Activating Mutations in the Tyrosine Kinase Domain of the Epidermal Growth Factor Receptor Are Associated with Improved Survival in Gefitinib-Treated Chemorefractory Lung Adenocarcinomas

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Abstract

Purpose: Activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) confer a strong sensitivity to gefitinib, a selective tyrosine kinase inhibitor of EGFR.

Experimental Design: We examined *EGFR* mutations at exons 18, 19, and 21 in tumor tissue from 68 gefitinib-treated, chemorefractory, advanced non – small cell lung cancer patients from the United States, Europe, and Asia and in a highly gefitinib-sensitive non – small cell lung cancer cell line and correlated their presence with response and survival. In addition, in a subgroup of 28 patients for whom the remaining tumor tissue was available, we examined the relationship among *EGFR* mutations, CA repeats in intron 1 of *EGFR*, *EGFR* and *caveolin-1* mRNA levels, and increased *EGFR* gene copy numbers.

Results: Seventeen patients had *EGFR* mutations, all of which were in lung adenocarcinomas. Radiographic response was observed in 16 of 17 (94.1%) patients harboring *EGFR* mutations, in contrast with 6 of 51 (12.6%) with wild-type *EGFR* ($P < 0.0001$). Probability of response increased significantly in never smokers, patients receiving a greater number of prior chemotherapy regimens, Asians, and younger patients. Median survival was not reached for patients with *EGFR* mutations and was 9.9 months for those with wild-type *EGFR* ($P = 0.001$). *EGFR* mutations tended to be associated with increased numbers of CA repeats and increased *EGFR* gene copy numbers but not with *EGFR* and *caveolin-1* mRNA overexpression (P = not significant).

Conclusions: The presence of *EGFR* mutations is a major determinant of gefitinib response, and targeting EGFR should be considered in preference to chemotherapy as first-line treatment in lung adenocarcinomas that have demonstrable *EGFR* mutations.

Platinum-based chemotherapy as first-line treatment in advanced non – small cell lung cancer (NSCLC) yields limited survival benefit. A retrospective analysis of advanced NSCLC patients showed that response rates decreased with each successive chemotherapy regimen: first line, 21%; second line, 16%; third line, 2%; fourth line, 0% (1). Aberrant epidermal growth factor receptor (EGFR) signaling limits sensitivity to anticancer agents, and ligand-independent tyrosine kinase activation of EGFR, often caused by EGFR mutations in the extracellular domain, has been observed in various tumor types, including glioblastoma multiforme (2). Pharmacologic inhibitors of EGFR, such as gefitinib (Iressa), disrupt EGFR activity by binding the ATP pocket within the catalytic domain containing a critical ATP-binding site, Lys^{745} (K745). Gefitinib and related tyrosine kinase inhibitors occasionally yield dramatic and durable "Lazarus responses" (3), yet response rates are variable, with higher rates in patients with adenocarcinoma, female gender, Asian origin, and never-smoker status (4, 5).

The value of EGFR inhibitors as an NSCLC treatment approach has been limited by the lack of reliable methods for predicting which patients are likely to respond. The logical supposition that tumors overexpressing EGFR would respond best to EGFR inhibitors has not been borne out either in preclinical models(6, 7) or in clinical trials (8, 9). However, recent discoveries of *EGFR* mutations in the tyrosine kinase

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domain have shed light on the relationship between EGFR and sensitivity to both gefitinib and the related kinase inhibitor erlotinib. Accumulated data from three studies (10–12) show that 25 of 31 (81%) tumors from NSCLC patients with partial response or marked clinical improvement contained mutations in the *EGFR* tyrosine kinase domain. In contrast, none of 29 specimens from patients refractory to EGFR inhibitors had such mutations ($P < 0.0001$). The mutations included small in-frame deletions (746-750) adjacent to K745 (ELREA amino acids) and missense mutations, mainly L858R adjacent to the DFG motif in the COOH-terminal lobe in the activation loop of the kinase (10–12). These *EGFR* mutations are bona fide somatic mutations in NSCLC and have not been identified in other primary tumor types (10, 13, 14), with the exception of colorectal tumors. One of 293 tumors contained a G719S point mutation (15) that had previously been reported in NSCLC (11), and recently, 4 of 33 tumors harbored point mutations in exons 19 and 20 (16). *In vitro* studies of lung cancer cell lines with endogenous *EGFR* mutations displayed elevated activation of downstream antiapoptotic targets like AKT and signal transducer and activation of transcription (STAT5 and STAT3), conferring enhanced gefitinib sensitivity and increased cisplatin resistance (17).

The transcription activity of the *EGFR* gene is closely related to the enhancer region in intron 1 that is located near a polymorphic CA single sequence repeat containing 14 to 21 CA dinucleotides. Decreased numbers (<19) of CA dinucleotides in this CA sequence correlate with increased *EGFR* transcription (18, 19), and in breast cancer, this CA sequence is a frequent target for *EGFR* gene alterations (20). Moreover, interethnic studies have found that Japanese breast cancer patients carry increased numbers (>19) of CA dinucleotides than Caucasian patients (20). It has been shown that the number of repeats itself affects the mutation rate of nucleotide repeats (21).

A variety of cell surface receptors, including EGFR, as well as intracellular signaling molecules, are concentrated in specialized plasma membrane domains known as caveolae (22). *Caveolin-1* mRNA expression is elevated in multidrug-resistant cultured cancer cells (23), and up-regulation of caveolin-1 and caveolae organelles has been observed in drug-resistant human and ovarian cancer cell lines (24). In addition, high *caveolin-1* mRNA expression has been observed in potentially chemoresistant NSCLC cell lines established from metastatic NSCLCs (25). We therefore hypothesized that tumors harboring EGFR mutations might be associated with higher levels of *caveolin-1* mRNA.

