Epidermal Growth Factor Receptor Gene and Protein and Gefitinib Sensitivity in Non–Small-Cell Lung Cancer

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Background: **Gefitinib is a selective inhibitor of the epidermal growth factor (EGFR) tyrosine kinase, which is overexpressed in many cancers, including non–small-cell lung cancer (NSCLC). We carried out a clinical study to compare the relationship between EGFR gene copy number, EGFR protein expression, EGFR mutations, and Akt activation status as predictive markers for gefitinib therapy in advanced NSCLC.** ***Methods:*** **Tumors from 102 NSCLC patients treated daily with 250 mg of gefitinib were evaluated for EGFR status by fluorescence in situ hybridization (FISH), DNA sequencing, and immunohistochemistry and for Akt activation status (phospho-Akt [P-Akt]) by immunohistochemistry. Time to progression, overall survival, and 95% confidence intervals (CIs) were calculated and evaluated by the Kaplan–Meier method; groups were compared using the log-rank test. Risk factors associated with survival were evaluated using Cox proportional hazards regression modeling and multivariable analysis. All statistical tests were two-sided.** ***Results:*** **Amplification or high polysomy of the EGFR gene (seen in 33 of 102 patients) and high protein expression (seen in 58 of 98 patients) were statistically significantly associated with better response (36% versus 3%, mean difference = 34%, 95% CI = 16.6 to 50.3; *P*<.001), disease control rate (67% versus 26%, mean difference = 40.6%, 95% CI = 21.5 to 59.7; *P*<.001), time to progression (9.0 versus 2.5 months, mean difference = 6.5 months, 95% CI = 2.8 to 10.3; *P*<.001), and survival (18.7 versus 7.0 months, mean difference = 11.7 months, 95% CI = 2.1 to 21.4; *P* = .03). EGFR mutations (seen in 15 of 89 patients) were also statistically significantly related to response and time to progression, but the association with survival was not statistically significant, and 40% of the patients with mutation had progressive disease. In multivariable analysis, only high EGFR gene copy number remained statistically significantly associated with better survival (hazard ratio = 0.44, 95% CI = 0.23 to 0.82). Independent of EGFR assessment method, $EGFR^+$/$P\text{-}Akt^+$ patients had a statistically significantly better outcome than $EGFR^-$, $P\text{-}Akt^-$, or $EGFR^+$/$P\text{-}Akt^-$ patients.** ***Conclusions:*** **High EGFR gene copy number identified by FISH may be an effective molecular predictor for gefitinib efficacy in advanced NSCLC.**
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Non–small-cell lung cancer (NSCLC) is the leading cause of cancer death worldwide *(1)*. Although chemotherapy has produced modest survival benefits in patients with advanced-stage disease, standard two-drug combinations generate considerable toxicity and require intravenous administration *(2–4)*. Progress in lung cancer biology led to the development of small-molecule inhibitors of target proteins involved in proliferation, apoptosis, and angiogenesis. The epidermal growth factor receptor (EGFR) superfamily, including the four distinct receptors EGFR/erbB-1, HER2/erbB-2, HER3/erbB-3, and HER4/erbB-4, was identified early as a potential therapeutic target in solid tumors. After ligand binding, these receptors homo- and heterodimerize and their tyrosine kinase domain is activated, initiating a cascade of events implicated in the development and progression of cancer through effects on cell cycle progression, apoptosis, angiogenesis, and metastasis *(5–8)*. EGFR is overexpressed in many human epithelial malignancies, including NSCLC *(9,10)*.

Given the biologic importance of the EGFR molecular network in carcinomas, several molecules have been synthesized that inhibit the EGFR tyrosine kinase domain *(11,12)*. These inhibitors include gefitinib (Iressa; AstraZeneca, Macclesfield, UK) and erlotinib (Tarceva; OSI Pharmaceuticals Inc., Melville, NY), both of which are orally active, selective EGFR tyrosine kinase inhibitors that produce objective response rates of 12%–27% in previously treated or untreated advanced NSCLC *(13–16)*. Recently, the Canadian trial BR.21 reported a survival benefit for erlotinib versus placebo as a second- or third-line therapy that was not confined to objective responders or to a single sex or histology *(17)*.

Ways to identify NSCLC patients who are most likely to respond to EGFR tyrosine kinase inhibitors are being investigated. Interestingly, data from retrospective studies suggest that the level of EGFR protein expression is not associated with gefitinib response *(18–20)*. Activating mutations cause ligand-independent activity of receptor tyrosine kinases, and recent reports show that specific missense and deletion mutations in the tyrosine kinase domain of the EGFR gene *(21–23)* are associated with EGFR tyrosine kinase inhibitor sensitivity and with female sex, adenocarcinoma histology, and never-smoking status—all clinical characteristics that are known to be related to tyrosine kinase

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inhibitor sensitivity *(13–16,24)*. Although these EGFR mutations can account for almost all objective responses obtained with tyrosine kinase inhibitors, the clinical benefit observed with these drugs and the survival benefit identified in the BR.21 trial cannot be explained only by the presence of mutations.

Among other important players in EGFR tyrosine kinase inhibitor sensitivity, the activation status of the Akt protein has been highlighted in preclinical and clinical studies *(25,26)*. Akt is a serine/threonine kinase that acts downstream of EGFR to regulate many cellular processes, including cell survival, proliferation, and growth, and it is activated by phosphorylation at amino acids Thr308 and Ser473 *(27)*. Sordella et al. *(25)* showed that gefitinib-sensitizing EGFR mutations activate antiapoptotic pathways involving Akt in lung cancer cell lines, and we have previously shown *(26)* that the activation status of Akt is associated with gefitinib sensitivity of NSCLC patients, in terms of response and time to progression, but not in terms of survival. The lack of association with survival could be explained by the presence of a subset of phosphorylated (P)-Akt-positive patients who are resistant to gefitinib therapy as a consequence of Akt activation by a non–EGFR-dependent mechanism *(28–30)*.

Gene amplification is another molecular mechanism responsible for oncogene overexpression, and this phenomenon has been associated with mutations in the EGFR gene in glioblastoma *(31)* and in the HER2 gene in breast cancer *(32)*. The NSCLC cell line NCI-H3255 has been reported to carry the $EGFR^{L858R}$ mutation, which is found in patients who respond to gefitinib, and has gene amplifications as well, and is reported to be sensitive to gefitinib in vitro *(33)*. However, the role of EGFR genomic gain in predicting gefitinib sensitivity in patients and its association with mutation remains to be clarified.

Here we investigated the status of EGFR at the genomic, mRNA, and protein levels using fluorescence in situ hybridization (FISH), DNA sequencing, quantitative reverse-transcription–polymerase chain reaction, and immunohistochemistry in 102 advanced NSCLC patients treated with gefitinib. We compared the results with P-Akt status and with clinical features and outcome.

Subjects and Methods

Study Design and Patient Characteristics

Patients included in this study were accrued from a prospective study of gefitinib *(26)* and the Expanded Access Study of gefitinib conducted at Bellaria Hospital (Bologna), Scientific Institute University Hospital San Raffaele (Milan), and Policlinico Monteluce (Perugia). Complete clinical information and tissue blocks were available from 80 of 106 patients enrolled in the Akt clinical trial *(26)* and from an additional 22 patients in the Expanded Access Study who were treated consecutively at the end of the Akt study and followed up in the same way as patients in the Akt trial. These studies were approved by the Bellaria Hospital institutional ethical review board, and written informed consent was obtained from each patient before enrollment. In the subgroup of patients participating in the Expanded Access Study of gefitinib, institutional review board approval was obtained according to Good Clinical Practice, and specific written informed consent was obtained from each patient (Expanded Access Study consent form, Italian version). Eligibility for both studies included histologically confirmed NSCLC with measurable, locally advanced or metastatic disease, progressing or relapsing after chemotherapy or with medical contraindications for chemotherapy. Patients had performance status ranging from grade 0 to 2. Performance status was defined according to Eastern Cooperative Oncology Group *(34)* and considered grade 0 when the patient was fully active and able to perform all predisease activities without restriction, grade 1 when the patient was restricted in physically strenuous activity but ambulatory and able to perform work of a light or sedentary nature, and grade 2 when the patient was ambulatory and capable of all self-care but unable to perform any work activities. Patients received gefitinib (250 mg/day) and were evaluated for response according to the Response Evaluation Criteria in Solid Tumors criteria *(35)*. Tumor response was assessed by computed tomography scan after 2 months, with a confirmatory evaluation to be repeated in responders and in patients with stable disease at least 4 weeks after the initial determination of response. Time to disease progression was calculated from the date of initiation of gefitinib treatment to the date of detection of progressive disease or to the date of last contact. Survival was calculated from the date of therapy initiation to the date of death or to the date of last contact.

