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Clinical Cancer Research

Predictive Biomarkers and Personalized Medicine

The Impact of *EGFR* T790M Mutations and *BIM* mRNA Expression on Outcome in Patients with *EGFR*-Mutant NSCLC Treated with Erlotinib or Chemotherapy in the Randomized Phase III EURTAC Trial

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Abstract

Purpose: Concomitant genetic alterations could account for transient clinical responses to tyrosine kinase inhibitors of the EGF receptor (EGFR) in patients harboring activating *EGFR* mutations.

Experimental Design: We have evaluated the impact of pretreatment somatic *EGFR* T790M mutations, *TP53* mutations, and Bcl-2 interacting mediator of cell death (*BCL2L11*, also known as *BIM*) mRNA expression in 95 patients with *EGFR*-mutant non–small-cell lung cancer (NSCLC) included in the EURTAC trial (trial registration: NCT00446225).

Results: T790M mutations were detected in 65.26% of patients using our highly sensitive method based on laser microdissection and peptide-nucleic acid-clamping PCR, which can detect the mutation at an allelic dilution of 1 in 5,000. Progression-free survival (PFS) to erlotinib was 9.7 months for those with T790M mutations and 15.8 months for those without, whereas among patients receiving chemotherapy, it was 6 and 5.1 months, respectively ($P < 0.0001$). PFS to erlotinib was 12.9 months for those with high and 7.2 months for those with low/intermediate *BCL2L11* expression levels, whereas among chemotherapy-treated patients, it was 5.8 and 5.5 months, respectively ($P = 0.0003$). Overall survival was 28.6 months for patients with high *BCL2L11* expression and 22.1 months for those with low/intermediate *BCL2L11* expression ($P = 0.0364$). Multivariate analyses showed that erlotinib was a marker of longer PFS (HR = 0.35; $P = 0.0003$), whereas high *BCL2L11* expression was a marker of longer PFS (HR = 0.49; $P = 0.0122$) and overall survival (HR = 0.53; $P = 0.0323$).

Conclusions: Low-level pretreatment T790M mutations can frequently be detected and can be used for customizing treatment with T790M-specific inhibitors. *BCL2L11* mRNA expression is a biomarker of survival in *EGFR*-mutant NSCLC and can potentially be used for synthetic lethality therapies. *Clin Cancer Res; 20(7); 2001–10. ©2014 AACR.*

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Introduction

A recent meta-analysis of patients with non–small-cell lung cancer (NSCLC) with activating mutations in the EGF receptor (*EGFR*) showed that EGFR tyrosine kinase inhibitors (TKI), mainly gefitinib and erlotinib, significantly delayed disease progression but had no effect on overall survival, although this may have been due to the confounding effect of crossover between randomization arms at the time of progression (1). In a large prospective study of *EGFR*-mutant patients treated with erlotinib, we observed similar overall survival in the first- and second-line settings (2).

In the randomized phase III EURTAC trial comparing erlotinib with chemotherapy in advanced *EGFR*-mutant patients with NSCLC, the interim analysis confirmed that

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Translational Relevance

Despite the recent paradigm shift in the treatment of patients with non–small-cell lung cancer (NSCLC), with a move toward biomarker-directed therapy, preclinical data have seldom been incorporated into clinical practice because the existence of multiple resistance mechanisms complicates the selection of optimal biomarkers. Here, we report possible causes of resistance to EGF receptor (EGFR) tyrosine kinase inhibitors (TKI) in *EGFR*-mutant NSCLC patients: the high coexistence of the pretreatment somatic T790M mutation, with a clear impact on progression-free survival, and the role of Bcl-2 interacting mediator of cell death (*BCL2L11*, also known as *BIM*) mRNA expression as an independent prognostic marker. Our findings show that the T790M mutation is present at clinically meaningful levels before treatment and provide the basis for the use of *EGFR* T790M mutations and *BCL2L11* mRNA levels to prospectively identify patients most likely to benefit from rational polytherapies designed to overcome innate and acquired resistance to EGFR TKI monotherapy.

EGFR mutations were a predictive biomarker of longer progression-free survival (PFS) to erlotinib (HR = 0.37; $P < 0.001$; ref. 3). However, response was limited to 58% of the patients in the erlotinib group and no impact on overall survival was observed.

