

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Rosell R, Moran T, Queralt C, et al. Screening for epidermal growth factor receptor mutations in lung cancer. *N Engl J Med* 2009;361:958-67. DOI: 10.1056/NEJMoa0904554.

METHODS

Patients

Chemotherapy-naïve patients or those who had received up to two prior chemotherapy regimens were eligible. Other eligibility criteria included measurable disease by Response Evaluation Criteria in Solid Tumors (RECIST), the availability of sufficient tumor tissue for EGFR mutation analysis, an Eastern Cooperative Oncology Group performance status (PS) of 0-2, and adequate hematologic, renal and hepatic function. Never-smoking was defined as less than 100 cigarettes. Exclusion criteria included active metachronous cancer, pulmonary fibrosis, severe heart disease or pregnancy.

Treatment and evaluation

Patients were treated with 150 mg of daily oral erlotinib continuously until progression or intolerable adverse effects (grade 4 non-hematological toxicities or any incidents of interstitial pneumonia). Dose interruptions were recommended for the initial management of treatment-related toxicities; in the case of recurrent toxicity, a dose reduction to 100 mg was allowed. Responses were categorized per RECIST and reported as best response achieved per patient. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria (version 3.0).

Cell culture

The PC-9 lung tumor cell line was kindly provided by Roche (Basel, Switzerland); the H1975 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). All tissue culture materials were obtained from Biologic Industries (Kibbutz Beit Haemek, Israel) or Invitrogen (Paisley, Scotland, UK).

Tissue samples and microdissection of tumor cells

Paraffin-embedded samples and slides were obtained by standard procedures. Fresh specimens were extended over an appropriate slide, fixed with 96% ethyl-alcohol and stained with Harris haematoxylin for 1 minute. Once the specimen was stained and rinsed in running water, a cover slide was placed over it to observe and mark the presence of malignant cells. Later, the cover slide could be removed and the sample kept in this stage for not more than 2 or 3 days. Tumor cells were identified by a

pathologist. For both fresh and paraffin-embedded samples, tumor cells were captured by laser microdissection (Palm, Oberlensheim, Germany) into extraction buffer (Ecogen, Barcelona, Spain), containing proteinase K, and incubated 4 hours to overnight at 60°C. In samples containing more than 400 tumor cells, microdissection was performed in 200 µl of buffer and DNA was extracted with phenol:chloroform and ethanol precipitation.¹ In samples containing less than 400 tumor cells, cells were microdissected into 10 µl of PCR buffer (Ecogen, Barcelona, Spain) plus proteinase K.² Proteinase K was inactivated at 95°C for 10 minutes, and the cell extract submitted directly to PCR.

Blood samples

Blood (15 mL) was collected from patients in three Vacutainer tubes (Becton Dickinson, Plymouth, UK), two for serum and one for plasma. Tubes were centrifuged twice at 2300 rpm for 10 min and the supernatant (serum or plasma) aliquoted. DNA was purified from 0.4 mL of serum or plasma by standard procedures, using the QIAamp DNA Blood Mini Kit (Qiagen), and resuspended in 40 µL of water. For each patient, DNA extraction and mutation analysis was performed per quadruplicate in two samples of serum and two samples of plasma.

For both blood and tissue samples, DNA from the cell line PC-9 was used as a mutated control for exon 19, and wild-type control for exons 20 and 21. DNA from the H1975 cell line was used as a wild-type control for exon 19, and mutated control for exons 20 and 21.

Laboratory procedures

In the case of DNA purified from tumor samples, EGFR status was determined by length analysis of fluorescently labeled PCR products (for exon 19) and TaqMan assay (for exons 20 and 21), using 2 µL of the DNA solution. All mutants were confirmed by PCR followed by DNA sequencing.¹

Extracts from samples containing a small quantity of tumor cells were submitted to a nested PCR followed by sequencing, using 2 µL of sample. Length analysis and Taqman assay were performed on 2 µL of the 1st PCR products.²

Finally, in the case of DNA from blood samples, exon 19 status was determined by length analysis, using a labelled primer, in presence of a PNA clamp designed to inhibit

the amplification of the wild type DNA. For exon 21 mutation, a PNA was added to the Taqman assay (method patent pending).

Length analysis of fluorescently labelled PCR products for EGFR deletions in exon 19

In tissue samples, amplification was performed with the following primers: forward 5'-ACTCTGGATCCCAGAAGGTGAG-3' and reverse 5'-FAM-CCACACAGCAAAGCAGAACTC-3'. Amplification was done for 30 seconds at 94°C, 30 seconds at 58°C, and 1 minute at 72°C in 50-µl volumes using 1 U of Ecotaq Polymerase, 250 µM dNTPs, 1 mM MgCl₂, and 0.5 pmol of each primer. One microliter of a 1/50 to 1/200 dilution of each PCR product was mixed with 0.5 µl of size standard (Applied Biosystems) and denatured in 9 µl formamide at 90°C for 5 minutes. Separation was done with a four-color laser-induced fluorescence capillary electrophoresis system (ABI Prism 3130 Genetic Analyzer, Applied Biosystems). The collected data were evaluated with the GeneScan Analysis Software (Applied Biosystems, Norwalk, CT). The number of cycles was as follows: 40 for DNA purified from tumor tissues, and 32 for the 1st PCR product from tissue samples with few tumor cells. In blood samples, PCR amplification was performed with the above-mentioned primers and the addition of a PNA clamp.