Tissue Preparation; EGFR Gene, mRNA, and Protein Analyses

Tumor specimens were obtained before any cancer therapy and embedded in paraffin. Serial sections (4 μm) containing representative malignant cells were stained with hematoxylin and eosin and classified based on the World Health Organization criteria *(36)*. Gene copy number per cell was investigated by FISH using the LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen probe (Vysis, Abbott Laboratories, IL), according to a published protocol *(10)*. Sections were incubated at 56 °C overnight, deparaffinized by washing in CitriSolv (Fisher Scientific, Pittsburgh, PA), and dehydrated in 100% ethanol. After incubation in 2× saline sodium citrate buffer (2× SSC; pH 7.0) at 75 °C for 15–25 minutes, sections were digested with proteinase K (0.25 mg/mL in 2× SSC; pH 7.0) at 37 °C for 15–25 minutes, rinsed in 2× SSC (pH 7.0) at room temperature for 5 minutes, and dehydrated using ethanol in a series of increasing concentrations (70%, 85%, 100%). The EGFR/CEP 7 probe set was applied per the manufacturer's instructions onto the selected area based on the presence of tumor foci on each slide, and the hybridization area was covered with a glass coverslip and sealed with rubber cement. The slides were incubated at 80 °C for 8–10 minutes for codenaturation of chromosomal and probe DNA and were then placed in a humidified chamber at 37 °C for 20–24 hours to allow hybridization to occur. Posthybridization washes were performed in 1.5 *M* urea and 0.1× SSC (pH 7.0–7.5) at 45 °C for 30 minutes and in 2× SSC for 2 minutes at room temperature. After the samples were dehydrated in ethanol as above, 4′,6′-diamidino-2-phenylindole (DAPI; 0.15 mg/mL in Vectashield mounting medium, Vector Laboratories, Burlingame, CA) was applied for chromatin counterstaining. FISH analysis was performed independently by two authors (F. Cappuzzo and M. Varella-Garcia) who were blinded to the patients' clinical characteristics and all other molecular variables.

Patients were classified into six FISH strata with ascending number of copies of the EGFR gene per cell according to the frequency of tumor cells with specific number of copies of the EGFR gene and chromosome 7 centromere: 1) disomy (≤2 copies

in >90% of cells); 2) low trisomy (≤2 copies in ≥40% of cells, 3 copies in 10%–40% of the cells, ≥4 copies in <10% of cells); 3) high trisomy (≤2 copies in ≥40% of cells, 3 copies in ≥40% of cells, ≥4 copies in <10% of cells); 4) low polysomy (≥4 copies in 10%–40% of cells); 5) high polysomy (≥4 copies in ≥40% of cells); and 6) gene amplification (defined by presence of tight EGFR gene clusters and a ratio of EGFR gene to chromosome of ≥2 or ≥15 copies of EGFR per cell in ≥10% of analyzed cells).

EGFR protein expression was evaluated by immunohistochemistry using methods and assessment criteria described elsewhere *(10)* with the mouse anti–human EGFR, clone 31G7 monoclonal antibody (Zymed Laboratories, San Francisco, CA). P-Akt was also detected by immunohistochemistry using the rabbit anti–mouse P-Akt (Ser 473) polyclonal antibody (Cell Signaling Technology, Beverly, MA), according to the manufacturer's protocol. P-Akt expression and EGFR expression were scored based on intensity and fraction of positive cells. The intensity score was defined as follows: 0 = no appreciable staining in the tumor cells, 1 = barely detectable staining in the cytoplasm and/or nucleus compared with the stromal elements, 2 = readily appreciable brown staining distinctly marking the tumor cell cytoplasm and/or nucleus, 3 = dark brown staining in tumor cells obscuring the cytoplasm and/or nucleus, or 4 = very strong staining of nucleus and/or cytoplasm. The score was based on the fraction of positive cells (0%–100%). The total score was calculated by multiplying the intensity score and the fraction score producing a total range of 0–400. For statistical analyses, scores of 0–200 were considered negative/low expression, and scores of 201–400 were considered positive/high expression. This cutoff level was based on consistency with previous studies from our group, in which we found a statistical correlation between increased EGFR protein expression and increased gene copy number *(10)*. Immunohistochemistry assays were scored jointly by two authors (W.A. Franklin and F.R. Hirsch); blinded to clinical, FISH, and EGFR mutation results; if discrepancies occurred, a consensus score was made by the two readers after discussion of the slide.

RNA was isolated, cDNA transcribed, and quantitative real-time polymerase chain reactions performed as described previously *(37)*. Microdissection of tumor cells was performed by manual or by laser capture technique using the PALM instrument (PALM Microlaser Technologies AG, Bernried, Germany), according to the manufacturer's guidelines. Primers and probes were as follows: forward EGFR primer: 5′-TCCGTCTCTTGCCGGGAAT-3′; reverse EGFR primer: 5′-GGCTCACCCTCCAGAACCTT-3′; EGFR Taqman probe: 5′-ACGCATTCCCTGCCTCGGCTG-3′ (GenBank accession number NM_005228). DNA for mutation analysis was also isolated from the same tumors. Fifty nanograms of genomic DNA was amplified for EGFR exons 18, 19, and 21 by touchdown heminested polymerase chain reaction *(38,39)* and sequenced in both sense and antisense directions. Exons 18, 19, and 21 were examined because they harbor 98% of the 56 EGFR mutations in NSCLC reported to date *(21–23)*.

Statistical Analysis

Differences between and among groups were compared using Fisher's exact test or Pearson's chi-square test for qualitative variables and using Student's *t* test or analysis of variance for continuous variables. Normality of the distribution was assessed using the Kolmogorov–Smirnov test *(40)*. Time to progression, overall survival, and 95% confidence intervals (CIs) were calculated and evaluated by the Kaplan–Meier method *(41)*; different groups were compared using the log-rank test. Association of risk factors associated with survival was evaluated using Cox proportional hazards regression modeling with a step-down procedure *(42)*. Only those variables with significant results in univariate analysis were included in the multivariable analysis. The criterion for variable removal was the likelihood ratio statistic, based on the maximum partial likelihood estimates (default *P* value of .10 for removal from the model). The study design guarantees independence of the observations. The proportional hazard assumption was tested by log-survival function analysis and found to hold. All statistical tests were two-sided, and statistical significance was defined as $P<.05$. All analyses were performed using the statistical package SPSS version 11.5 (SPSS Italia srl, Bologna, Italy).

Results

Patient Characteristics

The clinical outcome based on sex, stage, histology, performance status, and smoking status, most of which was reported in a previous publication *(25)*, is shown in Table 1. For the entire group, the objective response rate was 14%, the progression rate was 60%, the median time to progression was 2.9 months, the median survival was 9.4 months, and 1-year survival was 40.7%. Female sex (mean difference = 22.6%, 95% CI = 6.6 to 38.6; $P = .004$) and never-smoking status (mean difference = 30.8%, 95% CI = 5.3 to 56.3; $P = .006$) were statistically significantly associated with better response, and female sex (mean difference = 3.0 months, 95% CI = 4.5 to 10.5 months; $P = .03$), adenocarcinoma and bronchioloalveolar histology (mean difference = 5.0 months, 95% CI = 2.8 to 7.2 months; $P = .03$), and performance status 0–1 (mean difference = 7.4 months, 95% CI = 5.6 to 9.1 months; $P = .004$) were statistically significantly associated with longer survival.