Preclinical and limited clinical studies have identified genetic events that could regulate response to EGFR TKIs in NSCLC, including a primary drug resistance mutation in *EGFR* (T790M; refs. 4–8), dysregulation of *Bcl-2* interacting mediator of cell death (*BCL2L11*; refs. 9–12), and mutations in *TP53* (13). The *EGFR* T790M mutation promotes the assembly of an active kinase conformation with high affinity for ATP binding (14). The *EGFR* T790M mutation has been found in pretreatment fresh-frozen or paraffin-embedded tissue from *EGFR*-mutant, EGFR TKI-naïve patients with NSCLC at frequencies ranging from 2.7% to 79% (4, 5, 7, 15–18). This variation seems to be due to the use of different detection methods. For example, the recent study by Su and colleagues compared mass spectrometry with standard direct sequencing and found the T790M mutation in 31.5% and 2.7%, respectively (15). The presence of the T790M mutation has been related to shorter PFS to EGFR TKIs (4, 5, 15). In NSCLC cell lines harboring *EGFR* activating mutations, *EGFR* mutations led to impaired *BCL2L11* expression, mediated by the activation of the extracellular signal-regulated kinase (ERK) pathway (9–11, 19). In a study of *EGFR*-mutant patients treated with EGFR TKIs, nine patients with low *BCL2L11* mRNA levels had a significantly shorter PFS than 15 with high levels (4.7 vs. 13.7 months; $P = 0.007$; ref. 12). *TP53* mutations have not been examined in patients with *EGFR* mutations; however, preclinical data indicate that p53 could be associated with acquired resistance to EGFR TKIs (20).

Here, we present the updated efficacy results of the EURTAC trial (ref. 3; data cutoff, January 24, 2013). We have also examined somatic T790M and *TP53* mutations and *BCL2L11* (also known as *BIM*) mRNA expression in pretreatment tumor samples from 95 patients included in the trial to assess their potential role as predictive biomarkers of progression-free and overall survival.

Patients and Methods

Patients

The EURTAC study enrolled 173 patients with *EGFR* mutations who were randomized to receive erlotinib or standard intravenous chemotherapy with cisplatin or carboplatin plus docetaxel or gemcitabine (3). Pretreatment tumor specimens were available from 95 of these patients for further genetic analyses. The EURTAC was approved by the Institutional Review Board of each participating center, and written informed consent was obtained from all patients.

Molecular analyses

All analyses were carried out centrally at the Pangaea Biotech Laboratory of Oncology, an ISO 15189-certified laboratory, located in the Quirón Dexeus University Hospital (Barcelona, Spain). See the Supplementary Materials and Methods for complete details of all methodologies.

T790M mutations

For the assessment of the pretreatment somatic *EGFR* T790M mutation, cancer cells were microdissected directly into 15 μL of PCR buffer (Ecogen) plus proteinase K (40 μg/mL) and incubated overnight at 60°C. Proteinase was inactivated at 95°C for 10 minutes, and the amount of DNA present in the cell extract was quantified in a Nanodrop and diluted to 5 ng of DNA/μL. Two extracts were obtained from every tumor, each from one or more separate tumor areas (5). Cell extracts were directly analyzed by TaqMan in the presence of a peptide-nucleic acid (PNA) designed to inhibit the amplification of the wild-type (wt) allele, as previously described (5). Primers, probes, and PNA were as follows: forward primer, 5′-AGGCAGCCGAAGGGCA-3′, reverse primer 5′-CCTCACCTCCACCGTGCA-3′; probes 5′-VIC CTCATCACGCAGCTCATG - MGB-3′; and 5′-FAM-CTCATCATGCAGCTCATG- MGB′3; and PNA: N (5′)-TCATCACGCAGCTC-C (3′). Amplification was performed in 12.5-μL volumes using 1 μL of extract, 6.25 μL of TaqMan 2× PCR Master Mix (Applied Biosystems), 7.5 pmol of each primer, 2.5 pmol of probes, and 6.25 pmol of PNA. Samples were submitted to 50 cycles of 15 seconds at 94°C and 1 minute at 60°C in a 7900 HT Fast Real-Time PCR System (Applied Biosystems). All cell extracts were run in quadruplicate, so every tumor sample was run in octuplicate. In addition, all cell extracts were also assayed in the absence of PNA to confirm the presence of DNA. DNA from the DLD1 cell line at 5 ng/μL was used as a negative control and DNA from the H1975 cell line at 50 pg/μL as a mutated, positive control. The H1975 cell line is derived from an

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EGFR TKI-naïve patient and harbors both the T790M and L858R mutation (6). Extraction and non-template controls were also run. Upon completion of the TaqMan assay, to confirm that the amount of DNA in the samples was not higher than 5 ng/μL, we compared the C_t of the wt allele in the absence of PNA with the corresponding C_t of the DLD-1 cell line. Subsequently, a tumor was considered positive if at least one of the eight octuplicates showed amplification of the T790M allele in the presence of PNA.

We had previously evaluated the sensitivity of our T790M assay using serially diluted genomic DNA from the cell line H1975. Five pg of DNA per μL were successfully amplified and the T790M mutation detected. In contrast, results from cell lines not harboring the T790M were consistently negative in our assay, at concentrations up to 5 ng/μL (a thousand times higher than the concentration of H1975 needed to detect the T790M). The specificity of our assay was determined by diluting DNA from H1975 (10 pg/mL final) into increasing concentrations (1:10 to 1:20,000) of DNA from the DLD-1 cell line (wt for the T790M mutation). Our assay consistently detected the T790M allele at an allelic distribution of 1 in 5,000. For additional details on the validation of our technique, see Supplementary Materials Methods, Supplementary Tables S1–S3, and Supplementary Fig. S1.