In the present study, we examined *EGFR* mutations in tumor tissue from gefitinib-treated, chemorefractory, advanced NSCLC patients from the United States, Europe, and Asia and in a highly gefitinib-sensitive NSCLC cell line (26) and correlated their presence with response and survival. In addition, in a subgroup of patients for whom remaining tumor tissue was available, we examined the relationship among *EGFR* mutations, number of CA repeats, *EGFR* and *caveolin-1* mRNA levels, and increased *EGFR* gene copy numbers.

Materials and Methods

Patients. Patients with pretreated NSCLC received gefitinib, based on the attending oncologist's decision at the time of chemotherapy failure, at a daily dose of 250 mg given until disease progression. Patients were selected for the present study based on the availability of tumor tissue, without scoring tumor response at the time of selection. Acquisition of tissue specimens and examination of clinical records was approved by the ethics committees of participating institutions. A total of 68 patients were included: 32 Asians (19 Japanese and 13 Chinese) and 36 Caucasians (23 Spanish, nine German, three North American, and one English patient resident in Hong Kong). Assessment of *EGFR* mutations was done for all 68 patients. After this initial analysis, sufficient genomic DNA remained to perform additional related analyses in a subgroup of 28 patients.

Patients were divided into smokers and nonsmokers (having smoked <100 cigarettes in their lifetimes; ref. 27). Tumor response was defined according to the Response Evaluation Criteria in Solid Tumors (28). Survival was calculated from the start of gefitinib treatment. Follow-up was calculated from the start of gefitinib treatment; median follow-up was 11.4 months (range, 1.7-40.3 months).

Epidermal growth factor receptor sequencing. Pure tumor genomic DNA was derived from paraffin-embedded tissue obtained by laser capture microdissection (Palm, Oberlensheim, Germany). For isolation of DNA from deparaffinated, microdissected tissue, the material was incubated with proteinase K and DNA was extracted with phenol-chloroform and ethanol precipitation. Primers for PCR amplification in nested reactions for exons 18, 19, and 21 of EGFR (Genbank accession no. X00558) were as follows: exon 18 (first PCR, forward 5′-CAAATGAGCTGGCAAGTGCCGTGTC-3′ and reverse 5′-GAGTTTCCCA-AACACTCAGTGAAAC-3′; nested PCR, forward 5′-CAAGTGCCGTGTCC-TGGCACCCAAGC-3′ and reverse 5′-CCAAACACTCAGTGAAACAAAG-AG-3′); exon 19 (first PCR, forward 5′-GCAATATCAGCCTTAGGT GCGGCTC-3′ and reverse 5′-CATAGAAAGTGAACATTTAGGATGTG-3′; nested PCR, forward 5′-GTGCATCGCTGGTAACATCC-3′ and reverse 5′-TGTGGAGATGAGCAGGGTCT-3′); exon 21 (first PCR, forward 5′-CTAA-CGTTCGCCAGCCATAAGTCC-3′ and reverse 5′-GCTGCGAGCT-CACCCAGAATGTCTGG-3′; nested PCR, forward 5′-GCTCAGAGCCTGG-CATGAA-3′ and reverse 5′-CATCCTCCCCTGCATGTGT-3′). Sequencing was done using forward and reverse nested primers with the ABI Prism 3100 DNA Analyzer (Applied Biosystems, Foster City, CA). Electropherograms were analyzed for the presence of mutations using Seqscape v2.1.1 software in combination with Factura to mark heterozygous positions. The human NSCLC cell line (PC9) derived from an adenocarcinoma (Kyushu Cancer Center, Fukuoka, Japan) was also examined using the same methods.

CA repeats in intron 1. In the subgroup of 28 patients, genomic DNA from peripheral blood or adjacent normal lung tissue was used to determine the number of CA repeats in intron 1. PCR amplification was done with 50 ng of genomic DNA; the primer sequences specific for this microsatellite marker were as follows: forward 5′-FAMGGGCTCACAG-CAAACTTCTC-3′ and reverse 5′-AAGCCAGACTCGCTCATGTT-3′. One microliter of each PCR product was mixed with 0.5 μL of size standard (GenScan-350 Rox Standard, Applied Biosystems) and denatured in 18 μL of formamide at 95°C for 5 minutes. Separation was done with a four-color laser-induced fluorescence capillary electrophoresis system (ABI Prism 3100 DNA Analyzer, Applied Biosystems). The collected data was evaluated with the GeneScan Analysis Software (Applera, Norwalk, CT). DNA from the tumor cell line Hep-2 was used as a control for PCR amplified microsatellite fragment length.

Quantitative PCR. In the subgroup of 28 patients, total RNA was derived from paraffin-embedded tissue obtained by laser capture microdissection. After standard tissue sample deparaffinization using xylene and alcohols, samples were lysed in a Tris-chloride, EDTA, SDS, and proteinase K containing buffer. RNA was then extracted with phenol-chloroform-isoamyl alcohol followed by precipitation with isopropanol in the presence of glycogen and sodium acetate. RNA was resuspended in RNA storage solution (Ambion, Inc., Austin, TX) and treated with DNase I to avoid DNA contamination. cDNA was synthesized using Moloney murine leukemia virus retrotranscriptase enzyme. Template cDNA was added to Taqman Universal Master Mix (Applied Biosystems) in a 12.5-μL reaction with specific primers and