EGFR Gene Copy Number and Clinical Outcome

First we assessed EGFR gene copy number by FISH. Disomy for the EGFR gene was present in 35.3% of case patients, low trisomy in 16.7%, high trisomy in 2%, low polysomy in 13.7%, high polysomy in 19.6%, and gene amplification in 12.7% (Table 2; Fig. 1, A–D). Patients with gene amplification and high polysomy had relatively high response rates (54% and 25%), long time to progression (9.1 and 6.3 months), and high 1-year survival rates (48.8% and 62.3%). Patients with high trisomy also had a high response rate (100%), a long time to progression (14 months), and a high 1-year survival (100%), but this group included only two patients. In contrast, among the patients with tumors in the disomy, low trisomy, and low polysomy categories, none were responders, more than 70% had progressive disease, time to progression was 3.5 months or less, and median survival was 7 months or less. For further analyses, patients with high gene copy numbers (gene amplification or high polysomy) were combined because of similar outcome and designated $FISH^+$, and patients in all other categories (disomy, low trisomy, high

Table 1. Characteristics of the non–small-cell lung cancer patients and gefitinib outcome*

Patient characteristic	No. of patients/%	Objective response total/%†	Progressive disease total/%	Median time to progression (mo)	Median survival (mo)	1-year % cumulative survival ± SD, %
Total	102/100	14/14	62/60	2.9	9.4	41 ± 5
Sex						
Male	67/66	4/6	46/69	2.7	8.3	37 ± 6
Female	35/34	10/29	16/46	5.2	11.3	48 ± 9
P		.004‡	.03§	.004‖	.03‖	.22‖
Stage						
III	14/14	1/7	6/43	6.0	8.3	36 ± 13
IV	88/86	13/15	56/64	2.7	9.5	42 ± 6
P		.7‡	.15§	.3‖	.9‖	.77‖
Histology						
Adenocarcinoma[A]	54/53	8/15	34/63	3.2	11.3	45 ± 7
Bronchioloalveolar[A]	9/9	3/33	5/56	3.0	16.5	67 ± 16
Squamous cell[B]	26/26	2/8	14/54	2.2	6.5	22 ± 9
Large cell[B]	2/2	0	2/100	0.8	0.8	0 ± 0
Undifferentiated[B]	11/11	1/9	7/64	2.1	9.0	46 ± 15
P ([A] vs [B])		.2‡	.7§	.3‖	.03‖	.04‖
Performance status¶						
0	49/48	5/10	32/65	2.6	10.1	41 ± 7
1	41/40	7/17	22/54	4.2	10.9	47 ± 8
2	12/12	2/17	8/67	2.1	2.7	22 ± 13
P (0 + 1 vs 2)		.7‡	.7§	.2‖	.004‖	.007‖
Smoking status						
Never	15/15	6/40	6/40	5.3	10.9	47 ± 14
Former	33/32	5/15	17/52	3.6	13.8	55 ± 9
Current	54/53	3/6	39/72	2.3	4.5	30 ± 6
P (Never vs others)		.006‡	.7§	.07‖	.25‖	.35‖

*Characteristics of 102 patients with histologically confirmed non–small-cell lung cancer with measurable, locally advanced or metastatic disease, progressing or relapsing after chemotherapy, or medical contraindications for chemotherapy who were subsequently treated with 250 mg of gefitinib daily. Percentages may not add to 100% due to rounding.

†Objective response = partial and complete response.

‡*P* values (two-sided) calculated using Fisher's exact test.

§*P* values (two-sided) calculated using Pearson's chi-square test.

‖*P* values (two-sided) calculated using the log-rank test.

¶Performance status was defined as 0 = fully active, able to carry on all pre-disease performance without restriction; 1 = restricted in physically strenuous activity but ambulatory and able to perform work of a light or sedentary nature, e.g., light housework, office work; and 2 = ambulatory and capable of all self care but unable to perform any work activities, and up and about more than 50% of waking hours [Eastern Cooperative Oncology Group criteria *(34)*].

trisomy, and low polysomy) were categorized as $FISH^-$. $FISH^+$ patients represented 32.4% of the total group, and $FISH^-$ patients represented 67.6% of the patients.

Next, we compared EGFR FISH categories with clinical outcome. $FISH^+$ patients had higher response rates (36% versus 3%, mean difference = 33.5%, 95% CI = 16.6 to 50.3; $P<.001$) and lower rate of disease progression (33% versus 74%, mean difference = 40.6%, 95% CI = 21.5 to 59.7; $P<.001$) compared with $FISH^-$ patients. $FISH^+$ patients also had longer median time to progression (9.0 versus 2.5 months, difference = 6.5 months, 95% CI = 2.8 to 10.3 months; $P<.001$), longer median survival and higher 1-year survival rate (18.7 versus 7.0 months, mean difference = 11.7 months, 95% CI = 2.1 to 21.4 months, and 57.2% versus 33.2%, mean difference = 24.0%, 95% CI = 20.7 to 27.3, respectively; $P = .03$) (Table 2; Fig. 2, A–B). $FISH^+$ patients were more likely to be female ($P = .04$) and never smokers ($P = .001$) than $FISH^-$ patients, although the association with histology was not statistically significant ($P = .78$) (Table 3).

EGFR gene expression was evaluated by quantitative real-time polymerase chain reaction in 63 specimens. The relative gene expression was 2.90 (range = 0.17–28.0) in 40 specimens with low EGFR gene copy numbers (disomy to low polysomy) and 7.15 (range = 0.19–28.3) in 23 specimens with high EGFR gene copy numbers (high polysomy and gene amplification) and was particularly high among nine tumors with gene amplification (average = 8.46, range = 1.7–21.5). There was a statistically significant positive correlation between the relative expression and the gene copy number (Pearson $r = .33$; $P = .007$), indicating that specimens with gain in copy numbers had higher levels of gene expression. No association was observed between relative gene expression, clinical characteristics, and patient outcome (data not shown).

EGFR Protein Expression and Clinical Outcome

EGFR protein expression was evaluated by immunohistochemistry in 98 patients (Fig. 1, E–H), and the outcome of patients according to protein score is shown in Table 2 and Fig. 2, C–D. Patients with the lowest scores (0–99) had no response, and only one had stable disease. These patients had a short time to progression (median 2.1 months) and short median survival (4.5 months), and 27% had 1-year survival. Patients with scores of 100–199 also had a poor outcome, with a 65% rate of progressive disease, short time to progression (median 2.3 months), and poor survival (only 35% of the patients alive at 1 year). Because their outcomes were similarly poor, the 40 patients (41%) with scores less than 100 and in the range 100–199 were combined ($EGFR\ IHC^-$). Patients with EGFR immunohistochemistry scores of 200–299 and of 300–399 had much better outcomes than patients in the $EGFR\ IHC^-$ group, and because they had

Table 2. Objective response rate, disease control rate, time to progression, and survival analysis in groups of non–small-cell lung cancer patients by ascending number of copies of the epidermal growth factor receptor (EGFR) gene, level of protein expression, and mutation results*

EGFR markers	No. of patients/%	Objective response, N/%	Progressive disease, N/%	Median time to progression (mo)	Median survival (mo)	1-year cumulative survival ± SD, %
FISH status: total	102/100	14/14	62/61	2.9	9.4	41 ± 5
Disomy	36/35	0	27/75	2.4	7.0	36 ± 8
Low trisomy	17/16	0	12/71	3.5	10.9	41 ± 12
High trisomy	2/2	2/100	0	13.5	NR	NR
Low polysomy	14/14	0	12/86	2.0	3.3	8 ± 8
High polysomy	20/20	5/25	8/40	6.3	13.8	62 ± 11
Gene amplification	13/13	7/54	3/23	9.1	10.3	49 ± 15
EGFR $FISH^-$	69/67	2/3	51/74	2.5	7.0	33 ± 6
EGFR $FISH^+$	33/33	12/36	11/33	9.0	18.7	57 ± 9
P ($FISH^+$ vs $FISH^-$)		<.001†	<.001‡	<.001§	.03§	.03§
Protein status: Total	98/100	14/14	58/59	2.9	9.5	41 ± 5
Score 0–99	20/20	0	19/95	2.1	4.5	27 ± 10
Score 100–199	20/20	2/10	13/65	2.3	5.3	35 ± 11
Score 200–299	15/15	4/26	5/33	8.6	15.2	72 ± 12
Score 300–400	43/44	8/19	21/49	4.5	11.3	41 ± 8
EGFR IHC^- (<200)	40/41	2/5	32/80	2.3	5.0	31 ± 8
EGFR IHC^+(≥200)	58/59	12/21	26/45	5.2	11.5	48 ± 7
P (IHC^- vs IHC^+)		.03‡	<.001‡	.001§	.01§	.01§
EGFR mutations: Total	89/100	12/13	56/63	2.9	9.4	41 ± 5
Mutation absent	74/83	4/5	50/68	2.6	8.4	38 ± 6
Mutation present	15/17	8/53	6/40	9.9	20.8	57 ± 13
P		<.001†	.04‡	.02§	.09§	.22§