TaqMan assay for EGFR mutation in exons 20 (T790M) and 21 (L858R)

In tissue samples, primers and probes were as follows: exon 21 (forward primer, 5'-AACACCGCAGCATGTCAAGA-3', reverse primer 5'-TTCTCTTCCGCACCCAGC-3'; probes 5'-FAM-CAGATTTTGGGCGGGCCAAAC-TAMRA-3'; and 5'-VIC-TCACAGATTTTGGGCTGGCCAAAC-TAMRA-3') and exon 20 (forward primer, 5'-AGGCAGCCGAAGGGCA-3', reverse primer 5'-CCTCACCTCCACCGTGCA-3'; probes 5' VIC-TGAGCTGCGTGATGA-MGB-3'; and 5'-FAMTGAGCTGCATGATGA- MGB-3). Amplification was performed in 25-µl volumes using 2 µl of first PCR product, 12.5 µl of Ampli Taq Gold PCR Master Mix (Applied Biosystems), 0.6 pmol of each primer and 0.2 pmol of probes. Samples were submitted to 40 cycles of 15 seconds at 94°C and 1 minute at 60°C in an Applied Biosystems 7000 real-time cycler.

In blood samples, PCR amplification was performed with the above-mentioned primers and the addition of a PNA clamp.

Nested PCR and EGFR gene sequencing

Primers were as follows: exon 19 (first PCR, forward 5'-GCAATATCAGCCTTAGGTGCGGCTC- 3', and reverse 5'-CATAGAAAGTGAACATTTAGGATGTG- 3'; second PCR, forward 5'-GTGCATCGCTGGTAACATCC-3' and reverse 5'-TGTGGAGATGAGCAGGGTCT-3'); exon 21 (first PCR, forward 5'-CTAACGTTCCGCCAGCCATAAGTCC-3' and reverse 5'-GCTGCGAGCTACCCAGAATGTCTGG-3', second PCR, forward 5'-GCTCAGAGCCTGGCATGAA-3' and reverse 5'-CATCCTCCCCTGCATGTGT-3'); exon 20 (first PCR, forward 5'-ACTTCACAGCCCTGCGTAAAC-3' and reverse 5'-ATGGGACAGGCACTGATTTGT-3', second PCR, forward 5'-ATCGCATTCATGCGTCTTCA-3' and reverse 5'-ATCCCCATGGCAAACCTCTTG-3').

The first PCR was performed in 50- μ l volumes adding 2 μ l of sample, 2 U of Ecotaq Polymerase (Ecogen, Barcelona, Spain), 7.5 μ l of PCR buffer x10, 250 μ M dNTPs, 3.5 mM MgCl₂, and 0.5 pmol of each primer. Amplification was as follows: 20 cycles (for DNA purified from samples) or 25 cycles (for extracts from tumor cells) of 30 seconds at 94°C, 30 seconds at 64°C, and 1 minute at 72°C (exons 19 and 21), or 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 1 minute at 72°C (exon 20). For the nested PCR, amplification was done using 2 μ l (for exons 19 and 20) or 4 μ l (for exon 21) of first PCR product, 1.25 U of Ecotaq Polymerase, 250 μ M dNTPs, 1.5 mM MgCl₂, and 0.5 pmol of each primer. Cycles were as follows: for exon 19, 35 cycles of 30 seconds at 94°C, 30 seconds at 64°C, and 1 minute at 72°C; for exon 21, 35 cycles (for DNA purified from samples) or 40 cycles (for extracts from tumor cells) of 30 seconds at 94°C, 30 seconds at 64°C, and 1 minute at 72°C; for exon 20, 20 cycles of 30 seconds at 94°C, 30 seconds at 59°C, and 1 minute at 72°C .

PCR products were visualized on a 2% agarose gel. Sequencing was performed by standard procedures, using forward and reverse primers and the ABI Prism 3130 DNA Analyzer (Applied Biosystems, Foster City, CA).

RESULTS

Progression-free and overall survival

Differences in progression-free survival (PFS) were observed according to gender in patients harboring the L858R mutation; PFS was 16 months (95% CI, 10.3 to 21.7 months) in women vs. 6 months (95% CI, 3.2 to 8.8 months) in men ($P=0.004$) (Fig. 1C in the Supplementary Appendix). No differences in PFS were seen in patients with L858R mutations according to other characteristics, including age (Fig. 1E in the Supplementary Appendix). Differences in PFS were also observed according to gender in patients with PS 1; PFS was 16 months (95% CI, 11.6 to 20.4 months) in women vs. 8 months (2.6 to 13.4 months) in men ($P=0.03$) (Fig. 1G in the Supplementary Appendix).

Median survival for patients with PS 0 was not reached, while it was 23 months (95% CI, 19.7 to 23.3) for patients with PS 1 and 32 months (95% CI, 11.3 to 52.7 months) for patients with PS 2 ($P=0.03$) (Fig. 1D in the Supplementary Appendix). Differences in median survival were observed according to age in patients having the L858R mutation. Median survival was 29 months (95% CI, 3.3 to 54.6 months) for patients younger than 60 years, 27 months (95% CI, 21.1 to 32.9 months) for those between 60 and 70 years and 16 months (95% CI, 12.9 to 19 months) for those older than 70 years ($P=0.01$, Tarone-Ware $P=0.008$) (Fig. 1F in the Supplementary Appendix). Differences were observed according to gender in PS 1 patients; median survival was 28 months (95% CI, 23.9 to 32 months) in women vs. 16 months (95% CI, 9.3 to 22.7 months) in men ($P=0.01$) (Fig. 1H in the Supplementary Appendix).