probe for each gene. The primer and probe sets were designed using Primer Express 2.0 Software (Applied Biosystems). Quantification of gene expression was done using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Primers and probe for *EGFR* and *caveolin-1* mRNA expression analysis were designed according to the Ref Seq NM_005228 and NM_001753, respectively (http://www.ncbi.nlm.nih.gov/LocusLink). The primers and labeled fluorescent reporter dye probe were as follows: β-actin, forward 5′-TGAGCGCGGCTACAGCTT-3′, reverse 5′-TCCTTAATGTCACGCACGATTT-3′, probe 5′-FAMACCACCACGGCCGAGCGG-3′TAMRA; EGFR, forward 5′-GGAAATTACCTATGTGCAGAGGAATT-3′, reverse 5′-TAACCAGCCACCCCCTGGAT-3′, MGB probe 5′-FAMTGATCTTTCCTTCTTAAAGAC-3′; Caveolin-1, forward 5′-CGACCCTAAACACCTCAACGA-3′, reverse 5′-GGTTCTGCAATCACATCTTCAAAG-3′, MGB probe 5′-FAMCGTGGTCAAGATTG-3′. Relative gene expression quantification was calculated according to the comparative C_t method using β-actin as an endogenous control and commercial RNA controls (Stratagene, La Jolla, CA) as calibrators. Final results were determined as follows: $2^{-(\Delta Ct\ sample\ -\ \Delta Ct\ calibrator)}$, where ΔC_t values of the calibrator and sample are determined by subtracting the C_t value of the target gene from the value of the *β-actin* gene. In all experiments, only triplicates with a SD of the $C_t < 0.20$ were accepted. In addition, for each sample analyzed, a retrotranscriptase minus control was run in the same plate to assure lack of genomic DNA contamination.

To distinguish between high and low gene expression levels, median levels obtained were used as cutoffs: 3.28 for *EGFR* and 0.52 for *caveolin-1* mRNA expression.

Fluorescence* in situ *hybridization assay. For each patient in the subgroup of 28 patients, two sections of 3- to 5-μm paraffin-embedded tumor tissue were placed over silenized treated slides. Another section was stained with H&E and confirmed to contain tumor tissue components. The silenized slides were left overnight at 60°C; deparaffinized in two changes of xylene for 10 minutes; rehydrated in 100% ethanol, 90% ethanol, and 70% ethanol for 1 minute each; and left in deionized water for 5 minutes. After tissue hydration, sections were placed in citrate buffer and heated in a microwave twice for 5 minutes at 800 W each. Slices were then digested by proteinase K treatment for 15 minutes at 37°C, fixed with formalin solution (pH 7.5), and washed in 2× SSC buffer. The hybridization was done using Vysis probes (LSI EGFR/CEP 7 Dual Color, Downers Grove, IL) following the manufacturer's instructions. Briefly, 5 μL of the probe solution were added to each slide and covered by a coverslip. Slides and probes were denatured for 3 minutes at 85°C in a slide warmer plaque (Hybrite, Vysis) and left at 37°C overnight. The coverslips were removed and the slides washed in 2× SSC/0.3%NP40 solution for 2 minutes at 72°C followed by an additional wash in 2× SSC/0.3%NP40 solution for 10 seconds at room temperature. Finally, slides were counterstained using a 4′-6′-diamidine-2-phenylindole-containing medium that specifically binds to DNA. For each patient, 100 nuclei from the selected tumor region were analyzed in a fluorescence microscope. The ratio of the average number of EGFR spots/nucleus by the average number of CEP 7 (centromeric chromosome 7) spots/nucleus was used for the scoring criteria. EGFR status in tumors was scored as follows: (a) single copy, up to four specific signals of both EGFR and CEP 7 probes with a ratio equal to 1; (b) polysomy, more than four specific signals of both probes per nucleus and a ratio <2; (c) amplification, more than four specific signals of EGFR probe per nucleus compared with CEP 7 with a ratio >2. Tumors scored as polysomy and/or amplification were labeled as having increased EGFR copy numbers.

Statistical methods. The primary objective of this study was to compare clinical characteristics, response rates, and survival in gefitinib-treated patients with and without mutations in the EGFR tyrosine kinase domain. In the subgroup of 28 patients, further analyses were done to examine the correlation among EGFR mutations, the number of CA repeats in intron 1 of EGFR in normal tissue, *EGFR* and *caveolin-1* mRNA expression levels in tumor tissue, and *EGFR* gene copy numbers.

The nonparametric Mann-Whitney *U* test and one-way ANOVA test were used to analyze differences in EGFR mutation status, number of CA repeats in intron 1 of EGFR, EGFR and caveolin-1 mRNA expression, and *EGFR* gene copy numbers. Normality of the distribution of continuous variables was assessed with the Kolmogorov-Smirnov test. The χ^2 and Fisher's exact tests were used to compare differences in response according to EGFR mutation status, number of CA repeats in intron 1, EGFR and caveolin-1 mRNA expression, and gene copy numbers. Univariate Cox regression models were used to measure hazard ratios. To identify relevant variables of influence, a multivariable logistic regression model was used, and the fit of the models was evaluated with the Hosmer-Lemeshow likelihood ratio test. The Wald test was used to test the statistical significance of each variable in the model. Survival curves were drawn with the Kaplan-Meier product limit method and *P* values assessed with the Tarone-Ware test. All reported *P* values are two sided; $P < 0.05$ was considered statistically significant. SPSS software version 11.5 (SPSS, Inc., Chicago, IL) was used for all analyses.