*Characteristics of 102 patients with histologically confirmed non–small cell lung cancer with measurable, locally advanced or metastatic disease, progressing or relapsing after chemotherapy, or medical contraindications for chemotherapy who were subsequently treated with 250 mg of gefitinib daily. Objective response = partial and complete response; NR = not reached. Fluorescence in situ hybridization $(FISH)^+$ = high polysomy (≥4 copies in ≥40% of cells) or gene amplification (defined by the presence of tight EGFR gene clusters and a ratio of EGFR gene to chromosome of ≥2 or ≥15 copies of EGFR per cell in ≥10% of analyzed cells). $FISH^-$ = disomy (≤2 copies in >90% of cells), low trisomy (≤2 copies in ≥40% of cells, 3 copies in 10%–40% of the cells, ≥4 copies in <10% of cells), high trisomy (≤2 copies in ≥40% of cells, 3 copies in ≥40% of cells, ≥4 copies in <10% of cells), or low polysomy (≥4 copies in 10%–40% of cells). Protein status by immunohistochemistry (IHC) was defined as 0 = no appreciable staining in the tumor cells, 1 = barely detectable staining in the cytoplasm and/or nucleus as compared with the stromal elements, 2 = readily appreciable brown staining distinctly marking the tumor cell cytoplasm and/or nucleus, 3 = dark brown staining in tumor cells obscuring the cytoplasm and/or nucleus, or 4 = very strong staining of nucleus and/or cytoplasm. Frequency score was based on fraction of positive cells; 0–100%. The total score was calculated by multiplying the intensity score and the fraction score, making a total range of 0–400.

†*P* values (two-sided) calculated using Fisher's exact test.

‡*P* values (two-sided) calculated using Pearson's chi-square test.

§*P* values (two-sided) calculated using the log-rank test.

similar response rates, progression times, and survival, they were also grouped together (EGFR IHC^+). EGFR IHC^+ patients, compared with IHC^- patients, had statistically significantly higher objective response rate (21% versus 5%; $P = .03$), lower progression rate (44.8% versus 80%; $P<.001$), longer time to progression (5.2 versus 2.3 months; $P = .001$), and longer survival (11.5 versus 5.0 months; $P = .01$). Protein status was not associated with clinical characteristics (Table 3) but was statistically significantly correlated with gene copy numbers (Pearson $r = .28$, $P = .006$).

EGFR Mutation Analysis and Clinical Outcome

Mutation analysis for EGFR exons 18, 19, and 21 was performed in 89 case patients (60 microdissected and 29 nonmicrodissected specimens). EGFR mutations were found in 15 patients (EGFR mutation positive = 17%), 12 from microdissected and three from nonmicrodissected specimens ($P = .30$), and consisted of missense mutations in exon 21 ($n = 8$) or small in-frame deletions in codons 746–753 in exon 19 ($n = 7$) (Fig. 3). All of these mutations have previously been described *(11–13)*, with the exception of the missense mutation in exon 21 (valine 851 to isoleucine, V851→I), which occurred in a male patient experiencing progressive disease. The presence of EGFR mutations was associated with never-smoking history ($P = .007$). The associations with sex and histology were not statistically significant ($P = .10$ for both), although mutations were more frequent in women and in patients with adenocarcinoma (Table 3).

We also compared associations between EGFR mutation status, FISH status, and level of protein expression in each tumor with patient outcome (Table 4). EGFR mutations were statistically significantly associated with $FISH^+$ status ($P = .01$) but not with high protein expression ($P = .10$). Gene mutations were statistically significantly associated with better response (54% versus 5%, mean difference = 47.9%, 95% CI = 22.2 to 73.7; $P<.001$) and longer time to progression (9.9 versus 2.6 months, mean difference = 7.3 months, 95% CI = 2.1 to 16.7 months; $P = .02$) (Table 2; Fig. 2, E–F). Patients with EGFR mutations had better survival, although it was not statistically significant (median 20.8 versus 8.4 months, mean difference = 12.4 months, 95% CI = 1.7 to 26.4 months; $P = .09$). However, six of the 15 patients with mutations (40%), five of whom carried point mutations in exon 21 (patients 1, 2, 3, 16, and 100; Fig. 3) and one of whom had an exon 19 deletion (patient 41, Fig. 3) had progressive disease. Among the eight patients with EGFR mutations responding to the treatment, seven were also $FISH^+$, whereas four of six progressing patients with mutations were $FISH^-$ (disomy). Moreover, among the 21 patients with stable disease, only one presented EGFR mutations.