There were no differences in the distribution of metastases according to type of mutation, gender, smoking status, PS, or first- vs. second- or third-line erlotinib (data not shown). Patients younger than 60 years had a higher frequency of bone metastases (27 patients [40.9%]) than those 60-70 years (19 patients [25.7%]) or those older than 70 years (13 patients [17.3%]) ($P=0.007$) (data not shown). PFS was 14 months (95% CI, 11.3 to 16.7 months) for patients without brain metastases and 10 months (95% CI, 5.6 to 14.4 months) for those with brain metastases ($P=0.31$). Median survival was 28 months (95% CI, 21.5 to 34.4 months) for patients without brain metastases and 18 months (95% CI, 4 to 31.9 months) for patients with brain metastases ($P=0.008$). PFS was 16 months (95% CI, 11.8 to 20.2) for patients without bone metastases and 11

months (95% CI, 8.1 to 13.9 months) for patients with bone metastases (P=0.05).

Median survival was 29 months (95% CI, 25.4 to 32.6 months) for patients without bone metastases and 20 months (95% CI, 16.5 to 23.5 months) for patients with bone metastases (P=0.07).

Progression-free and overall survival according to response

PFS was not reached for the 24 patients who attained CR, while it was 15 months (95% CI, 12.2-17.8 months) for the 115 patients with PR, 9 months (95% CI, 5.9 to 12.1 months) for the 38 patients with SD, and 2 months (95% CI, 0 to 4.2 months) for the 20 patients with PD (P<0.001)(Fig. 2A in the Supplementary Appendix). For patients with CR, median survival was not reached and 3-year survival was 58.7%. For patients with PR, median survival was 28 months (95% CI, 21.7 to 34.3 months) and 3-year survival was 32.5%. For patients with SD, median survival was 18 months (95% CI, 10.6 to 25.4 months) and 3-year survival was 0%. For patients with PD, median survival was 9 months (95% CI, 6.6. to 11.4 months) (P < 0.001) (Fig. 2B in the Supplementary Appendix).

T790M at the time of disease progression

The T790M resistance mutation was assessed in 35 patients at the time of progression – eight in tumor rebiopsies and 27 in serum DNA drawn at the time of progression. Four patients harbored the T790M mutation in tumor and eleven in serum.

DISCUSSION

We plan to perform further genetic analyses on available remaining tumor samples in order to better understand the phenomena involved in progression-free survival.

T790M resistant mutation

The threonine-to-methionine substitution at amino acid position 790 (T790M) of the EGFR has been described in progressing non-small-cell lung cancers with acquired resistance to gefitinib or erlotinib.^{3,4} This mutation is analogous to an acquired resistant mutation in chronic myeloid leukemia, a threonine-to-isoleucine mutation at codon 315 (T315I) in the ABL kinase domain.⁵ In addition, the T790M confers enhanced kinase activity to primary activating EGFR alleles.⁶ Two studies of patients rebiopsied after progression identified the T790M mutation in almost 50% of cases. None of the tumors had K-ras mutations and the T790M was not present in the pre-treatments specimens.^{7,8} Intriguingly, however, Inukai et al discovered the T790M mutation in three of seven non-responders with gefitinib-sensitive EGFR mutations but in none of 19 responders.⁹ These findings suggest that the T790M mutation is sometimes present in a minor population of tumor cells and the detection of a small proportion of T790M mutant alleles could be useful for predicting gefitinib resistance of non-small-cell lung cancer with sensitive EGFR mutations.⁹ Equally tantalizing was the fact that the T790M mutation was detected in two tumors with K-ras mutations and in four with wild-type EGFR.⁹ Recently, a low proportion of T790M was detected in pretreatment tumor samples from 10 of 26 patients (38%). Again, the relatively high number of amplification cycles that were required to detect T790M indicates that the mutation is present in only a small number of cells.¹⁰ The presence of the resistance mutation at such a low frequency did not preclude significant responses to TKIs among patients with EGFR-mutant tumors, but it was associated with a striking difference in PFS, with a median of 7.7 months in patients with a detectable T790M allele, compared to 16.5 months in those without a detectable allele (hazard ratio for progression for the T790M allele, 11.5, $P < 0.001$).¹⁰

BIM expression

A common signaling cascade has been proposed for EGFR-mutant tumors and chronic myeloid leukemia.^{5, 11} For example, the BCR-ABL tyrosine kinase increases phosphorylation and transactivation of STAT5, which leads to increased expression of the antiapoptotic BCL-XL, Ras-Raf-ERK and AKT. Through deregulated AKT activity, BCR-ABL inhibits the forkhead transcription regulator FOXO3a, which leads to depletion of the cyclin-dependent kinase-2 inhibitor p27 and the BH3 domain-only-containing proapoptotic BIM protein.¹² Furthermore, BIM mediates imatinib-induced apoptosis of BCR-ABL leukemic cells,¹³ and gefitinib or erlotinib dramatically induces BIM levels in sensitive but not in resistant cell lines.^{14, 15} BIM was induced after erlotinib in drug-sensitive H3255 (L858R) and PC9 (del 19) cells but not in the drug-resistant H1650 (del19)¹⁶ or H1975 (L858 plus T790M)¹⁵ cells. No BIM upregulation was observed in A549 (EGFR wild-type and K-ras mutation) possibly because of sustained phosphorylation of AKT/ERK.¹⁴ Transcriptome analysis has revealed upregulation of BIM and p27 as well as downregulation of antiapoptotic MCL1, cyclin D1 and heat shock (HSP90) proteins in H1975 cells following effective treatment with a pan-inhibitor of tyrosine kinases.¹⁷

