Published OnlineFirst February 16, 2010; DOI: 10.1158/1535-7163.MCT-09-0665

Research Article

Molecular Cancer Therapeutics

Association of Polymorphisms in *AKT1* and *EGFR* with Clinical Outcome and Toxicity in Non–Small Cell Lung Cancer Patients Treated with Gefitinib

Elisa Giovannetti[1,4], Paolo A. Zucali[6], Godefridus J. Peters[1], Filippo Cortesi[1], Armida D'Incecco[7], Egbert F. Smit[2], Alfredo Falcone[5,7], Jacobus A. Burgers[3], Armando Santoro[6], Romano Danesi[4], Giuseppe Giaccone[8], and Carmelo Tibaldi[7]

Abstract

EGFR mutations are strongly predictive of epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor activity in non–small cell lung cancer (NSCLC), but resistance mechanisms are not completely understood. The interindividual variability in toxicity also points out to the need of novel pharmacogenetic markers to select patients before therapy. Therefore, we evaluated the associations between *EGFR* and *AKT1* polymorphisms and outcome/toxicity in gefitinib-treated NSCLC patients. Polymorphic loci in *EGFR*, and *AKT1*, and *EGFR* and *K-Ras* mutations were assessed in DNA isolated from blood samples and/or paraffin-embedded tumor from 96 gefitinib-treated NSCLC patients. Univariate and multivariate analyses compared genetic variants with clinical efficacy and toxicity using Fisher's, log-rank test, and Cox's proportional hazards model. *AKT1-SNP4* association with survival was also evaluated in 127 chemotherapy-treated/gefitinib-naive patients, whereas its relationship with *AKT1* expression and gefitinib cytotoxicity was studied in 15 NSCLC cell lines. *AKT1-SNP4 A/A* genotype was associated with shorter time-to-progression ($P = 0.04$) and overall survival ($P = 0.007$). Multivariate analyses and comparison with the gefitinib-nontreated population underlined its predictive significance, whereas the *in vitro* studies showed the association of lower *AKT1* mRNA levels with gefitinib resistance. In contrast, *EGFR*-activating mutations were significantly correlated with response, longer time-to-progression, and overall survival, whereas *EGFR −191C/A* ($P < 0.001$), *−216 G/T* ($P < 0.01$), and *R497K* ($P = 0.02$) polymorphisms were strongly associated with grade >1 diarrhea. *AKT1-SNP4 A/A* genotype seems to be a candidate biomarker of primary resistance, whereas *EGFR −191C/A, −216G/T*, and *R497K* polymorphisms are associated with diarrhea when using gefitinib in NSCLC patients, thus offering potential new tools for treatment optimization.
Mol Cancer Ther; 9(3); 581–93. ©2010 AACR.

Introduction

Non–small cell lung cancer (NSCLC) is the leading cause of cancer death worldwide. Chemotherapy increases survival in advanced disease, but overall prognosis remains poor.

Several new agents against critical molecular targets in cancer cell survival have been tested. Among these, inhibitors of the epidermal growth factor tyrosine kinase (EGFR-TKI), such as gefitinib and erlotinib, have shown antitumor activity in 4% to 27% of unselected NSCLC patients and have been registered in several countries for advanced NSCLC patients who failed chemotherapy (1–4).

Although no selection of patients was done in the pivotal studies, several clinical and biological signatures were shown to affect the sensitivity or resistance to EGFR inhibitors (5, 6). Many studies documented a relationship between female gender, adenocarcinoma histology, Asian ethnicity, and never having smoked and higher response rates to EGFR-TKIs (6). Specific activating mutations within the *EGFR-TK* domain are associated with these characteristics and are predictive of EGFR-TKI activity (7, 8). Prospective studies with gefitinib or erlotinib in patients harboring *EGFR*-activating mutations reported response rates ranging from 60% to 82%, suggesting that these drugs might be a valuable alternative to chemotherapy in selected patients (9, 10). However, in