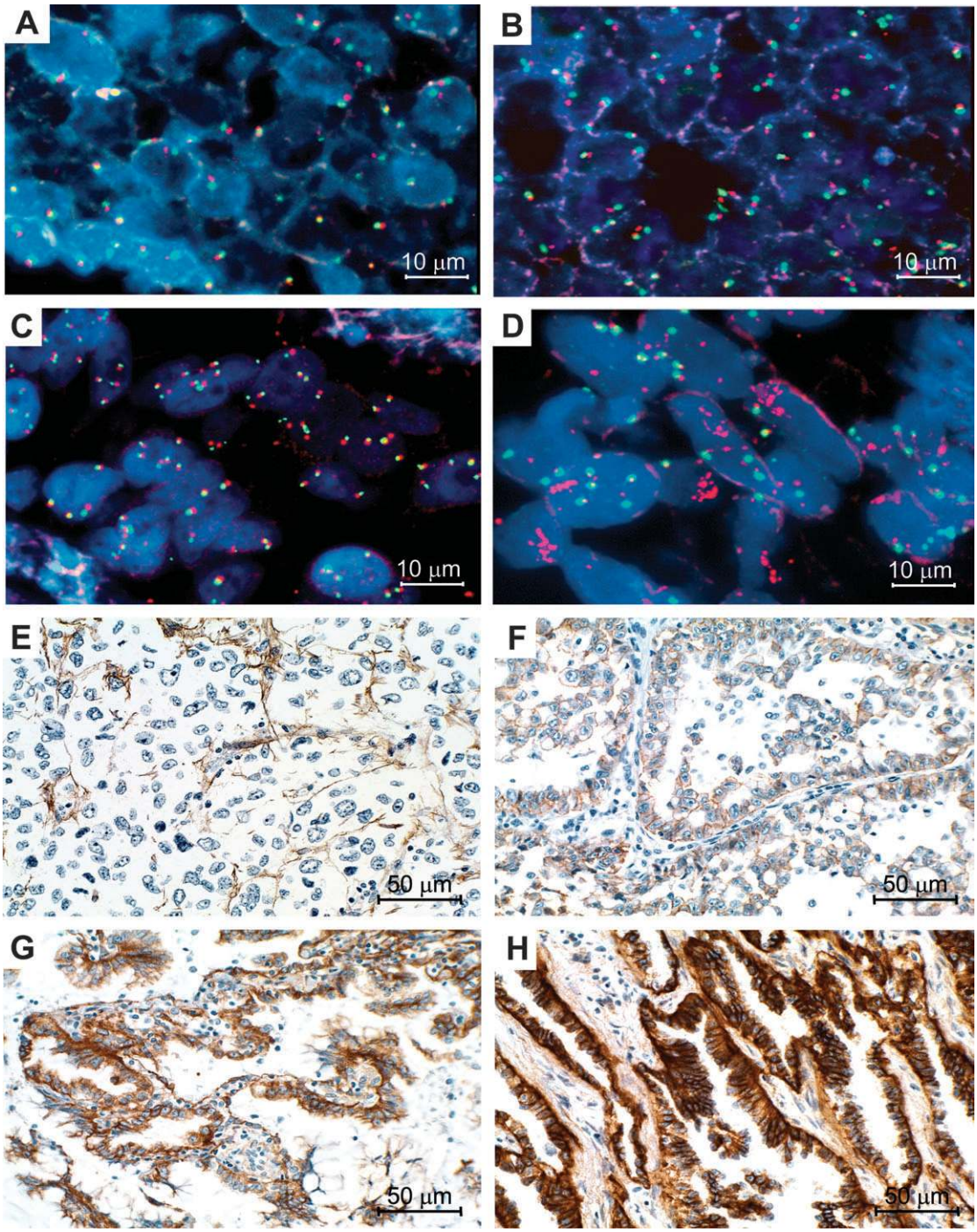

Fig. 1. EGFR content as determined by fluorescence in situ hybridization (FISH) and immunohistochemical staining. FISH was performed with the EGFR (**red**)/CEP 7 (**green**) probe (Vysis; Abbott Laboratories, IL). Panels illustrate specimens representing no (disomy = **A**) or low (trisomy = **B**) gain in gene copy number per cell (EGFR FISH negative); high (high polysomy = **C**; gene amplification = **D**) gain in gene copy number per cell (EGFR FISH positive). Total nuclear DNA was stained with 4′,6′-diamidino-2-phenylindole (**blue**). Immunohistochemistry was performed with a mouse anti–human EGFR monoclonal antibody (Zymed Labs, San Francisco, CA). A semiquantitative approach was used to generate a score for each tissue section. Percentage of stained cells (0%–100%) was multiplied by the dominant intensity pattern of staining, considering 1 as negative or trace, 2 as weak, 3 as moderate and 4 as strong. Therefore, the overall score ranged from 0 to 400. Specimens with a score of 200 or less were considered negative (EGFR protein negative), whereas a score greater than 200 was considered positive (EGFR protein positive). Panels illustrate specimens graded with score 0 (intensity pattern 0 in 100% of cells = **E**), 200 (pattern 2 in 100% of cells = **F**) score 300 (pattern 3 in 100% of cells = **G**), 400 (pattern 4 in 100% of cells = **H**).

EGFR Multivariable Analysis

To define which variables were predictive for survival, those factors that were statistically significant in the univariate analysis (sex, histology, performance status, FISH, and protein status) were included in a multivariable model. Mutation and smoking status were not included because they were not associated with survival ($P = .09$ and $P = .20$, respectively) in univariate analyses. Poor performance status (PS 2) remained statistically significantly associated with increased risk of death (hazard ratio [HR] = 3.27, 95% CI = 1.49 to 7.17; $P = .003$), whereas adenocarcinoma/bronchioloalveolar histologies (HR = 0.58, 95% CI = 0.35 to

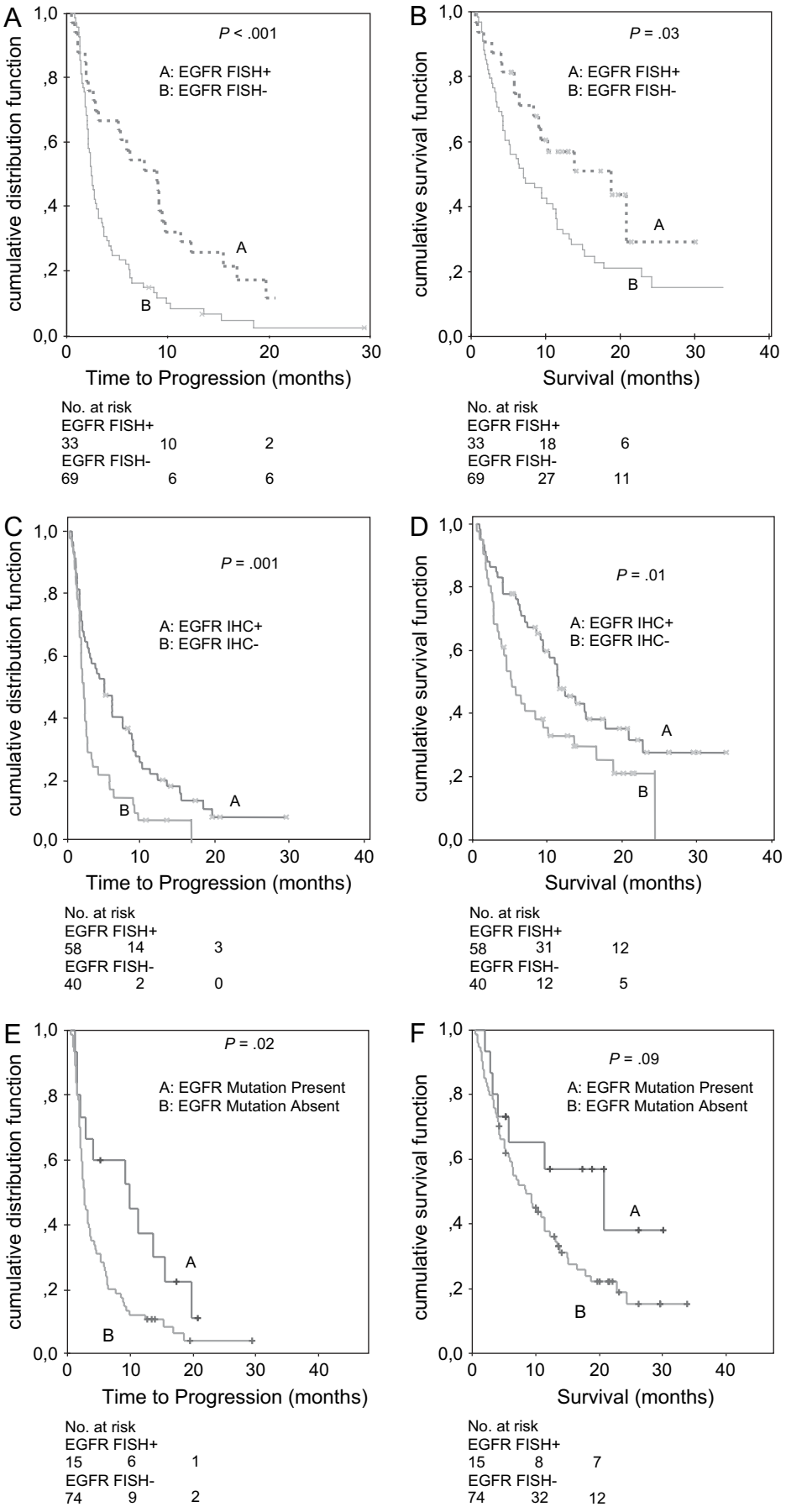

Fig. 2. Kaplan–Meier curves for time to disease progression and survival. Data were analyzed according to gene copy numbers (**A–B**), level of protein expression (**C–D**), and presence of mutations (**E–F**). Time to disease progression was calculated from the date of initiation of gefitinib treatment to the date of detection of progressive disease or to the date of last contact. Survival was calculated from the date of therapy initiation to the date of death or to the date of last contact. Median time to progression was 9.0 months (95% CI = 4.9 to 13.1 months) for EGFR FISH$^+$ patients and 2.5 months (95% CI = 2.1 to 2.8 months) for EGFR FISH$^-$, 5.2 months (95% CI = 2.8 to 7.7 months) for EGFR IHC$^+$ and 2.3 months (95% CI = 1.9 to 2.7 months) for EGFR IHC$^-$, 9.9 months (95% CI = 0 to 19.9 months) for EGFR mutation$^+$ and 2.7 months (95% CI = 2.1 to 3.2 months) for EGFR mutation$^-$. Median survival was 18.7 months (95% CI = 5.0 to 32.5 months) for EGFR FISH$^+$ patients and 7.1 months (95% CI = 2.9 to 11.1 months) for EGFR FISH$^-$, 11.5 months (95% CI = 95% 8.4 to 14.5 months) for EGFR IHC$^+$ and 5.0 months (95% CI = 3.3 to 6.8 months) for EGFR IHC$^-$, 20.8 months (95% CI = 3.5 to 38.2 months) for EGFR mutation$^+$ and 8.5 months (95% CI = 5.2 to 11.8 months) for EGFR mutation$^-$. Statistical significance of differences between groups was evaluated with the log-rank test.