Because the T790M mutation is present in an extremely low percentage of cells, many standard, commercially available techniques detect it only in a relatively small percentage of tumors, because they can detect the mutation at an allelic distribution of 1 in only 10 to 100. One of these assays, the T790M TaqMan Mutation Detection Assay (TMDA; Applied Biosystems), is based upon cast-PCR, where an allele-specific primer detects the mutant allele and an MGB oligonucleotide blocker suppresses the amplification of the wt allele. We have also examined the T790M mutation with the TMDA in our series of 95 patients.

TP53 mutations

We used high-resolution melting (HRM) to screen for mutations in exons 5, 6, 7, and 8 of *TP53*. The HRM products of all samples that appeared mutated or unclear in the dissociation curve analyses were subsequently sequenced. All mutated samples were reconfirmed by standard PCR followed by agarose gel electrophoresis and sequencing (Supplementary Tables S4–S6).

BCL2L11 mRNA expression

Gene expression analysis of *BCL2L11* was performed on RNA isolated from the tumor tissue specimens. RNA extraction, retrotranscription analysis, and real-time PCR were performed as previously described (5), and gene expression was examined by quantitative PCR using *β-actin* as housekeeping gene.

Statistical analyses

On 24 January 2013, 135 PFS events had occurred, and the results reported here are based on data analyses from that cutoff date. For the overall survival analysis, patients were not censored at crossover, whereas all patients were censored at crossover for the analysis of PFS. Progression-free and overall survival were estimated by means of the Kaplan–Meier method and compared with a nonparametric log-rank test. A multivariate Cox proportional hazard model was applied with treatment and potential risk factors as covariates, obtaining HRs and their 95% confidence intervals (CI). Response rates were compared with the χ^2 test or Fisher exact test, as required. The *U* Mann–Whitney test and the Kruskal–Wallis test were used to test associations between genotypes and clinical characteristics. Each analysis was performed with the use of a two-sided 5% significance level and a 95% CI. The statistical analyses were performed using SAS version 9.2, SPSS version 17.0, or S-PLUS version 6.1. The EURTAC study is registered with ClinicalTrials.gov, number NCT00446225.

Results

Table 1 shows patient characteristics of the patients included in the subanalysis of 95 patients from the EURTAC trial (3). Characteristics of the 95 patients were consistent with those of all 173 patients included in the trial. The pretreatment somatic *EGFR* T790M mutation was detected in 62 (65.26%) patients using our assay with laser microdissection and PNA-clamping PCR. The analysis of non-tumor tissue ruled out the presence of germline mutations. In contrast, the TMDA assay detected the T790M allele in only 23 of 95 samples (24.2%; Supplementary Table S7).

TP53 mutations were successfully assessed in 82 and detected in 23 (28.05%). Among the 83 patients in whom *BCL2L11* mRNA was successfully examined, *BCL2L11* expression was low (< 1.83) or intermediate (1.83–2.96) in 53 (63.96%) and high (≥2.96) in 30 (36.14%). There was no statistically significant association between T790M mutation status and *BCL2L11* expression levels. No differences in baseline characteristics were observed according to T790M or *TP53* mutations or *BCL2L11* expression levels (Table 1).

Progression-free survival

At January 24, 2013, median PFS for all 173 patients in the EURTAC study was 10.4 months for patients treated with erlotinib and 5.1 months for those treated with chemotherapy (HR = 0.33; 95% CI, 0.23–0.49; $P < 0.0001$).

Differences in PFS times for the 95 patients included in this subanalysis, based on treatment and T790M mutation status as determined by our method, were highly significant ($P < 0.0001$; Fig. 1A). Among patients treated with erlotinib, PFS was 15.8 months (95% CI, 8.8-NR) for those without the concomitant T790M mutation, compared with 9.7 months (95%CI, 6.9–12.9) for those with the T790M mutation ($P = 0.0185$), whereas among those treated with chemotherapy, PFS was 5.1 months (95% CI, 1.1–6.7) for those without the T790M mutation and 6 months (95% CI, 4.1–7.7) for those with the T790M mutation ($P = 0.2427$; Supplementary Fig. S2A–S2B). When tumor samples were classified as having low or high levels of T790M, using the

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Table 1. Patient characteristics of the 95 patients from the EURTAC trial included in the present study

