported by Chang et al.[5] They demonstrated a deletion mutation without T790M mutation in rebiopsied tumor sample from a patient who responded to erlotinib after gefitinib failure. Just as suggested by the authors, erlotinib might be able to overcome resistance due to these second mutations other than T790M mutation. It appears that precise position of mutation within the kinase domain of EGFR could be an important factor in determining which patients might respond to erlotinib. The tumor microenvironment and pharmacokinetics of the drug may influence the type of mutations.[4,6]

So, why does the tumor that becomes resistant to gefitinib respond to erlotinib? The molecular structure of gefitinib and erlotinib are similar, but not identical. Much lower IC_{50} value of erlotinib against wild-type EGFR compared with gefitinib might be translated into the higher antitumor effect.[7] Furthermore, in case of second mutations with less sterical hindrance for drug binding, small change in molecular structure might allow erlotinib to be fit in the catalytic pocket of EGFR and thus to overcome resistance to gefitinib. Regarding the authors' prediction that an increase in doses of gefitinib or switching to erlotinib would lead to clinical benefit, we prefer the switch to erlotinib rather than high-dose of gefitinib because of poor tolerability.

A similar example was described in chronic myeloid leukemia. Resistance frequently results from the emergence of point mutations within the kinase domain of the bcr/abl protein that reduce the binding affinity of imatinib.[8] Interestingly, the degree of resistance ranges from a few fold for some of the mutation to complete resistance for the T315I mutation which is analogous to T790M in EGFR. Overcoming resistance to imatinib can be achieved through several approaches. These include escalating the dose of imatinib or using new inhibitors, such as nilotinib. Due to structural modifications, nilotinib is more potent in the killing of wild-type bcr/abl–expressing cells and also maintains activity against imatinib-resistant mutant, except T315I.[9]

In our article, we proposed prior response to gefitinib as a predictive marker for subsequent erlotinib therapy. However, based on the authors' observation, molecular predictor can lead to better patient selection than clinical predictor. That is, although a tumor showed response to gefitinib and subsequently progressed, it may respond to erlotinib in the absence of T790M mutation. Therefore, it would be reasonable and probably important to perform rebiopsy of tumor tissue on acquisition of resistance in order to optimize and individualize subsequent targeted therapy. As a practical point, acquiring adequate tissue for EGFR mutation analysis, however, is often not feasible, particularly in heavily pretreated patients. We sincerely expect a highly sensitive and noninvasive method for the detection of EGFR mutation in serum DNA to become available as soon as possible.[10]

The authors' observation did not address why erlotinib produced a response in our patient with wt EGFR who had stable disease (SD) on gefitinib. This important conclusion in our study may imply that these tumors have resistant mechanisms that could be overcome by erlotinib. In the BR.21 study, a significant prolongation of survival was achieved despite response rate of less than 10%, perhaps because of high proportion of the patients had durable SD while receiving treatment.[11] Until today, however, little has been studied about the resistant mechanisms in this large group of patients. Further understanding of mechanism of resistance will facilitate more effective ways to overcome the acquired resistance.

Several published data are now available regarding the clinical activity of erlotinib after the failure of gefitinib. We summarized 16 patients who achieved clinical benefit to erlotinib in Table 1.[2,5,12-18] Consistent with our previous assertion, nine among 16 patients showed SD on gefitinib. Seven patients showed partial response (PR)

Effects of Erlotinib in *EGFR* Mutated Non-Small Cell Lung Cancers with Resistance to Gefitinib

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Abstract

Purpose: Most lung cancers with activating epidermal growth factor receptor (*EGFR*) mutations respond to gefitinib; however, resistance to this tyrosine kinase inhibitor (TKI) invariably ensues. The T790M mutation occurs in 50% and *MET* amplification in 20% of TKI-resistant tumors. Other secondary mutations (D761Y and L747S) are rare. Our goal was to determine the effects of erlotinib 150 mg/d in *EGFR* mutated patients resistant to gefitinib 250 mg/d, because the EGFR TKI erlotinib is given at a higher biologically active dose than gefitinib.

Experimental Design: Retrospective review of 18 *EGFR* mutated (exon 19 deletions, L858R, and L861Q) patients that were given gefitinib and subsequently erlotinib. Seven patients had tumor resampling after TKI therapy and were analyzed for secondary *EGFR* mutations and *MET* amplification.

Results: Most patients (14 of 18) responded to gefitinib with median progression-free survival of 11 months (95% confidence interval, 4-16). After gefitinib resistance (*de novo* or acquired), 78% (14 of 18) of these patients displayed progressive disease while on erlotinib with progression-free survival of 2 months (95% confidence interval, 2-3). Six of 7 resampled patients acquired the T790M mutation, and 0 of 3 had *MET* amplification. Only 1 gefitinib-resistant patient with the acquired L858R-L747S *EGFR*, which *in vitro* is sensitive to achievable serum concentrations of erlotinib 150 mg/d, achieved a partial response to erlotinib.

Conclusions: In *EGFR* mutated tumors resistant to gefitinib 250 mg/d, a switch to erlotinib 150 mg/d does not lead to responses in most patients. These findings are consistent with preclinical models, because the common mechanisms of TKI resistance (T790M and *MET* amplification) *in vitro* are not inhibited by clinically achievable doses of gefitinib or erlotinib. Alternative strategies to overcome TKI resistance must be evaluated.

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In 2008, lung cancer continues to lead cancer-related deaths in the United States for both men and women (1). Non-small cell lung cancers (NSCLC) comprise the majority of cases, and the prognosis of patients diagnosed with advanced NSCLC continues to be dismal (2). Use of palliative platinum-based chemotherapy has been the standard therapy for NSCLC (3). However, even the addition of the vascular endothelial growth factor monoclonal antibody bevacizumab (4) to chemotherapy can only achieve response rates of 30%, progression-free survival (PFS) of <8 months and the median overall survival (OS) barely reaches 12 months. Despite three Food and Drug Administration-approved second-line therapies for platinum-progressive NSCLC, which are docetaxel (5), pemetrexed (6), and erlotinib (7), very few patients survive for longer than 2 years. Nonetheless, there is great heterogeneity between patients and their clinical course and response to different anticancer therapies.