Results

Table 1 shows characteristics for all patients according to *EGFR* mutation status. Seventeen of the 68 patients harbored *EGFR* mutations in the tyrosine kinase domain. Mutations were not observed in DNA from peripheral blood or adjacent normal lung tissue, indicating that all mutations were somatic. All mutations were identified in adenocarcinomas (Table 1); 10 were heterozygous and six were homozygous (Table 2). Eleven tumors had in-frame nucleotide deletions in exon 19, adjacent to K745; five were delE746-A750, which was also observed in

Table 1. Characteristics of all patients according to *EGFR* mutation status

	EGFR mutation status		*P*
	Mutation	Wild-type	
No. patients	17	51	0.8
Age (range)	60 (34-84)	59 (39-86)	
Sex (%)			
Male	6 (35.3)	39 (76.5)	0.003
Female	11 (64.7)	12 (23.5)	
Histology (%)			
Adenocarcinoma	17 (100)	30 (58.8)	0.007
Large cell carcinoma	—	5 (9.8)	
Squamous cell carcinoma	—	11 (21.6)	
Other	—	5 (9.8)	
Smoking history (%)			
Smokers	3 (17.6)	43 (84.3)	0.0001
Nonsmokers	14 (82.4)	8 (15.7)	
No. prior regimens (range)	1 (0-3)	2 (0-6)	0.04
Response to gefitinib (%)			
Complete and partial response	16 (94.1)	6 (11.8)	<0.0001
Stable disease	1 (5.9)	8 (15.7)	
Progressive disease	—	34 (66.7)	
Not evaluable	—	3 (5.8)	
Duration of gefitinib treatment			
Months (range)	9.4 (1.1-23.1)	4.2 (0.2-41.9)	0.07

Table 2. Clinical characteristics and *EGFR* mutation status in 22 responders to gefitinib

Country of origin	Age	sex	Smoking status	Pathol	Prior regimens	Response	Overall survival (mo)	Survival status	*EGFR* mutation 1 AA sequence	*EGFR* mutation 2 AA sequence	Mutational status
Spain	60	F	Yes	ADC	1	PR	6.7	D	wt		wt
Spain	52	F	Yes	ADC	1	PR	8.3	D	wt		wt
Germany	65	M	Yes	ADC	1	PR	22.1	D	wt		wt
Japan	53	F	No	LCC	3	PR	18.4	D	wt		wt
Japan	76	M	Yes	ADC	3	PR	10.8	A	wt		wt
Japan	68	F	No	ADC	2	PR	24.3	D	wt		wt
Spain	71	F	No	ADC	1	PR	8.9	A	delE746.A750		Hetero
Spain	63	M	Yes	ADC	3	PR	17.8	A	delE746.T751InsA		Hetero
Germany	66	F	No	ADC	2	PR	13.7	D	delE746.S752InsV		Hetero
China	67	M	No	ADC	0	PR	22.0	A	delL747.P753InsS	L861Q	Hetero
China	34	F	No	ADC	2	PR	6.1	A	L858R		Homo
China	61	F	No	ADC	2	PR	14.3	A	L858R		Hetero
China	49	M	Yes	ADC	1	PR	25.4	A	L858R		Homo
China	37	M	No	ADC	0	PR	15.9	A	delE746.S752InsV		Hetero
Japan	71	F	No	ADC	1	PR	14.2	A	del719G(G)C to GC		Hetero
Japan	66	F	No	ADC	0	PR	9.5	A	delE746.T751		Homo
Japan	54	F	No	ADC	2	PR	18.7	D	delE746.A750		Homo
Japan	60	F	No	ADC	3	PR	15.3	A	L718P		Hetero
Japan	50	M	No	ADC	0	PR	18.4	A	delL747.T751InsF		Hetero
Japan	52	M	Yes	ADC	1	PR	8.9	A	delE746.A750		Homo
Japan	42	F	No	ADC	2	CR	18.9	A	delE746.A750		Homo
USA	84	F	No	ADC	1	PR	11.7	D	delE746.A750		Hetero

Abbreviations: Pathol, pathologic diagnosis; ADC, adenocarcinoma; LCC, large cell carcinoma; PR, partial response; CR, complete response; A, alive; D, dead; AA, amino acid; Homo, homozygogous; Hetero, heterozygous; wt, wild type.

the PC9 cell line; one was delE746-T751; four contained an amino acid insertion (one delE746-T751insF, one delE746-T751insA, and two delE746-752insV); and one tumor contained both an amino acid insertion (delL747-P753insS) and a missense mutation L861Q in exon 21. Four tumors contained an L858R mutation in exon 21. One tumor had an L718P mutation and another had a nucleotide deletion (guanine) in codon 719, both in exon 18. This second mutation was heterozygous. The G deletion affects the reading frame 5′ downstream of this position. The protein is nonfunctional, and the new sequence has a stop codon in codon 747 (TAA instead of TTA).

Twenty-two patients (32%) achieved a partial response to gefitinib. Table 2 shows the clinical characteristics of all responders. Sixteen of the 17 patients (94.1%) carrying EGFR mutations attained a partial radiographic response in contrast with 6 (12.6%) of the 51 patients with wild-type EGFR ($P < 0.0001$) Patients with *EGFR* mutations had 17.1 times greater probability of response ($P = 0.02$). Probability of response was also increased in nonsmokers, patients receiving a greater number of prior chemotherapy regimens, Asians, and younger patients (Table 3).