The role of MET

In addition to EGFR, ERBB2 and MET are hyperphosphorylated in human epithelial cells (HBECs) expressing mutant EGFR and in the adenocarcinoma cell line H1650, suggesting a role for lateral signaling or crosstalk between various receptor tyrosine kinases. IGF1R is also more activated in EGFR-mutant cells than in cells with wild-type EGFR or mutant K-ras.¹⁸ MET amplification was found in 22% of lung cancer samples that became resistant to gefitinib or erlotinib.^{19, 20} Four of nine EGFR-resistant tumors with MET amplification harbored the EGFR T790M mutation.²⁰ In addition, high levels of immunoreactivity for hepatocyte growth factor (HGF) are involved in resistance to gefitinib in lung adenocarcinomas harboring EGFR mutations. HGF reactivates the phosphatidylinositol-3-kinase/AKT signaling pathway via phosphorylation of MET.²¹ Only low levels of HGF immunoreactivity were detected in cancer cells of responding tumors, while high levels were detected in cancer cells of intrinsic resistant tumors and in one of two tumors with acquired resistance with neither the T790M mutation nor MET amplification.²¹

REFERENCES

1. Taron M, Ichinose Y, Rosell R, et al. Activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor are associated with improved survival in gefitinib-treated chemorefractory lung adenocarcinomas. *Clin Cancer Res* 2005;11(16):5878-85.
2. Molina-Vila MA, Bertran-Alamillo J, Reguart N, et al. A sensitive method for detecting EGFR mutations in non-small cell lung cancer samples with few tumor cells. *J Thorac Oncol* 2008;3(11):1224-35.
3. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005;352(8):786-92.
4. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2005;2(3):e73.
5. Carter TA, Wodicka LM, Shah NP, et al. Inhibition of drug-resistant mutants of ABL, KIT, and EGF receptor kinases. *Proc Natl Acad Sci U S A* 2005;102(31):11011-6.
6. Mulloy R, Ferrand A, Kim Y, et al. Epidermal growth factor receptor mutants from human lung cancers exhibit enhanced catalytic activity and increased sensitivity to gefitinib. *Cancer Res* 2007;67(5):2325-30.
7. Kosaka T, Yatabe Y, Endoh H, et al. Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and acquired resistance to gefitinib. *Clin Cancer Res* 2006;12(19):5764-9.
8. Balak MN, Gong Y, Riely GJ, et al. Novel D761Y and common secondary T790M mutations in epidermal growth factor receptor-mutant lung adenocarcinomas with acquired resistance to kinase inhibitors. *Clin Cancer Res* 2006;12(21):6494-501.
9. Inukai M, Toyooka S, Ito S, et al. Presence of epidermal growth factor receptor gene T790M mutation as a minor clone in non-small cell lung cancer. *Cancer Res* 2006;66(16):7854-8.
10. Maheswaran S, Sequist LV, Nagrath S, et al. Detection of mutations in EGFR in circulating lung-cancer cells. *N Engl J Med* 2008;359(4):366-77.
11. Sharma SV, Gajowniczek P, Way IP, et al. A common signaling cascade may underlie "addiction" to the Src, BCR-ABL, and EGF receptor oncogenes. *Cancer Cell* 2006;10(5):425-35.
12. Fiskus W, Pranpat M, Balasis M, et al. Cotreatment with vorinostat (suberoylanilide hydroxamic acid) enhances activity of dasatinib (BMS-354825) against imatinib mesylate-sensitive or imatinib mesylate-resistant chronic myelogenous leukemia cells. *Clin Cancer Res* 2006;12(19):5869-78.
13. Kuroda J, Puthalakath H, Cragg MS, et al. Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic. *Proc Natl Acad Sci U S A* 2006;103(40):14907-12.
14. Costa DB, Halmos B, Kumar A, et al. BIM mediates EGFR tyrosine kinase inhibitor-induced apoptosis in lung cancers with oncogenic EGFR mutations. *PLoS Med* 2007;4(10):1669-79; discussion 80.
15. Gong Y, Somwar R, Politi K, et al. Induction of BIM Is Essential for Apoptosis Triggered by EGFR Kinase Inhibitors in Mutant EGFR-Dependent Lung Adenocarcinomas. *PLoS Med* 2007;4(10):e294.