The identification of somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) gene in patients with NSCLC provided one of the first examples of

Translational Relevance

EGFR mutated NSCLC are sensitive to EGFR inhibitors in preclinical models. Clinical experience with the use of gefitinib/erlotinib in *EGFR* mutated patients indicates that many exon 19 deletion and L858R-bearing tumors display responses that sometimes reach a year; however, acquired resistance to EGFR TKIs invariably develops. The secondary T790M mutation occurs in 50% and amplification of *MET* in 20% of TKI-resistant tumors. Few other secondary mutations (D761Y and L747S) have been described. Few therapies have been studied for the expanding number of *EGFR* mutated tumors that become resistant to gefitinib. Our data indicate that in *EGFR* mutated patients with resistance to gefitinib 250 mg/d, a switch to erlotinib 150 mg/d does not lead to radiographic responses in most patients despite the higher biologically active dose of erlotinib. Only a patient with the acquired L858R-L747S responded to erlotinib. Preclinical models indicated that the two most common mechanisms of acquired resistance to gefitinib, EGFR-T790M and *MET* amplification, are highly resistant to achievable clinical concentrations of erlotinib, whereas L858R-L747S is sensitive to erlotinib at 150 mg/d. The correlation of our findings with the molecular understanding of sensitivity and resistance of *EGFR* mutated systems underlines the need for genotype-based clinical studies to advance our understanding of treatment of this representative patient cohort.

potential patient-tailored therapy in this disease (8–10). Large-scale sequencing efforts have consistently identified *EGFR* mutations in an enriched cohort of women, never smokers, adenocarcinomas, and East Asians (11). The most prevalent *EGFR* mutations consist of small in-frame deletions around the conserved LREA motif of exon 19 (residues 747-750) followed by a single point mutation (L858R) in exon 21 (12, 13). Both cell line and mouse models of *EGFR* mutations show that tumor cells that harbor such mutations are exquisitely sensitive to EGFR inhibition (9, 14, 15). The aforementioned models have identified that EGFR-driven lung cancers are "addicted" to EGFR signaling for their survival and proliferation. More so, *EGFR* mutations are oncogenic and alter the tyrosine kinase pocket of EGFR to a degree that enhances the sensitivity to ATP-competitive EGFR inhibitors (16). Both these factors make *EGFR* mutated NSCLC more sensitive to EGFR tyrosine kinase inhibitors (TKI).

Retrospective studies of thousands of patients treated with the two currently available anilinoquinazoline small-molecule EGFR TKIs, gefitinib and erlotinib, as second- or third-line therapies in NSCLC (17, 18), showed that a majority (close to 80%) of patients with classic *EGFR* mutant tumors attain radiographic and clinical responses to these oral agents. In some series, both PFS and OS were significantly better for EGFR TKI-treated patients with *EGFR* mutations when compared with wild-type cases (17). The evaluation of *EGFR* mutation as a prognostic and predictive marker is NSCLC is under way, with multiple phase II and III trials analyzing this biomarker. Seven prospective phase II trials have evaluated gefitinib monotherapy for patients selected based on their *EGFR* mutational status (19–21). These have confirmed that ~75% of patients with L858R or exon 19 deletion mutations achieve responses.

Despite the efficacy of gefitinib monotherapy for *EGFR*-mutant NSCLC, acquired resistance to EGFR TKI therapy is seen in most patients. In almost all prospective trials, the PFS did not exceed 12 months (19). The secondary resistant T790M mutation (22, 23) arises most often in *cis* to L858R or exon 19 deletions in ~50% of patients with radiographic progression (24, 25). The acquired amplification of the *MET* oncogene occurs in ~20% of gefitinib/erlotinib-resistant patients and in half of these cases in conjunction with T790M (26, 27). The mechanisms of resistance in the remaining tumors have not been completely clarified and very few other secondary mutations, such as L858R-D761Y (24) and L858R-L747S (28, 29), identified in gefitinib-progressive specimens.

The management of this growing population of EGFR TKI-resistant NSCLC is not established, but the success of any approach will likely be dependent on the mechanism of acquired resistance of the tumor. In other "oncogene-addicted" tumors, such as chronic myeloid leukemia and gastrointestinal stromal tumors, where the *BCR-ABL* translocation or c-*KIT* mutations, respectively, make these cancers sensitive to imatinib, it seems that the dose of the TKI matters (30). In both disorders, one clinical step when resistance emerges is to increase the dose of imatinib from 400 to ≥600 mg/d (31–33). This dose escalation maneuver is only effective in some patients, possibly by inhibiting secondary mutations with borderline resistance to imatinib or by affecting nonmutation dependent mechanisms, with short periods of disease control (31, 33). Second-generation ABL and KIT inhibitors have gained momentum and recently received Food and Drug Administration approval as alternative therapies (34, 35).

In *EGFR* mutated tumors, it is unknown if EGFR TKI dose escalations, in the face of acquired or *de novo* resistance, changes the course of TKI-progressive tumors. To evaluate the efficacy of such approach, we retrospectively studied the course of *EGFR* mutated patients that first received gefitinib 250 mg/d and on becoming gefitinib-resistant were exposed to erlotinib 150 mg/d. This gefitinib to erlotinib switch is predicted to expose patients to almost double the biologically active dose of an EGFR TKI (36, 37). Because EGFR-T790M and *MET* amplification lead to high level of *in vitro* resistance to both gefitinib and erlotinib (22, 27), we hypothesized that erlotinib should only alter the response of acquired borderline resistant clones carrying the rare L858R-D761Y or L858R-L747S gefitinib-resistant mutations.

Materials and Methods

Patient selection. Patients were identified from the databases of five academic medical centers: (*a*) Beth Israel Deaconess Medical Center, (*b*) Dana-Farber Cancer Institute, (*c*) Massachusetts General Hospital, (*d*) Memorial Sloan-Kettering Cancer Center, and (*e*) Yonsei University College of Medicine. Inclusion criteria to use the patient's data included signed informed consent for *EGFR* mutation analysis, an institutional approved protocol for human studies and genomic analysis of stored tumor tissue, a diagnosis of stage IV metastatic NSCLC with a proven *EGFR* mutation, and the exposure to both gefitinib and erlotinib. Gefitinib at an initial dose of 250 mg/d had to be given as the first EGFR TKI therapy and erlotinib at a starting dose of 150 mg/d subsequently to progression on gefitinib. We did not exclude patients that had received investigational compounds between gefitinib and erlotinib to maximize

the number of patients identified. Data were collected from the patient's medical records for baseline clinical, demographic, and pathologic characteristics. Radiographic data was reviewed by each center. Portions of the clinical characteristics and response to gefitinib and erlotinib monotherapy in some of these patients have been reported previously by our academic groups (21, 28, 29, 38–41).

EGFR genotype in the identified patients. Each institution performed *EGFR* genotypes using their own protocols as described previously (10, 13, 21, 22, 24, 28, 39, 41). The methods of DNA and RNA isolation from fresh tissue or paraffin-embedded tissue, and the technique used to enhance tumor-derived DNA, which included either microdissection or use of more sensitive PCR amplification techniques, was left to the discretion of each institution. All protocols either sequenced exons 18 to 21 of the *EGFR* gene or identified L858R and deletions in exon 19.

In patients who had tumor resampling after progression on gefitinib or erlotinib, DNA or RNA was isolated from the tumor tissue and the *EGFR* gene was sequenced as above. Specific attention was made to compare results to the original biopsy and identify the exon 20 T790M mutation (22).