	Total ($N = 95$) *N* (%)	Erlotinib ($N = 50$) *N* (%)	Chemotherapy ($N = 45$) *N* (%)	*P*
Gender				0.1112[a]
Female	71 (74.74)	34 (68.00)	37 (82.22)	—
Male	24 (25.26)	16 (32.00)	8 (17.78)	—
Age				0.9481[a]
<65 y	44 (46.32)	23 (46.00)	21 (46.67)	—
≥65 y	51 (53.68)	27 (54.00)	24 (53.33)	—
Smoking history				0.3120[b]
Never smoked	64 (67.37)	32 (64.00)	32 (71.11)	—
Former smoker	23 (24.21)	15 (30.00)	8 (17.78)	—
Current smoker	8 (8.42)	3 (6.00)	5 (11.11)	—
ECOG performance status				0.7263[a]
0	31 (32.63)	15 (30.00)	16 (35.56)	—
1	51 (53.68)	27 (54.00)	24 (53.33)	—
2	13 (13.68)	8 (16.00)	5 (11.11)	—
Histology				0.0193[b]
Adenocarcinoma	87 (91.58)	47 (94.00)	40 (88.89)	—
Bronchioalveolar adenocarcinoma	1 (1.05)	0 (0.00)	1 (2.22)	—
Large cell carcinoma	2 (2.11)	2 (4.00)	0 (0.00)	—
Squamous cell carcinoma	1 (1.05)	1 (2.00)	0 (0.00)	—
Other	4 (4.21)	0 (0.00)	4 (8.89)	—
Tumor stage				0.4381[b]
IIIB (malignant effusion)	7 (7.37)	5 (10.00)	2 (4.44)	—
IV	87 (91.58)	44 (88.00)	43 (95.56)	—
Unknown[c]	1 (1.05)	1 (2.00)	0 (0.00)	—
Lung or pleura metastasis				0.6017[b]
Yes	92 (96.84)	49 (98.00)	43 (95.56)	—
No	3 (3.16)	1 (2.00)	2 (4.44)	—
Bone metastasis				0.3139[a]
Yes	27 (28.42)	12 (24.00)	15 (33.33)	—
No	68 (71.58)	38 (76.00)	30 (66.67)	—
Brain metastasis				0.2505[a]
Yes	11 (11.58)	4 (8.00)	7 (15.56)	—
No	84 (88.42)	46 (92.00)	38 (84.44)	—
Type of *EGFR* mutation				0.7643[a]
del 19	64 (67.37)	33 (66.00)	31 (68.89)	—
L858R	31 (32.63)	17 (34.00)	14 (31.11)	—
T790M mutation				0.5548[a]
Detected	62 (65.26)	34 (68.00)	28 (62.22)	—
Not detected	33 (34.74)	16 (32.00)	17 (37.78)	—
TP53 mutations				0.5950[a]
Detected	23 (24.21)	10 (20.00)	13 (28.89)	—
Not detected	59 (62.11)	33 (66.00)	26 (57.78)	—
No data[d]	13 (13.68)	7 (14.00)	6 (13.33)	—
BIM expression				0.5418[a]
Low/intermediate	53 (55.79)	26 (52.00)	27 (60.00)	—
High	30 (31.58)	16 (32.00)	14 (31.11)	—
No data[d]	12 (12.63)	8 (16.00)	4 (8.89)	—
Response				<0.0001[b]
Complete response	1 (1.05)	1 (2.00)	0 (0.00)	—
Partial response	32 (33.68)	27 (54.00)	5 (11.11)	—
Stable disease	34 (35.79)	12 (24.00)	22 (48.89)	—
Progressive disease	14 (14.74)	6 (12.00)	8 (17.78)	—
Unknown[c]	14 (14.74)	4 (8.00)	10 (22.22)	—

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

[a]χ^2 test.

[b]Fisher test.

[c]Information not recorded (percentages are based on all 95 patients).

[d]Insufficient tumor tissue for analysis (percentages are based on all 95 patients).