16. Deng J, Shimamura T, Perera S, et al. Proapoptotic BH3-only BCL-2 family protein BIM connects death signaling from epidermal growth factor receptor inhibition to the mitochondrion. *Cancer Res* 2007;67(24):11867-75.
17. de La Motte Rouge T, Galluzzi L, Olaussen KA, et al. A novel epidermal growth factor receptor inhibitor promotes apoptosis in non-small cell lung cancer cells resistant to erlotinib. *Cancer Res* 2007;67(13):6253-62.
18. Guha U, Chaerkady R, Marimuthu A, et al. Comparisons of tyrosine phosphorylated proteins in cells expressing lung cancer-specific alleles of EGFR and KRAS. *Proc Natl Acad Sci U S A* 2008;105(37):14112-7.
19. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007;316(5827):1039-43.
20. Bean J, Brennan C, Shih JY, et al. MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. *Proc Natl Acad Sci U S A* 2007;104(52):20932-7.
21. Yano S, Wang W, Li Q, et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. *Cancer Res* 2008;68(22):9479-87.

Table 1 (Supplementary Appendix). Patient characteristics according to gender (N=217)

	Female N=158 N (%)	Male N=59 N (%)	p
Age, median (range)	68 (22-86)	65 (33-86)	0.18
Race			0.81
African & Asian	3 (1.9)	1 (1.7)	
Caucasian	155 (98.1)	58 (98.3)	
Smoking History			<0.0001
Ex-smoker	22 (13.9)	34 (57.6)	
Current smoker	6 (3.8)	7 (11.9)	
Never-smoker	130 (82.3)	18 (30.5)	
ECOG PS			0.34
0	41 (25.9)	10 (16.9)	
1	89 (56.3)	39 (66.2)	
≥2	28 (17.8)	10 (16.9)	
Histology			0.47
Adeno	131 (82.9)	45 (76.3)	
BAC	16 (10.1)	6 (10.2)	
LCC	11 (7)	8 (13.5)	
Stage			0.40
IIIB	8 (5.1)	4 (6.8)	
IV	150 (94.9)	55 (93.2)	
Erlotinib Treatment Line			0.36
First	86 (54.4)	27 (45.8)	
Second	72 (45.6)	32 (54.2)	
EGFR Mutation			0.90
del 19	98 (62)	37 (62.7)	
L858R	60 (38)	22 (37.2)	
Response			0.35
CR	20 (13.7)	4 (7.8)	
PR	85 (58.2)	30 (58.8)	
CR+PR	105 (71.9) (95%CI:64.1-78.6)	34 (66.7) (95%CI:52.8-78.1)	
SD	29 (19.9)	9 (17.6)	
PD	12 (8.2)	8 (15.7)	
NE	12	8	
Response			0.48
CR+PR	105 (71.9)	34 (66.7)	
SD+PD	41 (28.1)	17 (33.3)	
EGFR Mutation in Serum	74 (61.7)	23 (52.3)	0.28

Adeno=adenocarcinoma; BAC=bronchioloalveolar adenocarcinoma; LCC=large-cell carcinoma

Table 2 (Supplementary Appendix). Patient characteristics according to smoking history

	Ex-smoker N=56 N (%)	Current Smoker N=13 N (%)	Never-smoker N=148 N (%)	p
Age, median (range)	57 (26-88)	52 (22-78)	70 (40-85)	<0.0001
Gender				<0.0001
Male	34 (60.7)	7 (53.8)	18 (12.2)	
Female	22 (39.3)	6 (46.2)	130 (87.8)	
Race				0.53
African & Asian	2 (3.6)	0(0)	2 (1.3)	
Caucasian	54 (96.4)	13 (100)	146 (98.7)	
ECOG PS				0.86
0	12 (21.5)	4 (30.7)	35 (23.6)	
1	33 (58.9)	7 (53.8)	86 (58.2)	
≥2	11 (19.6)	2 (15.5)	27 (18.2)	
Histology				0.75
Adeno	44 (78.6)	13 (100)	119 (80.5)	
BAC	6 (10.7)	0 (0)	16 (10.8)	
LCC	6 (10.7)	0 (0)	13 (8.7)	
Stage				0.63
IIIB	2 (3.6)	0 (0)	10 (6.7)	
IV	54 (96.4)	13 (100)	138 (93.3)	
Erlotinib Treatment Line				0.24
First	27 (48.2)	4 (30.8)	80 (54.4)	
Second	29 (51.8)	9 (69.2)	68 (45.6)	
EGFR Mutation				0.24
del 19	38 (67.8)	10 (76.9)	87 (58.8)	
L858R	18 (32.2)	3 (23.1)	61 (41.2)	
Response				0.79
CR	5 (10.4)	1 (9.1)	18 (13)	
PR	25 (52.1)	6 (54.5)	84 (60.9)	
SD	12 (25)	2 (18.2)	24 (17.4)	
PD	6 (12.5)	2 (18.2)	12 (8.7)	
NE	8	2	10	
Response				0.28
CR+PR	30 (62.5) (95%CI: 48.2-74.9)	7 (63.6) (95%CI: 35.1-85)	102 (73.9) (95%CI: 65.9-80.6)	
SD+PD	18 (37.5)	4 (36.4)	36 (26.1)	
EGFR Mutation in Serum	24 (61.5)	8 (72.7)	65 (57)	0.56

Adeno=adenocarcinoma; BAC=bronchioloalveolar adenocarcinoma; LCC=large-cell carcinoma