MET amplification analysis. In the tumor specimens that were obtained after progression on TKIs, we attempted to identify the amplification of *MET* when enough material for studies was available. Levels of *MET* and endogenous control were evaluated using quantitative genomic PCR methods described previously (26, 27) in DNA samples. Fluorescence *in situ* hybridization was employed, as described previously (27), in tumor samples that had paraffin-embedded tissue available for analysis.

Treatment schedules, response, PFS assessment, and statistical analysis in the identified patients. All the identified patients had the same initial treatment schedule for gefitinib. This medication was given orally at a dose of 250 mg/d, and gefitinib was used until tumor progression and afterwards continued at the physician's discretion. Erlotinib was given orally at a dose of 150 mg/d and continued until radiographic tumor progression or overt clinical progression. Need for EGFR TKI dose reduction was determined by each treating physician based on the patient's tolerance and side-effect profile.

The objective tumor response was determined by Response Evaluation Criteria in Solid Tumors (42). It was left at the discretion of each institution and physician to determine when to obtain reimaging radiographs. PFS and OS were calculated from the date of starting the EGFR TKI until the date of radiographic tumor progression or overt clinical progression (for PFS) and death (for OS). PFS and OS estimates were made using the Kaplan-Meier method (43), and the 95% confidence interval (95% CI) for the median was based on the sign test. Exploratory differences in response rate and PFS were compared by Fisher's exact test and the log-rank test.

Results

Patient characteristics. After a review of *EGFR* genotyped patients in our centers from 2004 to 2008, we identified 18 *EGFR* mutated patients that had received gefitinib and erlotinib. Clinical, demographic, pathologic, and molecular characteristics of this cohort are displayed in Table 1. Sixty-one percent of patients were women (11 of 18) and the majority never smokers (11 of 18). Ages varied between 43 and 80 years (Table 1). Almost all (16 of 18) patients had adenocarcinoma as the main histologic type of their tumor. These characteristics are similar to historic cohorts of *EGFR* mutated tumors (17). Exon 19 deletion-containing tumors were found in 13 (72%) patients, L858R mutations in 4 (22%) patients, and L861Q in 1 patient (Table 1).

Of the studied patients, 8 received gefitinib as their first anticancer therapy (44%) and 10 had received platinum-based chemotherapy previously (56%). Most patients (15 of 18, 83%) were not exposed to any other form of therapy between stopping gefitinib and before receiving erlotinib (Tables 1 and 2).

Initial response to gefitinib 250 mg/d. Fourteen of the 18 (78%) patients had radiographic responses to gefitinib (Table 2), a number that is compatible with retrospective and prospective data for *EGFR* mutated patients (11, 19, 44). Two (11%) patients had stable disease (SD), and another 2 patients had *de novo* resistance to gefitinib with progressive disease (PD) as best response.

The median PFS was 11 months, with a 95% CI of 4 to 16 months (Fig. 1). Five patients had responses that lasted more than 16 months (Table 2). All patients eventually displayed radiographic and clinical progression that required discontinuation of gefitinib. PFS was similar between patients that were chemotherapy-naive or had received chemotherapy previously (Table 2).

Response to erlotinib 150 mg/d. Patients were given erlotinib at an initial dose of 150 mg/d after their tumors had become gefitinib-resistant. The majority of patients had no additional systemic therapy between gefitinib and erlotinib (Table 1).

Fourteen of the 18 (78%) patients had PD as the best response to erlotinib monotherapy, an additional 3 (16%) patients had brief periods of SD as best response, and only 1 (6%) patient had a radiographic partial response (PR; Tables 2 and 3).

Median PFS was 2 months, with a 95% CI of 2 to 3 months (Fig. 2). Only 2 (11%) patients, one each with PR and SD, remained on erlotinib without progression for over 5 months and no patient had a PFS of over 6 months (Table 2). PFS was similar for patient that had or had not received chemotherapy

Table 1. Clinical, pathologic, demographic, and molecular characteristics of the studied *EGFR* mutated patients

Characteristics	No. patients (%)
Age (y)	
Median	63
Range	43-80
Sex	
Female	11 (61)
Male	7 (39)
Smoking history	
Never smoker	11 (61)
Former smoker	5 (28)
Smoker	2 (11)
Histology	
Adenocarcinoma	16 (89)
NSCLC-not otherwise specified	2 (11)
EGFR mutation	
Exon 19 deletion*	13 (72)
L858R	4 (22)
L861Q	1 (6)
Therapy before gefitinib	
Platinum-based chemotherapy	10 (56)
No prior therapy	8 (44)
Therapy in between gefitinib and erlotinib	
Experimental agent	3 (17)
No therapy	15 (83)

*Specific *EGFR* sequences of the exon 19 deletions are detailed in Table 2.

Table 2. Clinical, pathologic, demographic, and molecular characteristics, response to therapy, PFS, and OS in the studied patients

Patient	Site	Age (y)/sex	Histology	Smoking history	Type of *EGFR* mutation	Therapy before gefitinib	Response, gefitinib 250 mg/d
1	BIDMC	71/M	Adenocarcinoma	Former (40 pack-years)	delL747-S752	Platinum-doublet	CR
2	BIDMC	74/F	Adenocarcinoma	Never smoker	L858R	Platinum-doublet	PR
3	BIDMC	75/F	Adenocarcinoma	Never smoker	delE746-A750	None	PR
4	BIDMC	77/F	Adenocarcinoma	Never smoker	L858R	None	PR
5	YCC	45/F	Adenocarcinoma	Never smoker	delL747-751InsP	Platinum-doublet	PR
6	YCC	56/F	Adenocarcinoma	Never smoker	delL747-751InsP	Platinum-doublet	PR
7	YCC	47/M	Adenocarcinoma	Smoker	delE746-T751InsA	Platinum-doublet	PD
8	YCC	49/F	Adenocarcinoma	Never smoker	delL747-751InsP	Platinum-doublet	PR
9	YCC	70/M	Adenocarcinoma	Smoker	delE746-A750	Platinum-doublet	PD
10	DFCI	43/M	NSCLC-not otherwise specified	Never smoker	L858R	None	PR
11	MGH	64/M	NSCLC-not otherwise specified	Former (30 pack-years)	L858R	None	PR
12	MGH	64/F	Adenocarcinoma	Never smoker	delE746-A750	None	PR
13	MGH	69/F	Adenocarcinoma	Former (20 pack-years)	L861Q	None	SD
14	MGH	60/F	Adenocarcinoma	Former (5 pack-years)	delE746-A750	None	PR
15	MSKCC	60/M	Adenocarcinoma	Never smoker	delE746-A750	Platinum-doublet	PR
16	MSKCC	52/F	Adenocarcinoma	Never smoker	delE746-A750	Platinum-doublet	PR
17	MSKCC	62/F	Adenocarcinoma	Never smoker	delE746-A750	Platinum-doublet	PR
18	MSKCC	80/M	Adenocarcinoma	Former (2 pack-years)	delE746-A750	None	SD

Abbreviations: BIDMC, Beth Israel Deaconess Medical Center; YCC, Yonsei Cancer Center; DFCI; Dana-Farber Cancer Institute; MGH, Massachusetts General Hospital; MSKCC, Memorial Sloan-Kettering Cancer Center; ND, not done; CR, complete response.
*Latest survival data collected June 1, 2008.
†*EGFR* resequence and/or *MET* amplification were obtained after exposure to gefitinib and erlotinib.

as their first line of systemic therapy (Table 2). Four of 14 (29%) gefitinib responders had PR or SD after erlotinib compared with 0 of 4 gefitinib nonresponders. All 4 of the gefitinib nonresponders progressed on erlotinib by 2 months, whereas half of the gefitinib responders had not progressed by 2 months.