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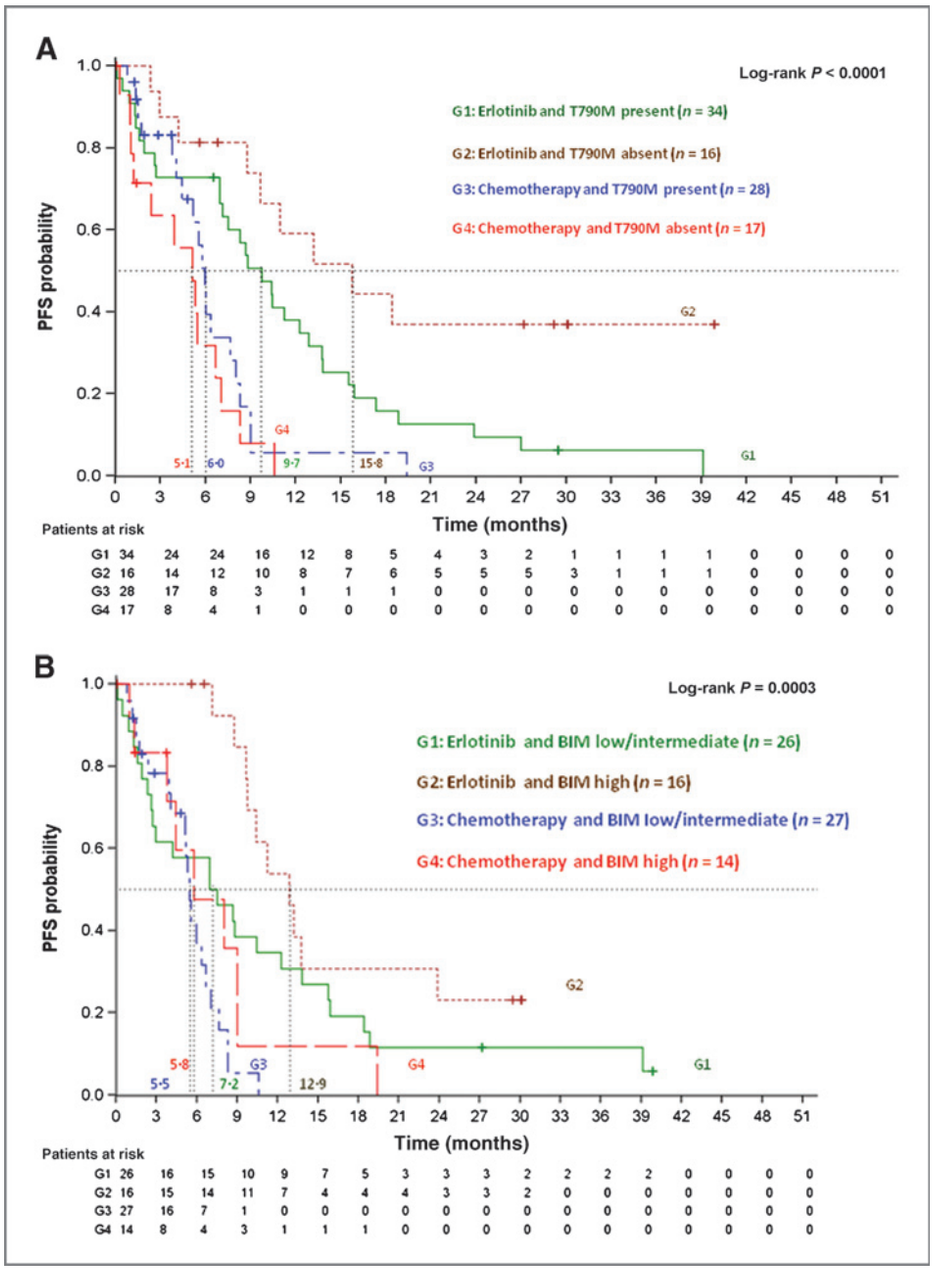

Figure 1. PFS according to treatment group and T790M mutation status (A) and treatment group and *BCL2L11* (also known as *BIM*) mRNA expression levels (low/intermediate vs. high; B).

median (0.0073) as the cutoff, PFS to erlotinib was 10.4 months in patients with low levels and 8.7 months in those with high levels ($P = 0.058$). When T790M mutation status was determined by the TMDA, PFS was similar in patients in whom the T790M mutation was detected (10.8 months) and in those in whom the mutation was not detected (10.4 months; Supplementary Fig. S3).

PFS to erlotinib was 7.2 months (95% CI, 2.6–12.3) for those with low/intermediate *BCL2L11* levels, compared with 12.9 months (95% CI, 9.7–23.9) for those with high *BCL2L11* levels, whereas among patients treated with chemotherapy, PFS was 5.5 months (95% CI, 3.9–6.7) for those with low/intermediate *BCL2L11* levels and 5.8 months (95% CI, 1.3–9.0) for those with high levels ($P = 0.0003$; Fig. 1B).

PFS to erlotinib was 9.7 months (95% CI, 0.5–15.9) for those with *TP53* mutations and 11 months (95% CI, 8.7–13.7) for those without *TP53* mutations, whereas among patients treated with chemotherapy, PFS was 5.3 months (95% CI, 1.3–7.7) for those with mutations and 6 months (95% CI, 3.9–8.3) for those without ($P = 0.0001$).

In the univariate analysis, only erlotinib (HR = 0.32; 95% CI, 0.19–0.53; $P < 0.0001$) and high *BCL2L11* expression (HR = 0.54; 95% CI, 0.31–0.93; $P =$

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Table 2. Univariate analyses of progression-free and overall survival in 95 patients from the EURTAC trial included in the present study