Table 3 (Supplementary Appendix). Patient characteristics according to age

	≤60 years N=68 N (%)	60-70 years N=74 N (%)	>70 years N=75 N (%)	p
Gender				0.18
Male	24 (35.3)	18 (24.3)	17 (22.7)	
Female	44 (64.7)	56 (75.7)	58 (77.3)	
Race				0.06
African & Asian	4 (5.9)	0 (0)	0 (0)	
Caucasian	64 (94.1)	74 (100)	75 (100)	
Smoking History				<0.0001
Ex-smoker	29 (42.6)	16 (21.6)	11 (14.7)	
Current smoker	9 (13.2)	2 (2.7)	2 (2.6)	
Never-smoker	30 (44.2)	56 (75.7)	62 (82.7)	
ECOG PS				0.21
0	20 (30.3)	20 (27.4)	11 (14.2)	
1	35 (53)	42 (57.5)	48 (64)	
≥2	11 (16.7)	11 (15.1)	16 (21.3)	
Histology				0.42
Adeno	60 (88.3)	60 (81.2)	56 (74.7)	
BAC	6 (8.8)	7 (9.4)	9 (12)	
LCC	2 (2.9)	7 (9.4)	10 (13.3)	
Stage				0.89
IIIB	5 (7.4)	3 (4)	4 (5.3)	
IV	63 (92.6)	71 (96)	71 (94.7)	
Erlotinib Treatment Line				0.002
First	23 (33.8)	44 (59.4)	46 (61.3)	
Second	45 (66.2)	30 (40.6)	29 (38.7)	
EGFR Mutation				<0.0001
del 19	57(83.8)	42 (56.8)	36 (48)	
L858R	11(16.2)	32 (43.2)	39 (52)	
Response				0.21
CR	7 (11.7)	10 (14.5)	7 (10.3)	
PR	31 (51.7)	46 (66.7)	38 (55.9)	
SD	14 (23.3)	11 (15.9)	13 (19.1)	
PD	8 (13.3)	2 (2.9)	10 (14.7)	
NE	18	5	7	
Response				0.05
CR+PR	38 (63.4) (95%CI:50.6-74.5)	56 (81.2) (95%CI:70.3-88.7)	45 (66.2) (95%CI:54.2-76.4)	
SD+PD	22 (36.6)	13 (18.8)	23 (33.8)	
EGFR Mutation in Serum	29 (61.7)	30 (52.6)	38 (63.3)	0.46

Adeno=adenocarcinoma; BAC=bronchioloalveolar adenocarcinoma; LCC=large-cell carcinoma

Table 4 (Supplementary Appendix). Patient characteristics according to ECOG PS

	ECOG 0 N=51 N (%)	ECOG 1 N=128 N (%)	ECOG ≤2 N=38 N (%)	p
Age, median (range)	62 (22-85)	70 (35-88)	69 (33-86)	0.03
Gender				0.34
Male	10 (19.6)	39 (29.7)	10 (26.3)	
Female	41 (80.4)	89 (70.3)	28 (73.7)	
Race				0.21
African & Asian	0 (0)	2 (1.6)	2 (5.3)	
Caucasian	51 (100)	126 (98.4)	36 (94.7)	
Smoking History				0.86
Ex-smoker	12 (23.5)	33 (25.8)	11 (28.9)	
Current smoker	4 (7.8)	8 (6.2)	1 (2.6)	
Never-smoker	35 (68.7)	87 (68)	26 (68.5)	
Histology				0.86
Adeno	42 (82.3)	105 (82)	29 (76.4)	
BAC	6 (11.8)	12 (9.4)	4 (10.5)	
LCC	3 (5.8)	11 (8.6)	5 (13.1)	
Stage				0.51
IIIB	3 (5.9)	8 (6.3)	1 (2.6)	
IV	48 (94.1)	120 (93.7)	37 (97.4)	
Erlotinib Treatment Line				0.44
First	23 (45.1)	68 (53.1)	22 (57.9)	
Second	28 (54.9)	60 (46.9)	16 (42.1)	
EGFR Mutation				0.86
del 19	33 (64.7)	79 (61.7)	23 (60.5)	
L858R	18 (35.3)	49 (38.3)	15 (39.5)	
Response				0.23
CR	9 (20.4)	11 (9)	4 (12.9)	
PR	27 (61.4)	71 (58.2)	17 (54.8)	
SD	6 (13.6)	24 (19.7)	8 (25.8)	
PD	2 (4.6)	16 (13.1)	2 (6.5)	
NE	7	6	7	
Response				0.12
CR+PR	36 (81.8) (95%CI:67.5-90.5)	82 (67.2) (95%CI:58.4-75)	21 (67.7) (95%CI:39.6-70)	
SD+PD	8 (18.2)	38 (32.8)	10 (32.3)	
EGFR Mutation in Serum	15 (44.1)	61 (58.7)	21 (80.8)	0.02

Adeno=adenocarcinoma; BAC=bronchioloalveolar adenocarcinoma; LCC=large-cell carcinoma

Table 5 (Supplementary Appendix). Patient characteristics according to treatment line