EGFR resequencing after progression on EGFR TKI therapy and subsequent response to erlotinib. Seven of the 18 patients had their tumors sampled after progression on EGFR TKI therapy: 3 after gefitinib therapy and the other 4 after gefitinib and erlotinib. Of these patients, 6 of 7 (86%) had acquired the T790M *EGFR* mutation in association with their initial activating exon 19 deletions. Five of the 6 (83%) T790M-carrying tumors displayed PD to erlotinib therapy (Table 2). One patient with exon 19 deletion (delE746-A750)-T790M had 6 months of SD on erlotinib; however, because a biopsy was obtained after gefitinib and erlotinib, we cannot excluded the possibility that T790M was acquired while on erlotinib therapy.

One patient had acquired the L747S secondary mutation in association with the activating L858R *EGFR* after exposure to gefitinib. The patient carrying L858R-L747S had a partial radiographic response to erlotinib 150 mg/d that lasted 6 months (Table 2).

MET amplification after progression on EGFR TKI therapy. Of the 7 patients that had their tumors sampled after progression on EGFR TKI therapy, 3 had sufficient material for analysis of *MET* amplification. None of these 3 had amplification of the *MET* oncogene. Patients 2 and 18 were analyzed by quantitative PCR methods and patient 6 was analyzed by fluorescence *in situ* hybridization (Table 2).

OS from start of gefitinib. The median OS of all 18 patients from start of gefitinib therapy to death was 30 months, 95% CI of 19 to 39 months. This is similar to OS reported for other series of *EGFR* mutated patients (17, 19).

Discussion

EGFR mutated cancers comprise a subset of NSCLC that are intrinsically sensitive to small-molecule EGFR inhibitors (12, 15, 17). The current clinical experience with the use of gefitinib and erlotinib in *EGFR* mutated patients indicates that exon 19 deletion and L858R-bearing tumors commonly display radiographic responses to these drugs with disease control durations that sometimes reach a year or longer (17, 19). Despite this unprecedented disease control rate, acquired resistance to EGFR TKIs invariably develops over the course of therapy and is becoming the main obstacle for management of this patient population (12). The first mechanism of acquired resistance described was the acquisition of the T790M *EGFR* mutation (22, 23). The methionine residue at position 790 generates a bulkier side chain that either affects binding of TKIs or enhances the affinity of the EGFR tyrosine kinase pocket to ATP, and this enhanced ATP affinity decreases the effective binding of gefitinib and erlotinib to the tyrosine kinase pocket of EGFR (22, 45). There is a great deal of similarities among structures of tyrosine kinase receptors and some analogous acquired resistance mutations fall exactly in the same amino acid residue. This is the case of the T315I, T670I, and T790M mutations in ABL1, KIT, and EGFR, respectively, in chronic myeloid leukemia, gastrointestinal stromal tumors, and *EGFR* mutated NSCLC (46). Our groups have shown in multiple *in vitro* and *in vivo* models that T790M in *cis* to an activating mutation (either L858R or exon 19 deletions) negates the sensitivity to achievable doses of gefitinib or erlotinib (23, 38).

Table 2. Clinical, pathologic, demographic, and molecular characteristics, response to therapy, PFS, and OS in the studied patients (Cont'd)

PFS, gefitinib (mo)	*EGFR* resequence	*MET* amplification	Therapy before erlotinib	Response, erlotinib 150 mg/d	PFS, erlotinib (mo)	Survival from gefitinib (mo)
24	delL747-S752+T790M	ND	Cetuximab, experimental Raf inhibitor	PD	1	30
40	L858R+L747S	No	Experimental EGFR inhibitor	PR	6	>62 (alive)*
26	ND	ND	None	PD	2	35
14	ND	ND	None	PD	3	27
16.5	ND	ND	None	SD	3.7	39
12	delL747-751InsP+T790M	No	None	PD	2	19
1	ND	ND	None	PD	2	3
3	ND	ND	None	PD	2	16
2	ND	ND	None	PD	2	7
4	ND	ND	None	PD	2	9
4	ND	ND	None	PD	2	21
7	ND	ND	Experimental heat shock protein 90 inhibitor	PD	1	>20 (alive)*
4	ND	ND	None	PD	2	9
17	ND	ND	None	SD	5	>49 (alive)*
16	delE746-A750+T790M †	ND	None	PD	4	32
10	delE746-A750+T790M †	ND	None	PD	3	>39 (alive)*
11	delE746-A750+T790M †	ND	None	SD	6	>44 (alive)*
11	delE746-A750+T790M †	No †	None	PD	2	32

The *in vitro* concentrations of gefitinib/erlotinib that can inhibit T790M-EGFR and T790M-carrying cells exceed 5 to 10 μmol/L (22, 23, 38, 46). Very few other secondary *EGFR* mutations have been described (24, 28). These have only been seen in patients receiving gefitinib who carried the L858R mutation. L858R-761Y (24) and L858R-L747S (28) *in vitro* shift the sensitivity curves for gefitinib and erlotinib when compared with L858R alone; however, both mutations are 100-fold less "resistant" than L858R-T790M or exon 19 deletion-T790M. Most *in vitro* data would suggest that L858R-D761Y and L858R-L747S would be inhibited if the EGFR TKI dose reached 1 to 2 μmol/L (24, 28), which is achievable with 150 mg/d erlotinib but not with 250 mg/d gefitinib. The clinical dose of gefitinib of 250 mg/d is far less than its maximum tolerated dose of 1,000 mg/d. The mean steady-state serum concentration of gefitinib following 225 mg/d varied from 0.03 to 0.32 μg/mL in a phase I trial (36), with an average of 0.16 μg/mL or 0.358 μmol/L. The mean concentration increases to 0.24 μg/mL at 300 mg/d and to 1.1 μg/mL or 2.461 μmol/L at 1,000 mg/d gefitinib (36). Erlotinib is used clinically at a dose of 150 mg/d (7), which is its maximum tolerated dose. The steady-state trough concentrations at this dose ranged from 0.33 to 2.64 μg/mL in the phase I trial (37), with a median of 1.26 μg/mL or the equivalent to 2.930 μmol/L.