Table 3. Epidermal growth factor receptor (EGFR) and characteristics of the non–small-cell lung cancer patients*

Patient characteristics	EGFR FISH status		EGFR protein status		EGFR gene mutation	
	Positive, N/%	Negative, N/%	Positive, N/%	Negative, N/%	Present, N/%	Absent, N/%
Total	33/32	69/68	58/59	40/41	15/17	74/83
Sex						
Male	17/51	50/72	37/64	27/67	7/47	51/69
Female	16/48	19/28	21/36	13/32	8/53	23/31
P	.04†		.70†		.10†	
Histology						
Adenocarcinoma[A]	18/54	36/52	29/50	22/55	10/67	40/54
Bronchioloalveolar[A]	3/9	6/9	4/7	5/12	2/13	6/8
Squamous cell[B]	9/27	17/25	18/31	8/20	1/7	20/27
Large cell[B]	1/3	1/1	1/2	1/2	0	1/1
Undifferentiated[B]	2/6	9/13	6/10	4/10	2/13	7/9
P ([A] vs [B])	.78†		.29†		.10†	
Performance status						
0	13/39	36/52	27/47	20/50	8/53	35/47
1	13/39	28/40	27/47	12/30	5/33	31/42
2	7/21	5/7	4/7	8/20	2/13	8/11
P (0 + 1 vs 2)	.053‡		.06‡		.60‡	
Smoking status						
Never	11/33	4/6	10/17	5/12	6/40	7/9
Former	8/24	25/36	21/36	11/27	5/33	24/32
Current	14/42	40/58	27/47	24/60	4/26	43/58
P (Never vs others)	.001‡		.52†		.007‡	

*Characteristics of 102 patients with histologically confirmed non–small-cell lung cancer patients with measurable, locally advanced or metastatic disease, progressing or relapsing after chemotherapy, or medical contraindications for chemotherapy who were subsequently treated with 250 mg of gefitinib daily Performance status was defined as 0 = fully active, able to carry on all predisease performance without restriction; 1 = restricted in physically strenuous activity but ambulatory and able to perform work of a light or sedentary nature, e.g., light housework, office work; and 2 = ambulatory and capable of all self-care but unable to perform any work activities, and up and about more than 50% of waking hours [Eastern Cooperative Oncology Group criteria, *(34)*]. FISH = fluorescence in situ hybridization.

†*P* values (two-sided) calculated using Pearson's chi-square test.

‡*P* values (two-sided) calculated using Fisher's exact test.

0.96; $P = .035$) and FISH status (HR = 0.44, 95% CI = 0.23 to 0.82; $P = .01$) were statistically significantly associated with better survival. Protein status (HR = 0.60, 95% CI = 0.36 to 1.01; $P = .056$) and sex (HR = 1.43, 95% CI = 0.79 to 2.6; $P = .20$) were not statistically significantly associated with survival. To demonstrate that EGFR adds to the predictive power of the other variables, we fit the multivariable model with and without EGFR and compared the two models by likelihood ratio method. The −2 log likelihood for the first model without EGFR was 505.16. The −2 log likelihood for the second model with EGFR was 500.20. The difference between the two models is 4.96 ($P<.05$). Statistical significance is based on the chi-square test with 1 degree of freedom.

Association Between EGFR and P-Akt

Evaluation of the P-Akt protein was successful in 98 patients (Table 4). P-Akt–positive status was significantly associated with better response rate (21% versus 0%, mean difference = 20.6%, 95% CI = 11.0 to 30.2; $P = .004$), disease control rate (50% versus 22%, mean difference = 28.1%, 95% CI = 9.5 to 46.7; $P = .008$), longer time to progression (4.2 versus 2.1 months, mean difference = 2.1 months, 95% CI = 0.7 to 3.4 months; $P = .01$), but not with survival (11.4 versus 9.4 months, mean difference = 2.0 months, 95% CI = 1.3 to 5.3 months; $P = .20$). P-Akt–positive status was also statistically significantly associated with EGFR gene gain ($FISH^+$ Pearson $r = .30$; $P = .01$) and high level of protein expression (EGFR IHC^+ Pearson $r = .27$; $P = .01$), but not with EGFR mutation ($P = .08$).

Combining FISH and P-Akt data (Table 5), we observed that double-positive patients (EGFR $FISH^+$/$P\text{-}Akt^+$) had a statistically significantly higher response rate (41% versus 3%, mean difference = 38.5%, 95% CI = 20.1 to 56.8; $P<.001$) and disease control rate (72% versus 28%, mean difference = 44.9%, 95% CI = 26.6 to 65.3; $P<.001$), longer time to progression (9.0 versus 2.5 months, mean difference = 6.5 months, 95% CI = 3.3 to 9.8 months; $P<.001$) and survival (18.7 versus 9.4 months, mean difference = 9.3 months, 95% CI = 4.7 to 13.9 months; $P = .04$) compared with EGFR $FISH^-$ patients and/or $P\text{-}Akt^-$ patients. Similar findings were observed when EGFR immunohistochemistry and mutation data were combined with P-Akt data. Compared with $EGFR^-$ and/or $P\text{-}Akt^-$ patients, EGFR IHC^+/$P\text{-}Akt^+$ patients had a statistically significantly better response rate (29% versus 4%, mean difference = 25.8%, 95% CI = 10.9 to 40.4; $P<.001$), disease control rate (66% versus 23%, mean difference = 43.1%, 95% CI = 23.9 to 60.6; $P<.001$), longer time to progression (6.2 versus 2.3 months, mean difference = 3.9 months, 95% CI = 1.5 to 6.3 months; $P = .001$), and longer survival (14.9 versus 8.3 months, mean difference = 6.6 months, 95% CI = 4.0 to 9.2 months; $P = .03$). EGFR $mutation^+$/$P\text{-}Akt^+$ patients had a statistically significantly better response rate (67% versus 6%, mean difference = 61.2%, 95% CI = 34.0 to 88.4; $P<.001$), disease control rate (75% versus 32%, mean difference = 43.5%, 95% CI = 16.8 to 70.2; $P = .008$), longer time to progression (11.2 versus 2.6 months, mean difference = 8.6 months, 95% CI = 3.3 to 14.0 months; $P = .004$), and longer survival (20.8 versus 9.3 months, mean difference = 11.5 months, 95% CI = 1.1 to 24.2 months; $P = .044$) than EGFR $mutation^-$ and/or $P\text{-}Akt^-$ patients.

Independent of the method of EGFR assessment, patients who were EGFR positive and P-Akt negative did not respond to gefitinib treatment (Table 5). The group of patients EGFR IHC^+/$P\text{-}Akt^-$ had a statistically significantly worse outcome than

Exon 19 deletions	
EGFR protein	739 K I P V A I K E L R E A T S P K A N 756
EGFR gene	2215 AAA ATT CCC GTC GCT ATC AAG GAA TTA AGA GAA GCA ACA TCT CCG AAA GCC AAC 2268
Patient 15	AAA ATT CCC GTC GCT ATC AAG … … … … … … TC T CCG AAA GCC AAC
Patients 19, 30, 41 and 53*	AAA ATT CCC GTC GCT ATC AA . … … … … ..A ACA TCT CCG AAA GCC AAC
Patient 57	AAA ATT CCC GTC GCT ATC AAG GAA T.. … … … … .CT CCG AAA GCC AAC
Patient 75[#]	AAA ATT CCC GTC GCT ATC AAG … … … … … ACA TCT CCG AAA GCC AAC
Notes	* Similar to patient 1 in (11) [#] Similar to the Del-1b (12)
Exon 21 mutations	
EGFR protein	850 H V K I T D F G L A K L L G 863
EGFR gene	2538 CAT GTC AAG ATC ACA GAT TTT GGG CTG GCC AAA C TG C TG GGT 2589
Patients 1, 2, 16, 26, 31, 38[†], 100 (substitution 2573 T>G)	H V K I T D F G **R** A K L L G CAT GTC AAG ATC ACA GAT TTT GGG C**G**G GCC AAA CTG CTG GGT
Patient 3 (Substitution 2541 G>A)[‡]	H **I** K I T D F G L A K L L G CAT ATC AAG ATC ACA GAT TTT GGG CTG GCC AAA C TG C TG GGT
Notes	[†]Patient 38 predominantly mutant. [‡]Patient 3 mutation has not been reported in SNP database.
Primers used for Mutation Analysis	
Exon 18 forward	GACCCTTGTCTCTGTGTTCTTGT
Exon 18 reverse outside	TATACAGCTTGCAAGGACTCTGG
Exon 18 reverse inside	CCAGACCATGAGAGGCCCTG
Exon 19 forward	CACAATTGCCAGTTAACGTCTTC
Exon 19 reverse outside	AGGGTCTAGAGCAGAGCAGC
Exon 19 reverse inside	GCCTGAGGTTCAGAGCCAT
Exon 21 forward	CATGATGATCTGTCCCTCACAG
Exon 21 reverse outside	CTGGTCCCTGGTGTCAGGAA
Exon 21 reverse inside	GCTGGCTGACCTAAAGCCACC

Fig. 3. Somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) gene in non–small-cell lung cancer (NSCLC) patients. Mutation analysis for EGFR exons 18, 19, and 21 was performed in a total of 89 patients (60 from microdissected and 29 from nonmicrodissected specimens). Tumor areas were microdissected by manual or by laser capture technique using the PALM instrument (PALM Microlaser Technologies AG, Bernried, Germany), according to the manufacturer's guidelines. Fifty nanograms of genomic DNA was amplified for EGFR exons 18, 19, and 21 by touchdown heminested polymerase chain reaction and sequenced in both sense and antisense directions.

the group positive for both proteins, in terms of response rate (0% versus 29%, mean difference = 29.3%, 95% CI = 15.3 to 43.2; $P = .012$), disease control rate (29% versus 66%, mean difference = 36.5%, 95% CI = 10.4 to 62.5; $P = .011$), and had a non–statistically significantly shorter time to progression (1.8 versus 6.2 months, mean difference = 4.4 months, 95% CI = 2.3 to 6.4 months; $P = .08$) and survival (9.4 versus 14.9 months, mean difference = 5.5 months, 95% CI = 1.6 to 9.3 months; $P = .21$). No comparisons were made with EGFR FISH and EGFR mutation because of the small number of patients (i.e., four and two, respectively) in the group positive for EGFR and negative for P-Akt.