	First-Line N=113 N (%)	Second-Line N=104 N (%)	p
Age, median (range)	70 (26-88)	62 (22-85)	<0.0001
Gender			0.20
Male	27 (23.9)	32 (30.8)	
Female	86 (76.1)	72 (69.2)	
Race			0.14
African & Asian	4 (3.5)	0 (0)	
Caucasian	109 (96.5)	104 (100)	
Smoking History			0.24
Ex-smoker	28 (24.8)	28 (26.9)	
Current smoker	4 (3.5)	9 (8.7)	
Never-smoker	81 (71.7)	67 (64.4)	
ECOG PS			0.44
0	23 (20.3)	28 (26.9)	
1	68 (60.2)	60 (57.7)	
≥2	22 (19.5)	16 (15.4)	
Histology			0.11
Adeno	88 (77.9)	88 (84.6)	
BAC	15 (13.3)	7 (6.7)	
LCC	10 (8.8)	9 (8.7)	
Stage			0.05
IIIB	6 (5.3)	6 (5.8)	
IV	107 (94.7)	98 (94.2)	
EGFR Mutation			0.57
del 19	68 (60.2)	67 (64.4)	
L858R	45 (39.8)	37 (35.6)	
Response			0.70
CR	13 (12.7)	11 (11.6)	
PR	62 (60.8)	53 (55.8)	
SD	19 (18.7)	19 (20)	
PD	8 (7.8)	12 (12.6)	
NE	11	9	
Response			0.35
CR+PR	75 (73.5) (95%CI:64.1-81.2)	64 (67.4) (95%CI:57.3-76)	
SD+PD	27 (26.5)	31 (32.6)	
EGFR Mutation in Serum	49 (57.6)	48 (61.5)	0.63

Adeno=adenocarcinoma; BAC=bronchioloalveolar adenocarcinoma; LCC=large-cell carcinoma

Table 6 (Supplementary Appendix). Metastatic sites

	N (%)
Lung	142 (66)
Lymph nodes	54 (25.1)
Pleura	43 (20.1)
Skin	3 (1.4)
Brain	30 (13.8)
Bone	59 (27.4)
Liver	20 (9.3)
Pericardium	4 (1.8)
Adrenal	6 (2.7)
Median no. metastases	2 (1-6)

Table 7 (Supplementary Appendix). Toxicities

	All Toxicities N (%)	Grade 3-4 Toxicities N (%)
Skin	151 (69.6)	16 (7.4)
Diarrhea	95 (43.8)	8 (3.7)

Supplementary Figure 1. Progression-free and overall survival in subgroups of patients.

1A. Progression-free survival according to gender (blue, female; red, male)

1B. Survival according to gender (blue, female; red, male)

1C. Progression-free survival in patients with L858R according to gender (blue, female; green, male)

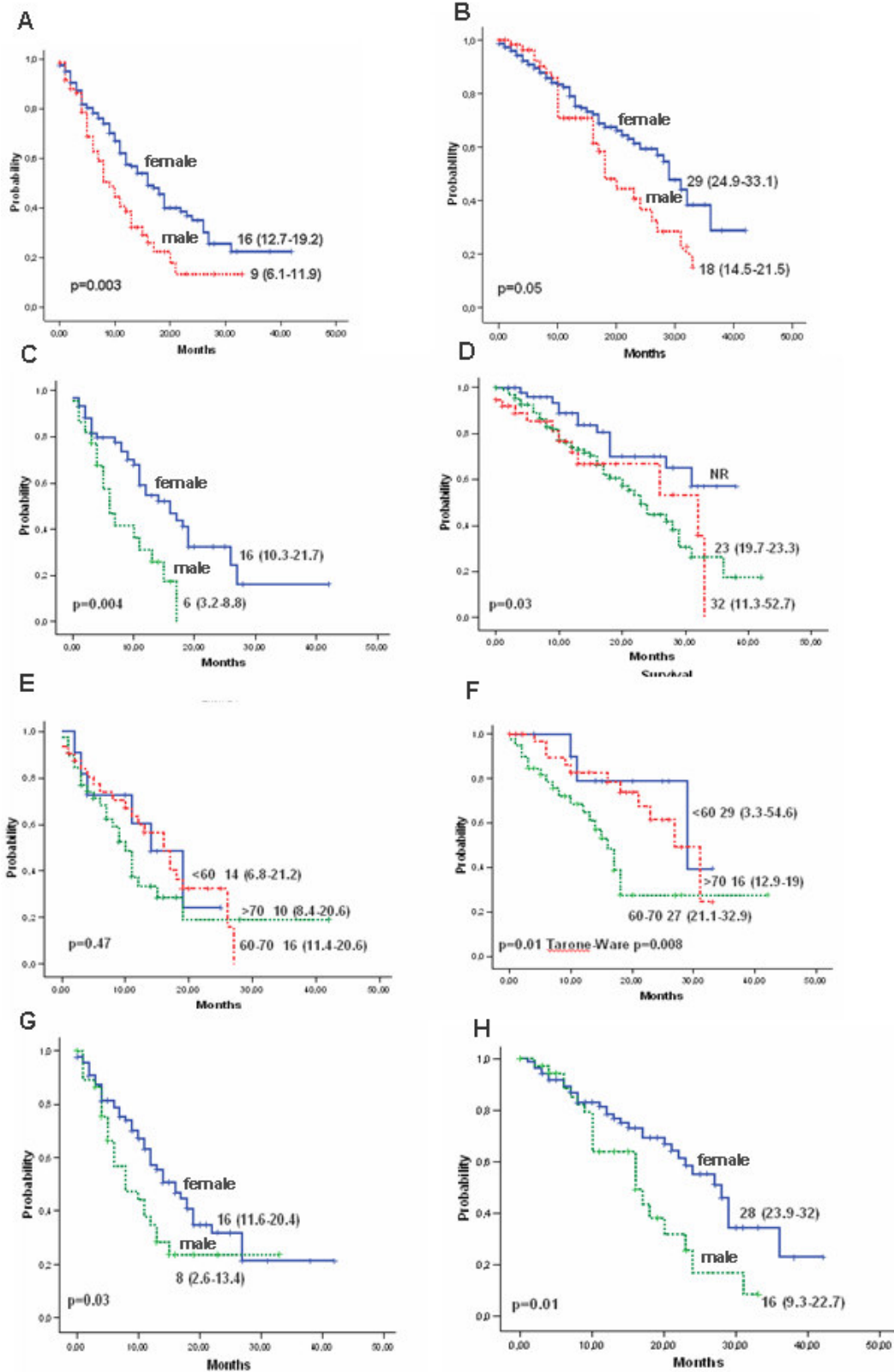
1D. Survival according to ECOG PS (blue, PS0; red, PS1; green, PS2)