In addition to secondary *EGFR* mutations, another mechanism of acquired resistance is an "oncogene switch" model. Our groups have recently shown that the acquired amplification of the *MET* oncogene occurs in ~20% of *EGFR* mutated patients with acquired resistance to gefitinib or erlotinib (26, 27). MET couples with other ErbB members and activates downstream signals that bypass the inhibited EGFR (27, 47). The *in vitro* resistance to erlotinib and gefitinib in this model was also in the range of 5 to 10 μmol/L. Dual inhibition of EGFR and MET with TKIs is able to overcome *MET* amplified EGFR TKI-resistant tumors (27). Of interest, in almost half of the patients with *MET* amplification, T790M was identified either in the same biopsy specimen or in biopsy specimens from other sites within the patient (26, 27). This indicates that T790M will continue to be the most prevalent form of EGFR TKI resistance. Other oncogenes, such as the insulin-like growth factor-I receptor, may also play a role in resistance to EGFR TKIs in non-*EGFR* mutated cells (48). Despite a rapidly growing understanding of the molecular mechanisms of acquired resistance to EGFR inhibitors, there is no standard therapy for the expanding number of *EGFR* mutated tumors that become resistant to gefitinib. Because, in an unselected population of platinum-refractory NSCLC patients, gefitinib was not statistically better than placebo in controlling disease progression (49), the Food and Drug Administration restricted its use for

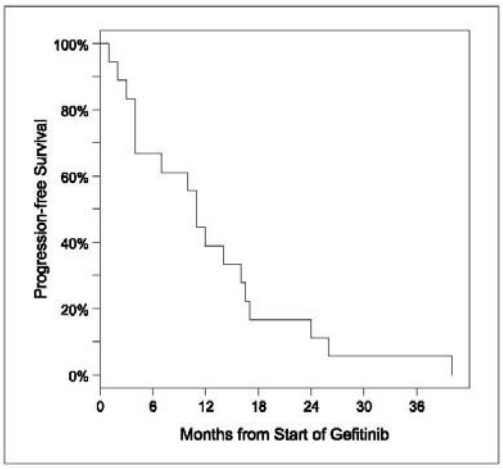

Fig. 1. Kaplan-Meier curve for PFS of the *EGFR* mutated patients during gefitinib therapy.

Table 3. Response and PFS of *EGFR* mutated gefitinib-resistant patients on erlotinib monotherapy

	PR	SD	PD
Best radiographic response, no. patients (%)	1 (6)*	3 (16)	14 (78)
PFS, mo (95% CI)	2 (2-3)		

*The only patient with PR had the L858R-L747S *EGFR* mutation, which *in vitro* is sensitive to achievable serum levels of erlotinib 150 mg/d.

patients previously benefiting from treatment or participating in clinical trials. Nonetheless, in the same phase III trial, the never smoker and Asian group of patients had a clear clinical benefit (49). Gefitinib continues to be widely used in East Asian countries and in *EGFR* genotyped patients (19, 50). Erlotinib is approved for use in unselected patients after failure of platinum-based therapy (7), and it, like gefitinib, has excellent efficacy in *EGFR* mutated patients in retrospective and prospective series (12, 17).

One question that remains unanswered is if gefitinib-resistant *EGFR* mutated patients could benefit from a switch to erlotinib. To address this, we retrospectively analyzed the clinical course of 18 *EGFR* mutated NSCLC that were treated with gefitinib and, on resistance, erlotinib. The patient characteristics, type of *EGFR* mutations (almost all had L858R or exon 19 deletions), and initial response to gefitinib 250 mg/d were consistent with previous experience in *EGFR* mutated patients (17, 19). Our clinical observation was that the majority (>83%) of the gefitinib-resistant patients given erlotinib 150 mg/d had radiographic progression within the first 2 to 4 months of exposure. This is consistent with our preclinical observations, because we expected gefitinib-resistant tumors to predominantly harbor T790M and/or *MET* amplification, which are cross-resistant to both EGFR TKIs as described above.

We had a second biopsy specimen in 7 of the 18 patients, and in 6 of them, the T790M secondary mutation was identified together with the initial activating exon 19 deletion. None of the 3 patients analyzed had *MET* amplification (Table 2). Almost all of these gefitinib-resistant patients had rapid progression on erlotinib. Only 1 patient achieved a partial radiographic response on switching to erlotinib (29). This patient had acquired the rare L747S mutation after exposure of the initial L858R-carrying tumor to gefitinib. As reported previously by our group, L858R-L747S is less sensitive to gefitinib and erlotinib than L858R *in vitro* (28). However, this compound mutation can be inhibited by increasing concentrations of gefitinib or erlotinib at a level that is clinically achievable for the later drug (29). We were not able to measure pharmacokinetic variables of either gefitinib or erlotinib during the course of therapy in this patient; however, the observed skin-related side effects (rash and pruritus) while on erlotinib 150 mg/d exceed in grade the effects while the patient was on gefitinib 250 mg/d (29), likely indicating a higher biologically active dose of the former compound in this individual. However, even in this patient, the duration of response was relatively short and radiographic progression was noted after 6 months. Further biopsies were not available to test if the tumor had acquired additional mechanisms of resistance, such as T790M or *MET* amplification.

Two recent reports have described the clinical experience of using erlotinib following gefitinib failure in Asian patients. The first was a phase II trial of erlotinib 150 mg/d in patients with either primary or acquired resistance to gefitinib (41). In the initial report, none of the *EGFR* mutated patients had a radiographic response to erlotinib. All of the *EGFR* mutated patients from that study were included in our analysis and we report updated clinical data in their response to both gefitinib and erlotinib. The second study evaluated 14 unselected patients that had failed gefitinib, and 5 harbored *EGFR* mutations (51). Of the *EGFR* mutated patients, a clinical and radiographic response was described for 2 patients after exposure to erlotinib. However, in 1 of these cases, the patient progressed on erlotinib within the first 2 months of therapy. In the 5 *EGFR* mutated patients, the time to progression on erlotinib averaged 3 months, whereas the initial time to progression on gefitinib exceed 8 months (51). No molecular data were available for these patients after progression on gefitinib. Anecdotal reports of the use of erlotinib after failure of gefitinib have been published by many investigators (52–57) and recently summarized by one of us (58). Combining all reports and the data presented here by us, it seems that most of the patients that harbored an *EGFR* mutation, when the genotype was available, did not benefit significantly from erlotinib after they had received and progressed on gefitinib. In almost all patients that harbored an acquired T790M mutation after gefitinib, rapid progression was noted on erlotinib.