	PFS		Overall survival	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Gender				
Female	1.00	—	1.00	—
Male	1.05 (0.63–1.76)	0.8487	0.955 (0.54–1.69)	0.8742
Treatment				
Chemotherapy	1.00	—	1.00	—
Erlotinib	0.32 (0.19–0.53)	< 0.0001	0.96 (0.59–1.57)	0.8651
Smoking history				
Current smoker	1.00	—	1.00	—
Never smoked	0.68 (0.29–1.60)	0.3774	0.55 (0.23–1.29)	0.1688
Former smoker	0.75 (0.30–1.90)	0.5424	0.56 (0.22–1.46)	0.2392
ECOG performance status				
1, 2	1.00	—	1.00	—
0	0.71 (0.43–1.18)	0.1834	0.70 (0.41–1.20)	0.1939
Bone metastasis				
No	1.00	—	1.00	—
Yes	1.45 (0.86–2.42)	0.1615	0.89 (0.51–1.57)	0.6915
Brain metastasis				
No	1.00	—	1.00	—
Yes	2.02 (0.80–5.10)	0.1347	1.11 (0.48–2.58)	0.8102
Type of *EGFR* mutation				
del 19	1.00	—	1.00	—
L858R	1.05 (0.65–1.71)	0.8330	1.41 (0.85–2.34)	0.1805
T790M mutation				
Not detected	1.00	—	1.00	—
Detected	1.36 (0.82–2.26)	0.2279	0.998 (0.59–1.69)	0.9941
TP53 mutations ($n = 82$)				
Not detected	1.00	—	1.00	—
Detected	1.67 (0.94–2.97)	0.0794	1.41 (0.78–2.53)	0.2546
BIM expression ($n = 83$)				
Low/intermediate	1.00	—	1.00	—
High	0.54 (0.31–0.93)	0.0261	0.55 (0.31–0.97)	0.0395

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

0.0261) were associated with longer PFS (Table 2). In the multivariate analysis, treatment with erlotinib (HR = 0.35; 95% CI, 0.20–0.62; $P = 0.0003$) and high *BCL2L11* expression (HR = 0.49; 95% CI, 0.28–0.86; $P = 0.0122$) was again markers of longer PFS.

Survival

Overall survival for all 173 patients included in the EURTAC study did not differ between treatment groups. At January 24, 2013, with a median follow-up of 40.7 months for the erlotinib group and 35.4 months for the chemotherapy group, median survival was 22.9 months in the erlotinib group and 22.1 months in the chemotherapy group (Supplementary Fig. S4).

Survival times were similar (25.8 vs. 23.9 months, respectively) for the 95 patients included in the present study. Importantly, however, the majority of patients had crossed over at the time of progression (Supplementary Table S8). Survival to erlotinib was 20.8 months (95% CI, 8.9–32.5) for those with low/intermediate *BCL2L11* levels, compared with 24.5 months (95% CI, 14-NR) for those with high *BCL2L11* levels, whereas among patients treated with chemotherapy, survival was 20.8 months (95% CI, 15–30.7) for those with low/intermediate *BCL2L11* levels and 35.8 months (95% CI, 18.1-NR) for those with high levels ($P = 0.2037$; Supplementary Fig. S5).

Table 2 shows the univariate analysis for survival. Among the 83 patients in whom *BCL2L11* mRNA expression was analyzed, overall survival was 28.6 months (95% CI, 19.8-NR) for those with high *BCL2L11* expression and 22.1 months (95% CI, 14.7–30.0) for those with low/intermediate *BCL2L11* expression ($P = 0.0364$; Fig.

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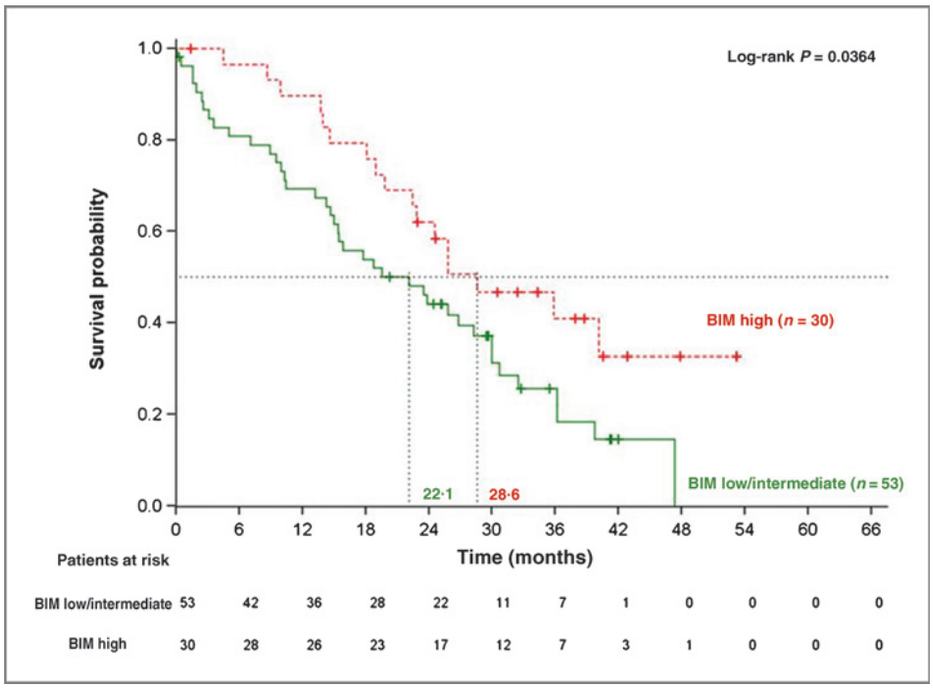

Figure 2. Overall survival in 88 patients according to *BCL2L11* (also known as *BIM*) mRNA expression (low/intermediate vs. high).