1E. Progression-free survival in patients with L858R according to age (blue, <60; green, 60-70; red, >70)

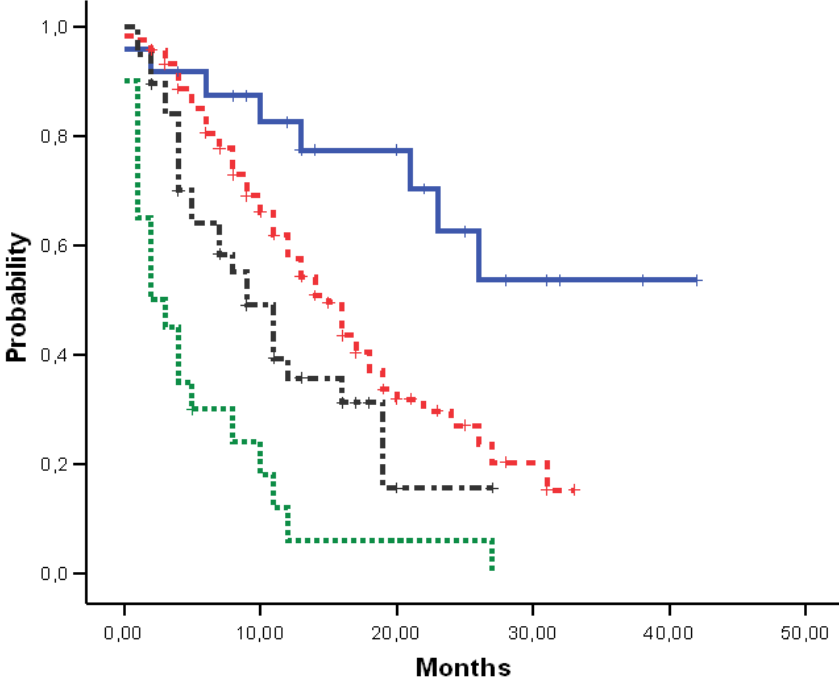
1F. Survival in patients with L858R according to age (blue, <60; green, 60-70; red, >70)

1G. Progression-free survival in PS1 patients according to gender (blue, female; green, male)

1H. Survival in PS1 patients according to gender (blue, female; green, male)

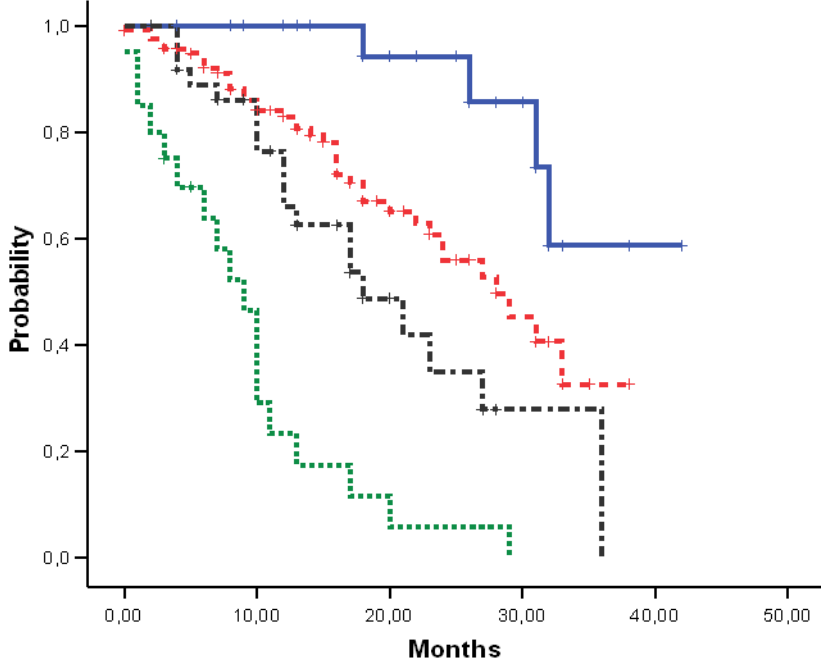


Supplementary Figure 2. Progression-free (2A) and overall (2B) survival according to response (blue, CR; red, PR; brown, SD; green, PD)



Patients at Risk

CR	24	17	11	5	1
PR	115	68	17	4	0
SD	38	14	1	0	0
PD	20	3	1	0	0



Patients at Risk

CR	24	24	14	7	1
PR	115	84	32	10	0
SD	38	26	7	1	0
PD	20	7	1	0	0