However, we cannot exclude the possibility that continued EGFR inhibition, either with the original EGFR TKI or with a different anilinoquinazoline, benefits *EGFR* mutant patients. The readministration of gefitinib or erlotinib in previously responsive patients that show radiographic progression has been reported to improve symptoms and the clinical course of patients (59, 60), suggesting a role for continued TKI use

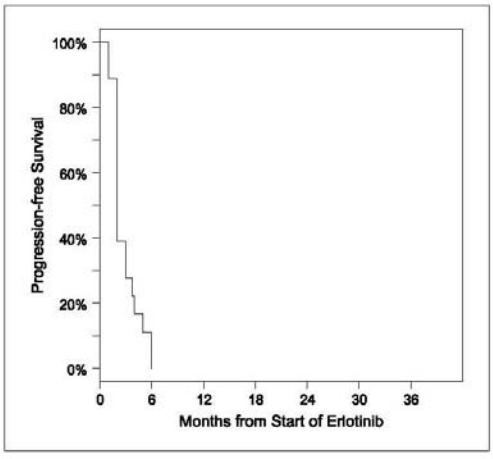

Fig. 2. Kaplan-Meier curve for PFS of the gefitinib-resistant *EGFR* mutated patients during erlotinib therapy.

to control the non-TKI-resistant clones of these "oncogene-addicted" cancers. Indeed, in our cohort of patients, we noted that patients with acquired resistance to gefitinib had modestly longer PFS on erlotinib than the ones that had *de novo* resistance, indicating that perhaps, in *EGFR* mutated patients with a prior response to TKIs, control of nonresistance clones is achievable and may improve clinical outcomes. Ongoing phase II randomized trials are attempting to confirm if maintaining some form of EGFR TKI therapy in addition to other lines of therapy is better than placebo in *EGFR* mutated patients with resistance to gefitinib or erlotinib.

Initial steps have begun to use preclinical data for rationale design of clinical trials of patients with acquired resistance to gefitinib or erlotinib. Our groups have shown that some irreversible and second-generation EGFR inhibitors *in vitro* can partially overcome the T790M mutation (22, 38, 46, 61). This knowledge has spawned phase II trials of the HKI-272 (ClinicalTrials.gov identifier: NCT00266877), BIBW-2992 (ClinicalTrials.gov identifier: NCT00656136), and XL-647 (ClinicalTrials.gov identifier: NCT00522145) compounds in this selected patient population. However, in recent *in vitro* cell line models and *in vivo* mouse models, HKI-272 used at doses achieved in the phase I clinical trial (62) actually induced the acquisition of EGFR-T790M (63) or was ineffective generating a radiographic response in L858R-T790M tumors (64). Thus, it is possible that at the achievable clinical concentrations of this, and other novel EGFR inhibitors, T790M will still not be inhibited. Continued development of alternative EGFR inhibitors that have a better profile against *EGFR* mutated tumors with T790M, such as PF00299804 (65), and development of MET inhibitors may one day help circumvent acquired resistance to EGFR-targeted therapy.

In summary our data indicate that, in *EGFR* mutated patients with acquired resistance to gefitinib at 250 mg/d, a switch to erlotinib at 150 mg/d does not lead to radiographic responses in most patients despite the higher biologically active dose of erlotinib (36, 37). The PFS was also short in these erlotinib-treated patients with a median of 2 months. These findings were expected, because preclinical models indicated that the two most common mechanisms of acquired resistance to gefitinib, EGFR-T790M and *MET* amplification, are highly resistant to achievable clinical concentrations of erlotinib (22, 23, 26, 27). As expected from our preclinical models, the only patient that achieved a radiographic response harbored the borderline resistant L858R-L747S mutation, which, similar to L858R-D761Y, can be overcome by increasing concentrations of either gefitinib or erlotinib at 150 mg/d (24, 28, 29).

Disclosure of Potential Conflicts of Interest

D.M. Jackman received consulting fees from Roche and Genentech; G.J. Riely received consulting fees from Boehringer-Ingelheim and Roche; P.A. Janne has received consulting fees from Roche and AstraZeneca and other remuneration from Genzyme; L.V. Sequist has received consulting fees from Genentech; and W. Pao has received remuneration from Molecular MD.

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4- DISCUSSÃO GERAL E CONCLUSÃO GERAL

A descoberta de que a elevada atividade da tirosina quinase poderia ser bloqueada em alguns tumores promoveu grandes avanços na terapia das doenças malignas. Tirosina quinases requerem ATP para sua atividade enzimática e por esse motivo moléculas semelhantes à ATP podem se ligar às quinases mutantes ou translocadas e torná-las inativas. O exemplo com aplicação clínica mais bem sucedido deste processo foi leucemia mielóide crônica (LMC), em que a translocação t(9;22) e a conseqüente atividade aumentada da tirosina quinase do BCR-ABL pode ser efetivamente inibida pelo imatinib. Este TKI oral revolucionou o tratamento da LMC e levou a remissões sustentáveis na maioria dos pacientes (Hehlmann et al., 2007). Um benefício clínico parecido foi observado em pacientes com CPNPC e as duas mutações ativadoras do *EGFR* mais comuns, L858R e deleção exon 19.

A. <u>Objetivo 1</u>: Coletar e analisar os dados clínicos de cinco ensaios clínicos prospectivos de gefitinib para pacientes com mutações do *EGFR*

Em nossa primeira publicação, resumimos e agrupamos os dados clínicos dos cinco primeiros ensaios clínicos prospectivos de monoterapia com gefitinib para pacientes com CPNPC e *EGFR* mutado (Costa et al., 2007b). Um total de 101 pacientes foi agrupado por estes estudos, e 59 receberam gefitinib como terapia de primeira linha e 42, após o recebimento de quimioterapia tradicional. A TR combinada nos 99 pacientes medidos foi de 80,8%. A TR para pacientes com deleção exon 19 e L858R foram parecidas. A SLP variou de 7,7 a 12,9 meses nos estudos identificados, e a sobrevivência geral não foi alcançada em 4 dos 5 relatórios com SMG de 15,4 meses em um deles. A administração de gefitinib foi segura, com menos de 50% dos pacientes desenvolvendo irritações cutâneas ou diarréia. Doença pulmonar intersticial só foi relatada em dois pacientes (2%), que sobreviveram. Concluímos, com essas observações, que a monoterapia com gefitinib leva a respostas objetivas na maioria dos pacientes com mutações do *EGFR*, e que pacientes com mutações L858R e deleção 19 apresentaram benefícios clínicos parecidos. Portanto, EGFR

TKIs como erlotinib e gefitinib são os novos paradigmas de tratamento para CPNPC com *EGFR* mutado.

B. <u>Objetivo 2</u>: Identificar o peptídeo pró-apoptótico chave requerido para os efeitos apoptóticos do gefitinib, erlotinib e outros inibidores EGFR TKI em células com CPNPC com *EGFR* mutado

Como já referido, a impressionante resposta inicial a inibidores de tirosina quinase constituídos por moléculas pequenas se origina do fenômeno do "vício oncogênico" e uma cascata de sinalização comum pode estar envolvida na apoptose induzida por múltiplos TKIs. A inativação rápida das formas fosforiladas de ERK, AKT, e STAT são comumente observadas em células dependentes dos oncogenes SRC-, BCR-ABL, e EGFR após exposição aos inibidores específicos SU6655, imatinib, e gefitinib, respectivamente (Sharma et al., 2006).