Authors' Affiliations: Departments of [1]Medical Oncology and [2]Pneumology, VU University Medical Center; and [3]Department of Thoracic Oncology, Nederlands Kanker Instituut, Amsterdam, the Netherlands; [4]Division of Pharmacology and Chemotherapy, Department of Internal Medicine, University of Pisa and [5]University of Pisa, Pisa, Italy; [6]Department of Medical Oncology and Hematology, Istituto Clinico Humanitas, IRCCS, Rozzano, Milan, Italy; [7]Division of Oncology, Department of Oncology, Azienda USL-6 of Livorno, Italy; and [8]Medical Oncology Branch, National Cancer Institute, Bethesda, Maryland

Note: E. Giovannetti and P.A. Zucali contributed equally to this study.

Corresponding Author: Elisa Giovannetti, Department of Medical Oncology, VU University Medical Center, Cancer Center Amsterdam-CCA 1.52, De Boelelaan 1117, 1081 HV Amsterdam, the Netherlands. Phone: 31-20-4442633; Fax: 31-20-4443844. E-mail: elisa.giovannetti@gmail.com, http://www.vumc.com/afdelingen/Medical-Oncology/

doi: 10.1158/1535-7163.MCT-09-0665

Published OnlineFirst February 16, 2010; DOI: 10.1158/1535-7163.MCT-09-0665

the BR.21 trial, the treatment with erlotinib resulted in statistically significant improvements in overall survival (OS) and quality of life in all of the subsets of patients (11, 12), and several studies reported that patients without mutations responded to EGFR-TKIs (13, 14). Similarly, some NSCLC cell lines with normal *EGFR* status are sensitive to these agents (15), suggesting that the question of who should receive EGFR-TKI therapy is still not completely answered and other molecular factors need to be identified.

Better outcome in gefitinib-treated patients was also associated with increased *EGFR* gene copy number (16, 17). In contrast, recent studies identified mutations in *EGFR* that are associated with resistance (18), and emerging data suggest that resistance to EGFR inhibition may be also due to the activation of proteins downstream of the receptor (K-Ras, mitogen-activated protein kinase, and signal transducers and activators of transcription 3), tumor dedifferentiation (so-called epithelial-mesenchymal transition), and other cell surface proteins, such as cMET (5, 6, 19–21). Nonetheless, all these changes do not completely explain the variable clinical outcomes, and identification of other biomarkers of EGFR-TKI sensitivity/resistance may help in optimal patient selection, especially in populations with a low frequency of *EGFR* mutations, such as Caucasians.

Germ line DNA variations of *EGFR* might also predict response to EGFR-TKIs. The length of a *CA*-repeat in intron-1 was associated with gefitinib response and/or time-to-progression(TTP)/OS in several retrospective studies (22–26). However, this polymorphism was not correlated with EGFR expression in cell lines and NSCLC tissues (24, 27), and no association with clinical outcome was observed in the largest pharmacogenetic analysis in NSCLC Caucasian patients treated with gefitinib (28). Interethnic differences in *CA*-intron-1-repeat frequency and linkage disequilibrium with other polymorphisms may explain these controversial results (29, 30). The variants *–216G/T* and *–191C/A* have been associated with increased *EGFR* promoter activity and gene expression, whereas a single nucleotide polymorphism (SNP) at codon 497 is associated with decreased EGFR activity (30, 31). A combination of *–216G/T* and *R497K* polymorphisms was weakly correlated with drug response in the NCI60 panel (27), whereas an integrated analysis of genotypic/pharmacokinetic variability showed a strong association between diarrhea in erlotinib-treated patients and the two polymorphisms in the *EGFR* promoter (32). Further studies are warranted to evaluate the interaction between mutations, gene amplification/expression, and polymorphisms in *EGFR* and how these factors might affect responsiveness to EGFR-TKIs.