In general, patients harboring *EGFR* mutations obtained dramatic responses. For example, a Japanese female with adenocarcinoma underwent three pulmonary resections between 1999 and 2002; two of the three resected tumors contained an EGFR mutation (delE746-T751). The patient developed multiple bilateral lung metastases and did not respond to several chemotherapy regimens. After 2 months of gefitinib treatment, almost complete response was attained and the patient remains in remission at the time of submitting this article (Fig. 1). A second patient, a Spanish

Table 3. Adjusted odds ratio for the joint effect on response of different covariates

	Odds ratio (95% confidence interval)	*P*
Odds ratio adjusted by covariates		
EGFR mutations	17.1 (5.1-58.8)	0.002
EGFR mutations by sex (female)	8.7 (2.2-34.6)	0.0001
EGFR mutations by smoking status (nonsmoker)	37.4 (3.1-426)	0.005
EGFR mutations by no. prior chemotherapy regimens	73.1 (7.6-462)	0.005
EGFR mutations by ethnicity	61.7 (5.9-639)	0.001
EGFR mutations by age	105.0 (11.4-981)	0.00001
Crude odds ratio		
Sex (female)	1.4 (0.6-3.4)	0.4
Smoking status (smoker)	0.6 (0.4-0.8)	0.001
No. prior chemotherapy regimens	0.7 (0.5-0.9)	0.003
Ethnicity (Asian)	4.0 (1.7-9.2)	0.001
Age	0.9 (0.98-0.99)	0.008

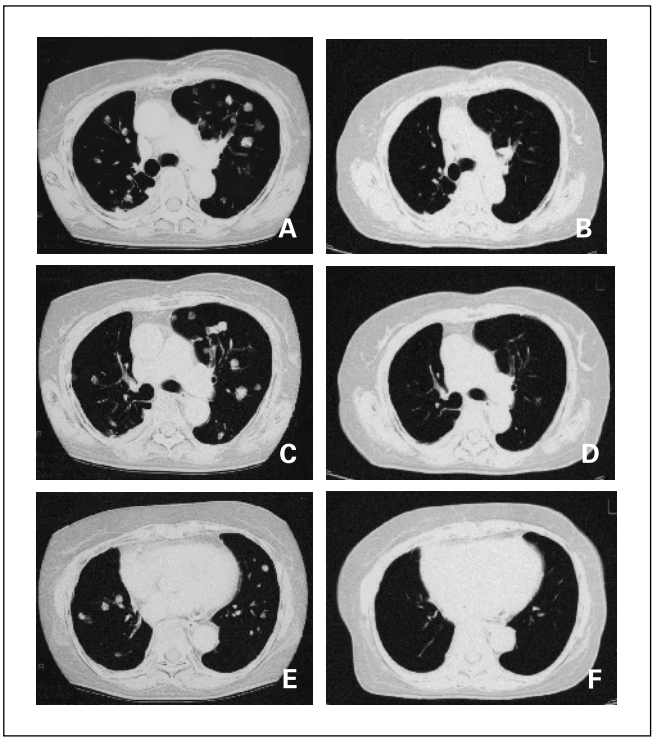


Fig. 1. Example of response to gefitinib in a representative patient with recurring NSCLC after three lines of chemotherapy. Computed tomography slices before gefitinib treatment (*A*, *C*, and *E*) and after 8 weeks of gefitinib treatment (*B*, *D*, and *F*).

female with relapsed lung adenocarcinoma underwent an upper left lobe lobectomy in 2002; the tumor contained an *EGFR* mutation (delE746-A750). One and a half years later, in 2004, the patient developed severe neurologic symptoms with impairment of walking, eating, and speaking and required a gastric feeding tube. The brain computed tomography showed multiple cystic, rim-enhancing supratentorial masses of various sizes (Fig. 2*A*). Brain biopsy was not done. Dexamethasone was given, without improvement, and brain irradiation was not indicated. One month later, gefitinib was given through the gastric feeding tube, and a rapid recovery of neurologic functions was observed, accompanied by a regression of the brain metastases (Fig. 2*B*). The patient is still in remission. A third patient, an 84-year-old North American female with lung adenocarcinoma underwent a lobectomy in 2003; the tumor contained an *EGFR* mutation (delE746-A750). The patient relapsed with bone and lung metastases; one cycle of chemotherapy was given, but she suffered a pulmonary embolism with a myocardial infarction. She recovered but did not receive additional chemotherapy. She developed a cardiac tamponade with clear evidence of progression of her lung metastases. Seven months later, in 2004, she started gefitinib treatment, and 3 weeks later she was clinically improved. New bone metastases were detected after 1 year and the patient died . Finally, a 42-year-old Japanese female with lung adenocarcinoma underwent a left upper lobectomy in 2001; the tumor contained not only a delE746-A750 mutation but also >20 *EGFR* gene copies by fluorescence *in situ* hybridization, elevated *EGFR* (47.3) and *caveolin-1* (0.9) mRNA expression, and increased number of CA repeats (20 of 21; Fig. 3). The patient developed brain metastases 9 months later, in 2002 and received stereotactic radiosurgery. Multiple lung metastases developed after 2 months, and six cycles of cisplatin/gemcitabine/vinorelbine were given. Eight months later, in 2003, the patient initiated gefitinib treatment. Before treatment, her carcinoembryonic antigen level was 257.2 ng/mL (normal level, <5 ng/mL). After 6 months of gefitinib treatment, her carcinoembryonic antigen level was 2.2 ng/mL. A complete remission of the lung metastases has been attained.

Median survival for patients carrying *EGFR* mutations was not reached, whereas it was 9.9 months (95% CI, 6.8-12.9) for those patients carrying wild-type *EGFR* ($P = 0.001$; Fig. 4).