2). In the multivariate analysis, only high *BCL2L11* expression (HR = 0.53; 95% CI, 0.30–0.95; P = 0.0323) was associated with longer survival.

Response

At April 11, 2012, the overall response rates in the EURTAC study were 65.1% in the erlotinib group and 16.1% in the chemotherapy group ($P < 0.0001$). Among the 95 patients included in the present study, response rates were 56% and 11.11%, respectively, ($P < 0.0001$). For the 83 patients with data on *BCL2L11* mRNA expression, erlotinib-treated patients with low/intermediate *BCL2L11* levels attained an overall response of 34.6%, compared with 87.5% for those with high *BCL2L11* levels. For patients receiving chemotherapy, overall response was 11.1% and 14.3%, respectively, ($P < 0.0001$). Response according to T790M mutation status is shown in Supplementary Table S9.

In the subgroup of 29 patients with the T790M mutation, the overall response to erlotinib was 80% when *BCL2L11* levels were high and 31.58% when *BCL2L11* levels were low ($P = 0.041$). A similar but nonsignificant difference in response rates was observed in the 13 patients without the T790M mutation (100% vs. 42.86%; $P = 0.07$).

Discussion

Through the analysis of a large prospective clinical cohort, our data establish novel clinical biomarkers and therapeutic targets that could be used to optimize the survival of patients with *EGFR*-mutant NSCLC. Although overall less than 60% of patients responded to erlotinib in the present study, the response rate was an unprecedented 100% in those with high *BCL2L11* mRNA expression and 75% in those without the T790M mutation. Moreover, in patients without the T790M mutation, PFS to erlotinib was 15.8 months, compared with 10.4 months for those with low levels of T790M and 8.7 months for those with high levels. In addition, the multivariate analyses showed that high *BCL2L11* expression was a marker of longer progression-free and overall survival, confirming preliminary data that total *BCL2L11* mRNA expression influences outcome of patients with *EGFR* mutations (12).

In H1650 cells (with an *EGFR* exon 19 deletion and low *BCL2L11* mRNA expression), the combination of gefitinib with the BH3 mimetic ABT-737 increased apoptosis (10). In addition, experiments with pharmacologic inhibitors indicated that blockade of ERK1/2 signaling, but not of phosphoinositide 3-kinase (PI3K), was crucial for *BCL2L11* activation (10). In *EGFR*-mutant cells (9, 11), *BCL2L11* expression is mainly regulated by the ERK pathway, and cross-talk between the ERK and the cyclic AMP (cAMP) pathway may be a key factor in innate resistance to EGFR TKIs. Phosphodiesterases 4 (PDE4) isoforms A and D inhibit protein kinase A (PKA), a main cAMP effector, and activate ERK (ref. 21; Fig. 3). The H1975 cell line, which overexpresses PDE4, was highly sensitive to the PDE4 inhibitor rolipram (21), suggesting that the use of PDE4 inhibitors in conjunction with EGFR TKIs could represent a novel synthetic lethality approach (19).

Although the expression and degradation of *BCL2L11* are regulated mainly by the ERK pathway (9, 10, 19), a variety of other mechanisms can also regulate BCL2L11 function, ranging from transcriptional and posttranscriptional regulation to posttranslational modification and epigenetic silencing (19, 22). For example, an inverse relationship has

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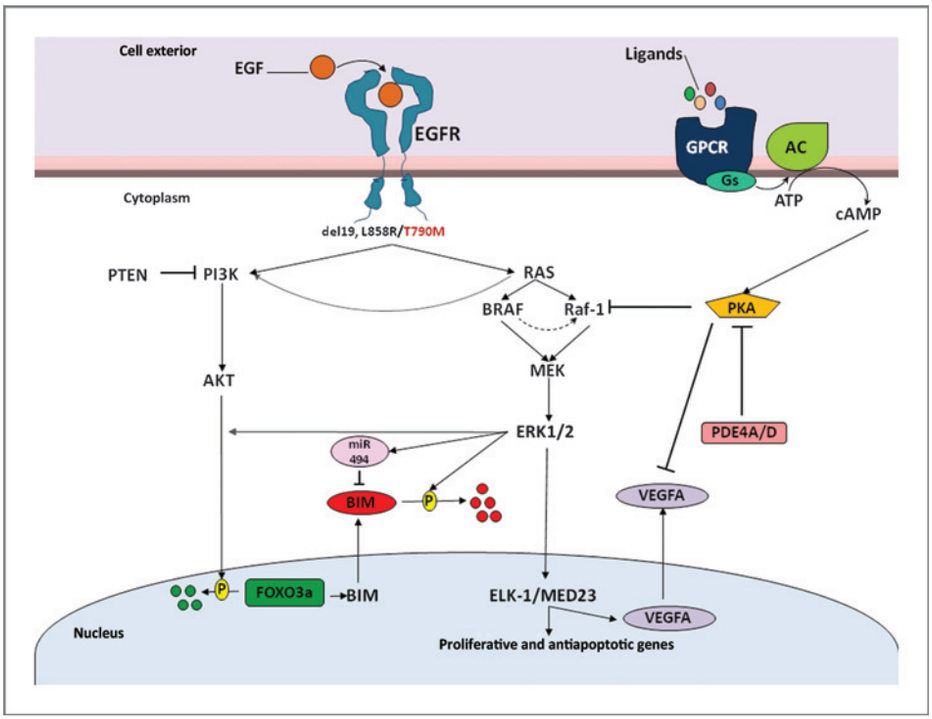