Unfavorable outcomes were also observed in the group of patients negative for EGFR but positive for P-Akt (Table 5). Compared with the double-positive group, the EGFR FISH$^-$/P-Akt$^+$ group had a statistically significantly worse response rate (5% versus 41%, mean difference = 36.1%, 95% CI = 16.8 to 55.4; $P<.001$), disease control rate (32% versus 72%, mean difference 40.8%, 95% CI = 3.7 to 9.1 months; $P = .001$) and a non–statistically significantly shorter survival (8.4 versus 18.7 months, mean difference = 10.3 months, 95% CI = 7.2 to 13.4 months; $P = .083$). Similar findings were observed when EGFR was evaluated by immunohistochemistry or for mutations. In both cases, the EGFR$^-$/P-Akt$^+$ group had a statistically significantly worse response rate ($P = .034$ and $P<.001$, respectively, for protein and mutation), disease control rate ($P = .002$ and $P = .025$), time to progression ($P = .010$ and $P = .009$) and had a non–statistically significantly worse survival ($P = .080$ and $P = .070$), compared with the double-positive group.

Discussion

In this study, we have shown that EGFR gene copy number, detected by FISH, is statistically significantly associated with response to gefitinib, and that gefitinib-treated patients carrying EGFR gene amplification or high polysomy had a statistically significant improvement in response, time to progression, and survival compared with patients with no or low genomic gain for EGFR. Multivariable analysis confirmed that EGFR gene amplification and high polysomy statistically significantly reduced the risk of death in patients receiving gefitinib (HR = 0.44, 95% CI = 0.23 to 0.82). Gefitinib sensitivity was also associated with high EGFR protein expression, and patients with low scores (<200) had an outcome as poor as those with low gene copy numbers or lacking mutations. In addition, in patients with positive EGFR status, the presence of Akt phosphorylation was significantly related to better response, disease control rate, time to progression, and survival. Our results indicate that high EGFR gene copy number identified by FISH may be an effective molecular predictive marker for gefitinib sensitivity in patients with advanced NSCLC.

Table 4. Epidermal growth factor receptor (EGFR) and phosphorylated (P)-Akt protein levels and outcome for non–small-cell lung cancer patients presenting EGFR mutation or gene amplification*

Patient	EGFR gene amplification	EGFR gene mutation	EGFR IHC	P-Akt	Response	Time to progression (mo)	Overall survival (mo)
1	–	L858R	–	+	PD	2.11	2.11
2	–	L858R	+	–	PD	2.18	+5.3
3	–	V852I	+	+	PD	4.05	4.05
4	+	ND	–	+	PD	2.2	2.73
12	+	None	–	+	SD	5.99	8.32
15	+	Exon 19 del	+	+	PR	+5.33	+5.33
16	–	L858R	+	–	PD	1.61	3.16
19	+	Exon 19 del	–	+	PR	9.18	+18.9
26	–	L858R	+	+	PR	13.6	+26.2
30	–	Exon 19 del	+	+	SD	9.87	11.5
31	+	L858R	+	+	PR	+17.4	+17.4
37	+	None	+	+	PD	2.66	4.05
38	+	L858R	+	+	CR	19.7	20.8
41	–	Exon 19 del	–	+	PD	2.89	5.72
51	+	ND	+	+	SD	7.7	+8.75
53	+	Exon 19 del	+	+	PR	+20.7	+20.7
57	–	Exon 19 del	+	+	PR	11.3	+12.2
75	+	Exon 19 del	+	+	PR	15.6	+30.2
91	+	ND	+	+	SD	5.16	8.098
100	–	L858R	–	ND	PD	1.55	2.86
101	+	ND	+	+	PR	9.05	10.3
102	+	None	+	+	PD	3.22	3.95

*Characteristics of 102 patients with histologically confirmed non–small-cell lung cancer with measurable, locally advanced or metastatic disease, progressing or relapsing after chemotherapy, or medical contraindications for chemotherapy who were subsequently treated with 250 mg of gefitinib daily. ND: not determined; PD = progressive disease, SD = stable disease, PR = partial response; CR = complete response. IHC = immunohistochemistry. EGFR gene amplification$^+$ = presence of gene amplification. EGFR gene amplification$^-$ = absence of amplification. EGFR IHC$^+$ = positive. EGFR IHC$^-$ = negative. P-Akt$^+$ = positive. P-Akt$^-$ = negative. Time to progression and survival$^+$ = censored.

Some limitations to the study must be taken into consideration. Our finding is so far based on a single retrospective study with a relatively small number of patients, and before any clinical recommendation can be made, our data need to be verified in a larger cohort of patients and prospectively. The EGFR status was determined on tumor tissue at time of primary diagnosis, and possible changes after chemotherapy were not determined in this study. Finally, both the classifications of the EGFR gene copy status and protein status represent a posthoc aggregation based on our previous reported studies *(10)*. However, although our group and others are exploring other methods to express the results of the EGFR expression, our classifications seems to be associated with a meaningful clinical outcome after gefitinib therapy.

The question could be raised whether increased EGFR copy number per se has a positive impact on prognosis, independent of the treatment. The converse appears to be the case; we have previously reported that NSCLC patients with resected tumors carrying high EGFR gene copy number have a tendency to a shorter survival *(10)*. Thus, similar to the findings in breast cancer for HER2 and trastuzumab (Herceptin; Genentech/Roche), increased EGFR gene copy number in NSCLC seems to be a poor prognostic feature but a good predictor for sensitivity to EGFR inhibitors.

The presence of EGFR gene mutations was also related to better response to gefitinib and time to progression, but the difference in survival did not reach statistical significance. An interesting finding was the association between EGFR mutations and increased gene copy number, a phenomenon that was recently described in the human lung cancer cell line H3255 *(32)* and that is probably relevant to gefitinib sensitivity. In fact, among the eight patients with EGFR mutations who responded to gefitinib therapy, seven were also FISH$^+$, and among the six nonresponding patients with EGFR mutations, four presented a disomic pattern. This observation suggests that the impact of genomic gain is critical for EGFR mutations to predict gefitinib sensitivity.

Another important finding was the virtual absence of EGFR mutations in patients with stable disease. Among the 21 patients with stable disease who were assessed for EGFR mutations, only one patient had an EGFR mutation. We defined stable disease as neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease, as confirmed by two consecutive observations no less than 4 weeks apart. The small number of mutations in patients with stable disease is of clinical relevance because data from the BR.21 trial *(17)* show that the survival benefit of gefitinib is not confined to responding patients. It is possible that survival improvement in the gefitinib-treated patients, as a whole, is due to the presence of a group of patients with an intermediate benefit from the treatment, such as those with stable disease, who would be excluded from tyrosine kinase inhibitor treatment if mutation analysis were established as the test of choice for patient selection. Moreover, although previous studies suggested that EGFR mutations are present in almost all responding patients *(21–23)*, in this study we observed that 40% of patients with EGFR mutations had progressive disease. These somewhat discrepant results could be explained by the fact that this is the first study conducted in a large and unselected cohort of gefitinib-treated patients, in whom clinical results are similar to those obtained in large clinical trials with gefitinib *(13,14)*.