Table 4 shows the characteristics of the 28 patients in whom we assessed CA repeats, *EGFR* and *caveolin-1* mRNA expression, and *EGFR* gene copy numbers. All patients with *EGFR* mutations also had increased numbers of CA repeats (≥19). The highly gefitinib-sensitive PC9 lung adenocarcinoma cell line, which harbored the deletion delE746-A750, also displayed increased numbers of CA repeats (20 of 20). There were no differences in median mRNA levels of *EGFR* or *caveolin-1* according to EGFR mutation status. Increased *EGFR* gene copy numbers were observed more frequently in patients with EGFR mutations. Gene amplification ranged widely from low to high levels, and in some patients,

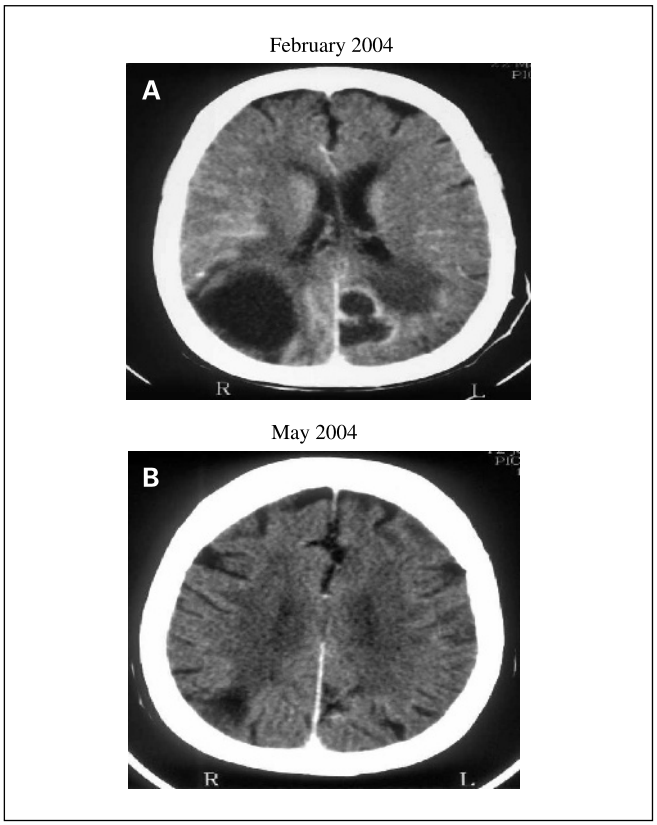


Fig. 2. Example of response to gefitinib in a lung adenocarcinoma patient with brain metastases. Computed tomography before gefitinib treatment (*A*) and after 8 weeks of gefitinib treatment (*B*). *A,* enlarged ventricles were observed in the pretreatment computed tomography. *B,* after treatment, with the disappearance of the periventricular brain metastases, ventricles were less visible.

amplification was seen in only 25% of the tumor cells examined. In this subset of 28 patients, the response rate for patients with increased gene copy numbers was 45%, in contrast with 89% for patients with *EGFR* mutations (P = 0.02). The response rate was 100% in patients with both *EGFR* mutations and gene amplification. Table 5 illustrates the levels of *EGFR* and *caveolin-1* mRNA according to *EGFR* mutation status and further broken down by gene copy numbers and number of CA repeats. The highest levels of *EGFR* mRNA were observed in the group of patients with both *EGFR* mutations and increased *EGFR* copy numbers. Patients with both *EGFR* mutations and low levels of *EGFR* or *caveolin-1* mRNA had a median survival of 13 months, whereas median survival has not been reached for those patients with *EGFR* mutations and high levels of *EGFR* or *caveolin-1* mRNA (data not shown).

Discussion

In the present study, we have observed that *EGFR* mutations are a strong predictor of gefitinib response in chemoresistant NSCLC patients. Sixteen of 17 patients (94.1%) with *EGFR* mutations attained an objective response, in contrast with only 6 of 51 patients (12.6%) with wild-type *EGFR* ($P <$ 0.0001). These results mirror accumulated data from three studies (10–12) in which 25 of 31 (81%) NSCLC patients with *EGFR* mutations attained an objective response, whereas none of 29 nonresponders had mutations. Furthermore, it has recently been shown that in 16 gefitinib-treated Taiwanese NSCLC patients, seven of nine responders had *EGFR* mutations (13). The delE746-A750 in the PC-9 cell line found in the present study has also been observed in a separate study (29), in which it also conferred hypersensitivity to gefitinib. *EGFR* mutations found in previous studies have mostly been heterozygous (10–12); however, Paez et al. (11) reported one homozygous mutation at exon 19 and Pao et al. (12) found homozygous mutations in two of seven gefitinib-treated patients, leading them to speculate that homozygosity may be the result of the selective amplification of the mutant gene or that mutations in general may be homozygous with the wild-type sequence originating from contaminating "normal" DNA. In the study by Huang et al. (13), 4 of 10 mutations were homozygous, and in the present study, 6 of 17 mutations were also homozygous. Contaminating "normal" cells with wild-type EGFR seems the most likely explanation for apparently heterozygous mutations, because even with microdissection, nonneoplastic tissue contamination cannot be completely ruled out. However, amplification of mutant *EGFR* could account for detection of only mutant sequences.

In the original studies (10, 11), only one mutation per tumor was detected. However, Pao et al. (12) found a tumor sample with two mutations, from a female never smoker with adenocarcinoma, treated with erlotinib for 13 months, and surviving 22 months. Furthermore, in the study by Huang et al. (13), two patients had two mutations in their tumors; one responded and one did not. In our study, one patient had two mutations: a 67-year-old Hong Kong Chinese female never smoker with adenocarcinoma. She attained a partial response and is still alive at 22 months (January 2005). It is not possible to draw definite conclusions from only four patients, and more data regarding the potential predictive value of two mutations in the same tumor is needed.