BIM é um dos principais efetores de apoptose mediada por imatinib em células leucêmicas com BCR-ABL. BIM pertence ao grupo "BH3-only" de proteínas que neutralizam vários membros da família antiapoptótica BCL2, impedindo sua atuação na inibição dos efeitos pró-apoptóticos do BAX e BAK no espaço mitocondrial intermembrana (Kuroda et al., 2006). BIM é regulado por múltiplos estímulos, incluindo as vias PI3K-AKT-FOXO e ERK1/2 MAPK. Remoção da citoquinas ou inibição da PI3K-AKT leva à desfosforilação e entrada nuclear do fator de transcrição FOXO-3A, o qual induz a expressão de mRNA *BIM* (Qi et al., 2006). Em adição, ERK1/2 regula a função do BIM por modificações pós-tradução do mRNA. Fosforilações dependentes do ERK1/2 antagonizam o BIM prevenindo a degradação proteossomal ou disrupção do complexo BIM-BAX (Qi et al., 2006).

Estas observações permitiram formular a hipótese de que o BIM teria um papel importante no apoptose induzida por gefitinib e outros TKIs que afetam o EGFR. Os resultados dos estudos referentes a esta possibilidade foram relatados em nossa segunda publicação (Costa et al., 2007a). Nossos dados indicam que a

via mitocondrial intrínseca está envolvida na morte celular induzida pelo gefitinib e que a ativação da regulação do polipeptídio pró-apoptótico BIM ocorre durante apoptose induzida por EGFR TKIs. Além disso, a ativação do EGFR, AKT, e/ou ERK causada pelas mutações resistentes T790M e L747S retardaram a ativação do BIM e apoptose nesses CPNPC. Mostramos também, em duas linhas distintas de CPNPC com mutações ativadoras de *EGFR*, que um bloqueio da produção gênica por uma estratégia de RNA de interferência (siRNA) do gene BIM levou à redução significante da quantidade de morte celular induzida por TKIs.

Estas observações sugerem que o Bim é um efetor chave no apoptose induzida por TKIs em tumores dependentes de EGFR e que a ativação do BIM pode ser um dos mecanismos comuns no qual células de tumores dependentes do "vício oncogênico" sofrem apoptose com TKIs. Mais estudos serão necessários para definir outras vias apoptóticas relevantes que estão envolvidas na apoptose induzida por gefitinib.

Três outros grupos publicaram dados similares após nossa publicação original (Cragg et al., 2007; Gong et al., 2007; Deng et al., 2007). Além de confirmar o BIM como mediador pró-apoptótico chave dos EGFR TKIs em CPNPC com EGFR mutado, estes investigadores notaram também que a adição de BH3-miméticos, como ABT-737, aumenta a atividade dos EGFR TKIs. Estas descobertas podem ser promissoras no futuro desenvolvimento de tratamentos para inibir EGFR e estimular a indução da cascata mediada pelo BIM.

C. <u>Objetivo 3</u>: Identificar e caracterizar novas mutações secundárias (L747S) que têm como conseqüência resistência adquirida à terapia EGFR TKI em CPNPC com *EGFR* mutado

A resistência adquirida ao imatinib ocorre em LMC e freqüentemente em GIST (Gorre et al., 2001; Rubin et al., 2007). Nas duas doenças, mutações secundárias, envolvendo os genes *ABL*, *KIT* e *PDGFRA,* foram identificadas como

fatores responsáveis principais para o restabelecimento da sinalização oncogênica nesses tumores. No caso da LMC, mais de 35 mutações foram descritas no domínio quinase do *BCR-ABL* em pacientes com resistência ao imatinib, e em GIST padrões similares foram vistos com mutações secundárias no domínio quinase de *KIT* e *PDGFRA* . Existem muitas semelhanças entre as estruturas de diversas tirosinas quinase, e algumas das mutações secundárias são localizadas exatamente em resíduos de aminoácidos similares. Este é o caso das mutações T315I, T670I, e T790M em *ABL*, *KIT*, e *EGFR*, respectivamente (Carter et al., 2005). Mutações destes resíduos chave podem prevenir o acesso dos inibidores à região da bolsa da quinase e atrapalhar ligações de hidrogênio e outras interações entre o inibidor e a quinase, e também podem afetar a afinidade ao ATP e reduzir a ligação com os TKIs (Carter et al., 2005; Yun et al., 2008). Apesar da similaridade entre as alterações na LMC, GIST, e CPNPC com mutações no *EGFR* nos padrões de resistência secundária, neste último somente as mutações secundárias T790M (Kobayashi et al., 2005a) e D761Y (Balak et al., 2006) haviam sido descobertas até 2007.

Em nossa segunda e terceira publicação (Costa et al., 2007a; Costa et al., 2008b), identificamos e caracterizamos uma nova mutação *EGFR* secundária, L747S. Nossos estudos *in vitro* demonstraram que as duas mutações secundárias inicialmente identificadas em nosso laboratório (T790M e L747S) conferiram vários graus de resistência aos sinais apoptóticos iniciados pelo gefitinib. EGFR, AKT, e ERK não foram inibidos por gefitinib ou erlotinib em nossos modelos celulares com T790M. O resíduo L747 é orientado em direção à região posterior da bolsa da fenda catalítica, tanto na conformação ativa e inativa do domínio tirosina quinase do EGFR. Baseado em estudos cristalográficos e simulações moleculares dinâmicas, a mutação L747S pode afetar a ligação ao ATP ou ao gefitinib devido à mudança no equilíbrio conformacional da quinase entre os estados ativos e inativos dos tumores com EGFR-L858R (Yun et al., 2007). Maiores estudos cristalográficos são necessários para compreender melhor a base estrutural dos efeitos da mutação L858R-L747S.

Nosso estudo mostrou que o EGFR-L858R-L747S apresenta um padrão de resistência menos acentuado ao gefitinib do que o observado com L858R-T790M. Estes resultados são similares aos observados na mutação secundária L858R-D761Y (Balak et al., 2006). A maioria dos dados *in vitro* sugere que L858R-D761Y e L858R-L747S seriam inibidos se a dose de EGFR TKI chegasse a 1-2 μM (Costa et al., 2007a; Balak et al., 2006), a qual é possível de ser alcançada com 150 mg/dia de erlotinib, mas não com 250 mg/dia de gefitinib. A dose clínica utilizada de gefitinib de 250 mg/dia é bem menor do que a dose máxima tolerada (DMT) de 1000 mg/dia. A concentração no plasma humano no "estado estável" do gefitinib 225 mg/dia foi medida ao redor de 0,16 μg/mL ou 0,358 μM em ensaios de fase I (Baselga et al., 2002). A concentração aumenta para 0,24 μg/mL com a ingestão de 300 mg/dia, e para 1,1 μg/mL ou 2,461 μM com 1000 mg/dia de gefitinib (Baselga et al., 2002). Erlotinib é usado clinicamente na dose de 150 mg/dia (Shepherd et al., 2005), que é a DMT. As concentrações da "fase estável" nesta dose variam entre 0,33 a 2,64 μg/mL nos ensaios de fase I, com uma média de 1,26 μg/mL ou o equivalente a 2,930 μM (Hidalgo et al., 2001).