Figure 3. A simplified hypothetical model of cross-talk circuits between signaling pathways that may downregulate *BCL2L11* (also known as *BIM*). EGFR downstream signaling pathways, including the mitogen—activated protein kinase (MAPK) and the PI3K pathway, are activated when the ligand binds to the receptor. ERK and AKT phosphorylate and directly downregulate *FOXO3a*. *FOXO3a* is responsible for the transcriptional upregulation of *BIM*, whereas ERK phosphorylates BIM and promotes BIM degradation posttranscriptionally. The ERK axis induces VEGF-A production. The overexpression of miR-494 through the MAPK pathway can increase resistance to EGFR TKI-induced apoptosis through *BIM* downregulation. Inhibition of the GPCR–cAMP–PKA pathway by PDE4A/D promotes Raf-1 kinase activity, downregulates *BIM*, and suppresses expression of VEGFA. MEK, MAP–ERK kinase.

been reported between miR-494 and *BCL2L11* expression (Fig. 3; ref. 23).

The T790M mutation was detected in 68% to 83% of rebiopsies of *EGFR*-mutant patients progressing to EGFR TKIs (15, 24) and is recognized as an acquired-resistance mutation in *EGFR*-mutant lung cancer (6, 25). However, the coexistence of the T790M mutation before treatment has been underappreciated despite several reports (4, 5, 15, 16). Several techniques have been used to detect the T790M mutation in pretreatment *EGFR*-mutant lung cancer samples with varying results. The mutation was detected in 2% to 3% of samples by Sanger sequencing (7, 26), in 4% by a mutant-enriched PCR assay (7), in 9% by targeted deep sequencing (27), in 31.5% by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (15), and in 79% by colony hybridization (ref. 18; Supplementary Table S7). We found the pretreatment T790M mutation at low dosage in 65% of our *EGFR*-mutant patients, which may be explained by three specific aspects of our method. First, we microdissect our samples to ensure the presence of at least 80% of tumor cells. Second, because the mutation is known to be heterogenously distributed within the tumor tissue, we analyze at least two separate tumor areas. Finally, we use a PNA clamp specifically designed to inhibit the amplification of the wt allele. To the best of our knowledge, only one other study has used the PNA-clamping PCR method for detection of low-level *EGFR* T790M mutations (28).

In the present study, the presence of the T790M mutation was related to shorter PFS. Because the combination of erlotinib with the VEGF inhibitor bevacizumab inhibited the growth of mouse xenograft H1975 tumors (29), the ongoing European phase II BELIEF trial (NCT01562028) is examining the efficacy of erlotinib plus bevacizumab in patients with *EGFR*-mutant NSCLC stratified according to the presence of the T790M mutation as detected by our method. T790M mutant-specific inhibitors are currently used at the time of acquired resistance, when other mechanisms of resistance can also be involved, including epithelial-to-mesenchymal transition (30), which can be induced by overexpression of AXL (31). However, these inhibitors warrant testing as first-line treatment when the pretreatment *EGFR* T790M mutation is detected.

The role of *TP53* mutations was not conclusive in the present study. Although our data indicate that these mutations are not predictive of outcome in patients with *EGFR*-mutant NSCLC, findings in trials of drugs that restore the transcriptional activity of mutant p53 (32) suggest that the impact of *TP53* mutations cannot be ruled out.

In conclusion, in this analysis of pretreatment tumor specimens from a large cohort of patients with *EGFR*-mutant NSCLC, the somatic *EGFR* T790M mutation was frequently detected before therapy, impacting PFS to erlotinib. Importantly, our findings reveal for the first time that *BCL2L11* mRNA expression is a unique biomarker of both progression-free and overall survival. The identification of the signaling pathways leading to abrogation of BCL2L11 can be crucial in the design of combinatory approaches for synthetic lethality and lead to novel therapeutic approaches to prevent innate and acquired resistance to EGFR TKI monotherapy.

Disclosure of Potential Conflicts of Interest

B. Massuti is a consultant/advisory board member for Roche. T.G. Bivona has received a commercial research grant from Daiichi Sankyo and Servier

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Clinical Cancer Research

The Impact of *EGFR* T790M Mutations and *BIM* mRNA Expression on Outcome in Patients with *EGFR*-Mutant NSCLC Treated with Erlotinib or Chemotherapy in the Randomized Phase III EURTAC Trial

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