In the present study, 6 of 51 patients with wild-type *EGFR* attained partial response to gefitinib. There were no differences in baseline clinical characteristics between responders with EGFR mutations and responders with wild-type *EGFR* (Table 2). However, only 16% of responders with wild-type *EGFR* remain alive at the time of submitting this article, in contrast with 81% of responders harboring *EGFR* mutations. In the series reported by Lynch et al. (10), one of nine gefitinib-sensitive patients did not have *EGFR* mutations. Along the same lines, Pao et al. (12) reported that 5 of 17 patients with partial response or clinical improvement to gefitinib or erlotinib had wild-type *EGFR* in exons 18 to 24.

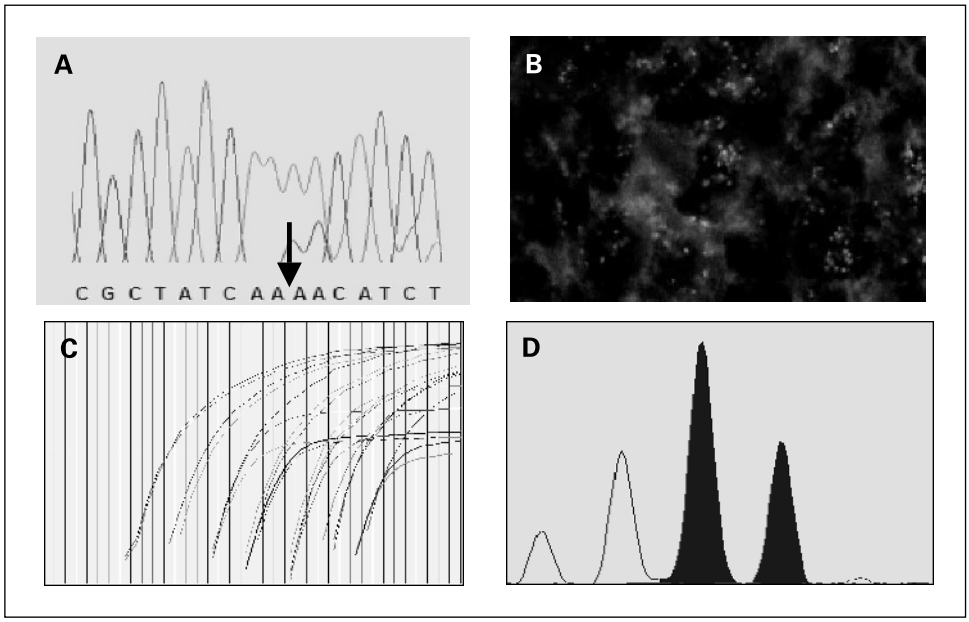
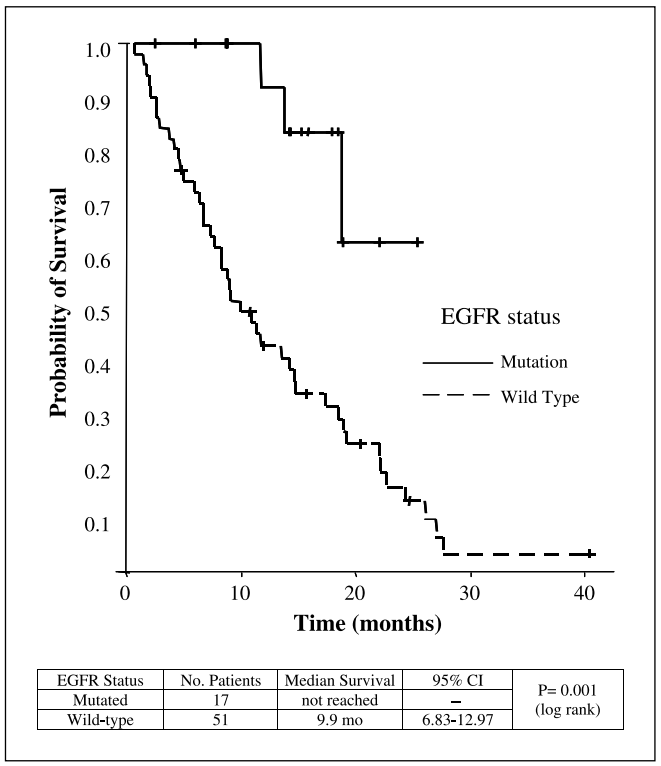


Fig. 3. Gefitinib responder showing, clockwise from top left: (*A*) an *EGFR* mutation (del E746-A750); (*B*) a high level of gene amplification (spots); (*C*) high *EGFR* and *caveolin-1* mRNA levels (superimposed one on the other); (*D*) and increased numbers of CA repeats. *C,* cDNAs for the gene of interest and an internal reference gene (*β-actin*) were quantified using a fluorescence-based real-time detection method. For each sample, parallel triplex Taqman PCR reactions were performed for the gene of interest and the *β-actin* reference gene to normalize for input cDNA. The expression of individual *EGFR* and *caveolin-1* was calculated using a relative quantification algorithm. In this patient, the *EGFR* mRNA level was 47 and the *caveolin-1* mRNA level was 0.98. *D,* number of CA repeats, determined by GeneScan analysis software (Applera). The number of CA repeats is determined by the mobility in the chromatogram. The shaded peaks represent the intensities of the two alleles. The left peak represents 20 CA repeats and the right peak represents 21 CA repeats. At submission, this patient has been in complete remission for 18.9 months.



EGFR Status	No. Patients	Median Survival	95% CI	
Mutated	17	not reached	–	P= 0.001 (log rank)
Wild-type	51	9.9 mo	6.83-12.97	

Fig. 4. Survival from the start of gefitinib treatment according to EGFR mutation status.