Em nossa terceira publicação mostramos que a suposição acima é verdadeira (Costa et al., 2008b). Reportamos que o paciente com mutação adquirida do *EGFR* (L858R-L747S) resistente ao gefitinib respondeu ao erlotinib por 6 meses. Não conseguimos medir os parâmetros farmacocinéticos de ambos gefitinib e erlotinib durante o curso de terapia no paciente com a mutação L858R-L747S, entretanto os efeitos colaterais observados relacionados à pele (erupção e prurido) durante o uso de erlotinib 150 mg/dia excederam os efeitos, enquanto o paciente estava com gefitinib 250 mg/dia (Costa et al., 2008b), indicando uma dose biológica ativamente maior do composto anterior neste individuo. Os pacientes com deleção do exon 19-T790M ou L858R-T790M não responderam ao erlotinib. Nosso grupo mostrou em múltiplos modelos *in vitro* e *in vivo* que T790M em *cis* com uma mutação ativadora (L858R ou deleção exon 19) produz resistência nas doses possíveis de serem obtidas com gefitinib ou erlotinib em pacientes (Kobayashi et al., 2005b; Pao et al., 2005). As concentrações *in vitro*

de gefitinib/erlotinib que podem inibir células portadoras de T790M-EGFR superam 5-10 μM (Kobayashi et al., 2005a; Kobayashi et al., 2005b; Carter et al., 2005; Pao et al., 2005).

Adicionalmente às mutações *EGFR* secundárias, o outro mecanismo de resistência adquirida aos EGFR TKIs é a amplificação adquirida do oncogene *MET* que ocorre em aproximadamente 20% dos pacientes com *EGFR* mutado com resistência adquirida ao gefitinib ou erlotinib (Bean et al., 2007; Engelman et al., 2007b). O MET se agrupa com outros membros ErbB (como ErbB3) e ativa a sinalização que contorna o EGFR inibido (Engelman et al., 2007b) (Figura 2). A resistência *in vitro* ao erlotinib e gefitinib neste modelo também é observada com doses de 5-10 μM. Inibição dupla do EGFR e MET com TKIs é capaz de bloquear o crescimento de células tumorais *MET* amplificadas resistentes aos EGFR TKIs (Engelman et al., 2007b). Em quase metade dos pacientes com amplificação *MET,* a mutação T790M foi identificada tanto na mesma amostra da biópsia quanto em amostras de biópsia de outros pontos no paciente (Engelman et al., 2007b; Bean et al., 2007). Isto indica que T790M continuará sendo a forma mais prevalente de resistência aos EGFR TKIs.

D. <u>Objetivo 4</u>: Analisar os efeitos clínicos e radiográficos do erlotinib 150 mg/dia em CPNPC com *EGFR* mutado e com resistência *de novo* ou adquirida ao gefitinib 250 mg/dia, e correlacionar as respostas radiográficas com os mecanismos moleculares de resistência

Apesar do rápido acúmulo de dados na compreensão dos mecanismos moleculares de resistência adquirida aos inibidores EGFR, não há ainda uma terapia para o número crescente de tumores que se tornam resistentes ao gefitinib. Uma pergunta que permanecia sem resposta era se os pacientes com *EGFR* mutado e resistentes ao gefitinib seriam beneficiados pela mudança para erlotinib. Abordamos esta questão em na nossa quarta publicação (Costa et al., 2008a). Nós analisamos retrospectivamente o curso clínico de 18

pacientes com CPNPC com *EGFR* mutado em cinco centros acadêmicos (nos EUA e Coréia) que foram tratados com gefitinib 250 mg/dia e após adquirirem resistência com erlotinib 150 mg/dia. As características dos pacientes, tipo de mutações *EGFR* (quase todos tinham L858R ou deleções exon 19) e resposta inicial ao gefitinib 250 mg/dia foram consistentes com a experiência anterior de pacientes com *EGFR* mutado (Costa et al., 2007b; Sequist et al., 2007a). Nossa observação clínica foi que a maioria (mais de 83%) dos pacientes resistentes ao gefitinib tiveram progressões radiográficas nos primeiros 2 a 4 meses de exposição ao erlotinib, 150 mg/dia. Isto é consistente com nossas observações pré-clínicas, que indicam que a maioria dos tumores resistentes ao gefitinib possui predominantemente T790M e/ou amplificações do *MET*, que são resistentes tanto ao gefitinib quanto erlotinib. Foi possível verificar uma segunda amostra de biópsia em 7 pacientes e 6 apresentavam T790M. Nenhum dos 3 pacientes analisados mostraram amplificação *MET*. Quase todos esses pacientes gefitinib resistentes tiveram uma progressão rápida com o erlotinib. Somente um paciente obteve uma resposta radiográfica parcial na mudança para erlotinib. Este paciente adquiriu a mutação rara L747S após exposição do tumor inicial L858R ao gefitinib. Esta mutação composta L858R-L747S pode ser inibida pelo erlotinib (Costa et al., 2008b). Entretanto, mesmo neste paciente a duração de resposta foi relativamente curta e a progressão radiográfica foi notada após 6 meses. Outras biópsias não estavam disponíveis para testar se o tumor adquiriu mecanismos de resistência adicionais, como T790M ou amplificação *MET*. Novas terapias são necessarias para tumores que se tornam resistentes ao gefitinib e erlotinib.

Alguns inibidores TKIs irreversíveis de segunda geração do EGFR *in vitro* podem superar parcialmente a mutação T790M (Kobayashi et al., 2005a; Kobayashi et al., 2005b; Carter et al., 2005; Kwak et al., 2005). Esta informação fez com que fossem iniciados ensaios clínicos de fase II dos compostos HKI-272, BIBW-2992 e XL-647 nesta população de pacientes com CPNPC. Merece ser citado que, em modelos recentes, utilizando linhagens celulares *in vitro* e modelos *in vivo* em ratos, o composto HKI-272 usado em doses possíveis de serem alcançadas nos ensaios clínicos de fase I (Wong, 2007) induziu à aquisição do

EGFR-T790M (Godin-Heymann et al., 2008) ou foi ineficaz na geração de resposta radiográfica em tumores L858R-T790M (Li et al., 2007). Portanto, é provável que nas concentrações clínicas alcançáveis deste (e outros inibidores) EGFR TKI irreversível, o crescimento celular de células com a mutação T790M não será inibido.

O desenvolvimento de inibidores EGFR TKIs alternativos que tenham um perfil melhor contra tumores com *EGFR* mutado, contendo T790M, como PF00299804 (Engelman et al., 2007a), e o desenvolvimento de inibidores de MET poderão, no futuro, contribuir para o tratamento da resistência adquirida à terapia alvo com EGFR TKIs em CPNPCs dependentes das vias celulares ativadas pelo EGFR.

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