In this study, gefitinib sensitivity was associated with high EGFR protein expression; outcomes in patients with low EGFR expression scores (<200) were as poor as those in patients with

Table 5. Association between epidermal growth factor receptor (EGFR) fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), and mutation with phosphorylated (P)-Akt in non–small-cell lung cancer patients*

Markers	No. of patients/%	Objective response, N/%	Disease control rate, N/%	Median time to progression (mo)	Median survival (mo)	1-year cumulative survival ± SD, %
EGFR FISH/P-Akt	98/100	14/14	40/40	4.5	11.5	47 ± 6
EGFR FISH$^+$/P-Akt$^+$	29/30	12/41	21/72	9.0	18.7	33 ± 9
EGFR FISH$^+$/P-Akt$^-$	4/4	0	1/25	1.1	13.8	75 ± 22
EGFR FISH$^-$/P-Akt$^+$	38/39	2/5	12/32	2.6	8.4	38 ± 8
EGFR FISH$^-$/P-Akt$^-$	27/28	0	6/22	2.4	6.0	57 ± 9
Any negative	69/70	2/3	19/28	2.5	9.4	37 ± 6
P (Any – vs +/+)		<.001†	<.001‡	<.001§	.041§	.075§
EGFR IHC/P-Akt	98/100	14/14	40/40	3.2	11.3	45 ± 6
EGFR IHC$^+$/P-Akt$^+$	41/42	12/29	27/66	6.2	14.9	29 ± 14
EGFR IHC$^+$/P-Akt$^-$	17/17	0	5/29	1.8	9.4	35 ± 12
EGFR IHC$^-$/P-Akt$^+$	26/27	2/8	7/27	2.3	6.4	38 ± 10
EGFR IHC$^-$/P-Akt$^-$	14/14	0	1/7	2.0	4.2	54 ± 8
Any negative	57/58	2/3	13/23	2.3	8.3	35 ± 7
P (Any – vs +/+)		<.001‡	<.001‡	.001§	.029§	.032§
EGFR mutation/P-Akt	85/100	12/14	32/38	2.9	10.1	43 ± 5
EGFR mutation$^+$/P-Akt$^+$	12/14	8/67	9/75	11.2	20.8	38 ± 10
EGFR mutation$^+$/P-Akt$^-$	2/2	0	0	1.1	3.1	40 ± 7
EGFR mutation$^-$/P-Akt$^+$	44/52	4/9	17/39	2.7	8.4	50 ± 35
EGFR mutation$^-$/P-Akt$^-$	27/32	0	6/22	2.4	9.4	65 ± 14
Any negative	73/86	4/5	23/31	2.6	9.3	39 ± 6
P (Any – versus +/+)		<.001†	.008†	.004†	.044§	.116§

*Characteristics of 102 patients with histologically confirmed non–small-cell lung cancer with measurable, locally advanced or metastatic disease, progressing or relapsing after chemotherapy, or medical contraindications for chemotherapy who were subsequently treated with 250 mg of gefitinib daily.

†*P* values (two-sided) calculated using Fisher's exact test.

‡*P* values (two-sided) calculated using Pearson's chi-square test.

§*P* values (two-sided) calculated using the log-rank test.

low gene copy numbers or lacking mutations, which is different from what has been observed in previous studies *(18–20)*. The lack of standardization in staining procedures and guidelines for interpretation of the EGFR assessment may be the major reason for the conflicting results across studies. The cutoff level used to classify a patient as "positive" and "negative" also varies from one study to another. In an earlier study *(18)*, we used the Zymed monoclonal antibody (Zymed Lab, San Francisco, CA) and scored the specimens according to the Herceptest criteria; tumors were classified as negative when the immunohistochemical staining score was 0 to 1+ and positive when the score was 2+ to 3+. Parra et al. *(20)*, using the DAKO EnVision visualization system (DAKO Corp, Glostrup, Denmark), scored staining intensity as negative to faint (0/1+) or medium to strong (2+/3+) and classified tumors with less than 20% immunoreactive cells as negative/low expressors and those with at least 20% immunoreactive cells as high expressors. In specimens from the IDEAL trials *(19)*, the pharmDx kit (DakoCytomation, Glostrup, Denmark) was used, and EGFR expression was measured for both membrane and cytoplasmic staining using a scoring system with values ranging from 0 to 3. In this study, we used the same antibody as we did in our previous study *(18)*, but a different scoring system. The final score of EGFR expression was made by multiplication of the percentage of positive cells (0%–100%) and the dominant pattern of intensity staining intensity (1 = negative or trace; 2 = weak; 3 = moderate; 4 = intense), creating a score ranging from 0 to 400.

The sampling size and selection of tissue material for immunohistochemical staining might also contribute to explain differences in results across the studies. For instance, tumors from only 43 and 50 patients were evaluated by Cappuzzo et al. *(18)* and Parra et al. *(20)*, respectively. In the retrospective immunohistochemical analysis of tumor tissue from the IDEAL trials, less than 40% of the total population of patients were studied *(19)*, whereas in our study more than 90% of patients had tissue available for immunohistochemical staining. Although the authors of the IDEAL trials have reported that the subgroup of patients who underwent EGFR analysis was representative of the whole study population in terms of sex, age, and histology, it is possible that the larger tumors, which have more tissue available for immunohistochemical staining analysis, represent distinct biology from that of small tumors, from which tumor tissue may not have been available. The impact on the outcome by using different antibodies and scoring systems for immunohistochemical staining assessment of EGFR should be addressed in future comparative investigations. To our knowledge, such data are not currently available either for NSCLC or for other tumors.

In this study, we also found an association between activated Akt pathway (e.g., expression of phosphorylated Akt) and gefitinib sensitivity, an association that has also been described and discussed by others *(25,26)*. The combinatorial analysis of EGFR and P-Akt status indicated that, independent of the method of EGFR assessment, when EGFR status was positive, the presence of Akt phosphorylation was statistically significantly related to better response, disease control rate, time to progression, and survival. Importantly, better outcome was observed not only when the subset of EGFR$^+$/P-Akt$^+$ patients was compared with all the other groups combined but also when this subset was compared with patients EGFR positive but P-Akt negative. These findings support the hypothesis that, when the gefitinib target is present but the antiapoptotic pathway is not activated, the patient is not sensitive to the inhibitory effects of gefitinib, as suggested by our previous study *(26)* and as demonstrated in preclinical models *(28,29)*. As expected, the EGFR$^+$/P-Akt$^+$ group also had a statistically significantly better outcome compared with the EGFR-negative and P-Akt–positive group, confirming preclinical

data indicating that aberrant, EGFR-independent Akt activation may lead to gefitinib resistance *(29,30)*. These data indicate that P-Akt–positive status is relevant in EGFR-positive patients for the identification of a subgroup of patients particularly sensitive to the drug. In EGFR-negative patients, P-Akt–positive status may identify a group of patients with a very low chance of benefiting from gefitinib treatment.

Information regarding the relationship between EGFR protein expression and Akt pathway activation would greatly advance the understanding of the mechanisms of gefitinib sensitivity. We compared EGFR protein and P-Akt expression in a subgroup of patients and, in general, we found expression of EGFR and P-Akt proteins in the same cell populations (data not shown), suggesting that the observed P-Akt was a result of EGFR activity. However, in some cases we found discrepancies in the expression (i.e., some cells expressed EGFR and not P-Akt and vice versa.). Reasons for discrepancies could be biological; for instance, Akt in EGFR-negative cell populations could be activated by other pathways. However, the discrepancies could also be explained by technical reasons, because the sections compared were not always in immediate proximity to each other. Future prospective studies should be undertaken to verify whether the same cells express both EGFR protein and activated Akt or whether the Akt activation for drug activity might also occur through other paracrine pathway mechanisms.

In conclusion, results from this study demonstrate that gefitinib is most effective in advanced NSCLC patients with high EGFR gene copy number, protein expression, or EGFR mutations. Because only high EGFR gene copy number was associated with prolonged survival in the multivariable analysis, and because FISH is a readily available clinical test, the EGFR FISH analysis represents an ideal test for selecting candidate NSCLC patients for gefitinib therapy. Because patients who had either high EGFR gene copy number or protein expression and P-Akt expression had a better response, disease control rate, time to progression, and survival, analysis of the activating status of the Akt protein may also be relevant for proper patient selection. Prospective studies are ongoing to validate these results.

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Notes

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