Mutations in these responders may not have been detected because they were below the detection rate of sequencing assays (30), or increased *EGFR* gene copy numbers in these responders may have conferred enhanced gefitinib sensitivity (12) in the absence of *EGFR* mutations. NSCLC cell lines with wild-type *EGFR* but with high levels of *EGFR*, *ErbB2*, or *ERbB3* mRNA have shown intermediate sensitivity to gefitinib and erlotinib (31).

The small number of patients examined in the present study limits the conclusions that can be drawn as to the role of CA repeats, *EGFR* and *caveolin-1* mRNA expression, and *EGFR* gene copy numbers. However, interethnic differences in the number of CA repeats warrant further investigation in Asian lung cancer patients, in whom increased numbers of CA repeats may be more frequently associated with the presence of *EGFR* mutations (19, 20). Amador et al. (32) found that head and neck cell lines with decreased numbers of CA repeats had higher expression of *EGFR* mRNA and were more sensitive to the inhibitory effects of erlotinib. In addition, in 19 gefitinib-treated colorectal cancer patients (32), 84% of those with decreased numbers of CA repeats developed skin toxicity, a feature related to the antitumor activity of EGFR inhibitors (33), compared with only 33% of those with increased numbers of CA repeats ($P = 0.04$; ref. 32).

In surgically resected NSCLC patients (13, 34), *EGFR* mutations were associated with well and moderately differentiated adenocarcinomas and smoking status but not with female gender. Dramatic clinical response to gefitinib is observed in only 10% to 19% of chemorefractory advanced NSCLC. Kris et al. (5) showed that female gender predicted response to gefitinib, whereas the number of prior chemotherapy regimens did not influence response. In our study, the number of prior chemotherapy regimens increased the probability of response in tumors containing *EGFR* mutations.

The strong correlation we observed between EGFR mutations and improved response and survival leads us to recommend the assessment of *EGFR* mutations in lung adenocarcinoma

Table 4. Patient characteristics of a subgroup of 28 patients according to *EGFR* mutation status, number of CA repeats in intron 1, *EGFR* and *caveolin-1* mRNA levels, and *EGFR* gene copy numbers

	Wild-type *EGFR*, *n* (%)	Mutated *EGFR*, *n* (%)	*P*
No. patients	19	9	
Age (y)			
<65	10 (52)	6 (66.6)	NS
≥65	9 (48)	3 (33.3)	
Sex			
Male	15 (79)	4 (45)	NS
Female	4 (21)	5 (55)	
Ethnicity			
Caucasian	8 (42)	4 (45)	NS
Asian	11 (58)	5 (55)	
Histology			
Adenocarcinoma	16 (85)	9 (100)	NS
Large cell carcinoma	1 (5)	0	
Squamous cell carcinoma	2 (10)	0	
Smoking status			
Smoker	15 (79)	3 (33.3)	0.035
Nonsmoker	4 (21)	6 (66.6)	
Response to gefitinib			
Yes	2 (11)	8 (88)	<0.0001
No	16 (84)	1 (12)	
Nonevaluable	1 (5)	—	
Duration of gefitinib response (wk)			
Median (range)	6.93 (0.2-27.6)	7.73 (1.05-15.63)	NS
CA repeats in intron 1			
<19	3 (20.5)*	0 (-)*	NS
≥19	11 (79.5)*	7 (100)*	
EGFR mRNA levels			
No. patients	15	8	0.087
Median (range)	2.61 (0.42-23.09)*	5.04 (1.79-47.37)*	
Caveolin-1 mRNA levels			
No. patients	14	8	NS
Median (range)	0.71 (0.06-2.16)*	0.55 (0.19-1.07)*	
EGFR gene copy numbers			
Increased	4 (21)	5 (55)	0.087
Normal	15 (79)	4 (45)	

Abbreviation: NS, not significant.
*Disparity between some figures is due to the lower availability of tumor tissue in some patients.

Table 5. *EGFR* and *caveolin-1* mRNA levels in patients with wild-type and mutated *EGFR*, further broken down according to number of CA repeats in intron 1 and *EGFR* gene copy numbers

	Wild-type *EGFR*				Mutated *EGFR*			
	EGFR mRNA		*Caveolin-1* mRNA		*EGFR* mRNA		*Caveolin-1* mRNA	
	No. patients	Median (range)	No. patients	Median (range)	No. patients	Median (range)	No. patients	Median (range)
CA repeats								
<19	2	14.24 (5.38-23.09)	1	2.16 (2.16-2.16)	0		0	
≥19	10	2.94 (1.54-6.44)	10	0.75 (0.19-1.47)	6	13.21 (3.0-47.37)	6	0.41 (0.19-1.07)
EGFR gene copy numbers								
Increased	4	5.12 (2.61-23.09)	3	1.23 (0.19-2.16)	5	20.31 (3.0-47.37)	5	0.61 (0.19-1.07)
Normal	11	2.04 (0.42-6.43)	11	0.57 (0.06-1.47)	3	3.01 (1.79-3.97)	3	0.46 (0.30-0.63)

patients to customize treatment. NSCLC cell lines containing *EGFR* mutations are chemoresistant but highly sensitive to gefitinib (17, 35). In the present study, we detected an unprecedented median survival in patients with *EGFR* mutations. The Spanish Lung Cancer Group is currently screening for *EGFR* mutations in metastatic lung adenocarcinomas to identify patients who could benefit from treatment with tyrosine kinase inhibitors.

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Activating Mutations in the Tyrosine Kinase Domain of the Epidermal Growth Factor Receptor Are Associated with Improved Survival in Gefitinib-Treated Chemorefractory Lung Adenocarcinomas

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