Clinical response to EGFR-TKIs may also be influenced by changes affecting downstream EGFR signal transducers. The serine-threonine kinase Akt is a central player in the phosphoinositide 3-kinase oncogenic pathway and is involved in antiapoptosis or in pro-cell proliferation effects. Retrospective studies showed that patients with phospho-Akt–positive tumors had a better response, disease control rate, and TTP, suggesting that gefitinib may be most effective with basal Akt activation (20, 33). However, in the ONCOBELL prospective trial, only the EGFR fluorescence *in situ* hybridization, and not Akt immunohistochemistry, predicted response to gefitinib (34). Recent studies discovered an oncogenic mutation in the *AKT1* subunit, which stimulates Akt signaling and induces cellular transformation, but its rare incidence suggests that it may not play a role in NSCLC development or response to EGFR-TKIs (35). A candidate gene approach focusing on apoptotic pathways identified two functional polymorphisms (*AKT1-SNP3* and *SNP4*) affecting the expression and activity of Akt (36). The haplotype including these polymorphisms was associated with lower protein levels in tissues from Caucasians and contributed to the lowest apoptotic response of EBV-transformed lymphoblastoids to radiation (36, 37).

A recent trial reported that other genetic variations in *AKT1* were associated with increased recurrence and significantly shorter survival in esophageal cancer patients treated with regimens including fluoropyrimidines, platinum compounds, and taxanes, but not with gefitinib, suggesting that genetic variations in the phosphoinositide 3-kinase/AKT pathway may be prognostic and/or predictive factors of drug response (38). However, no data are available on the effect of the *AKT1-SNP3* and *SNP4* polymorphisms on drugs targeting the EGFR/phosphoinositide 3-kinase/AKT signaling.

The purpose of this study was to retrospectively evaluate the associations between selected functional *EGFR* and *AKT1* polymorphisms and clinical outcomes in gefitinib-treated NSCLC patients.

Materials and Methods

Patients

Fifty-one and 45 patients were treated within the Expanded-Access-Program of gefitinib at Humanitas Clinical Institute (Rozzano-Milano, Italy) and Livorno Civil Hospital (Livorno, Italy), respectively. The selection was based on the diagnosis of histologically confirmed NSCLC with measurable, locally advanced, or metastatic disease, progressing/relapsing after chemotherapy, or with contraindications for chemotherapy. The clinical trial and the study on patients' specimens was approved by the Ethics Committees (ClinicalTrials.gov-ID-NCT00831454) and conducted in accordance with principles stated in the Declaration of Helsinki. Patients received 250 mg/d gefitinib orally and were evaluated for response after 2 mo according to the Response Evaluation Criteria in Solid Tumors criteria. Tumor response was assessed by computed tomography scan, with a confirmatory evaluation repeated in patients with complete/partial response (PR) and stable disease (SD) at least 4 wk after the initial determination of response. Toxicities were

Published OnlineFirst February 16, 2010; DOI: 10.1158/1535-7163.MCT-09-0665

assessed using the National Cancer Institute Common toxicity criteria manual version 3.0.[9]

Based on the analysis of the results obtained in the gefitinib-treated patients, an additional population of 127 chemotherapy-treated/gefitinib-nontreated NSCLC patients (39) was used to evaluate the prognostic versus predictive role of the polymorphic genotypes that were associated with OS.

DNA Isolation

DNA was isolated from blood or paraffin-embedded tumor samples using the microDNA-kit (Qiagen). DNA yields and integrity were checked at 260 to 280 nm with the NanoDrop-1000-Detector (NanoDrop-Technologies).

Mutations and Polymorphisms Analysis

Nested PCR to amplify *EGFR* (exons 18–21) and *K-Ras* (exon 1–2), and sequencing of PCR products on a ABI-3100 genetic analyzer (Applied Biosystems) was done as previously described (20). The *CA* repeat length in the intron-1 *(CA)n* and the *−216G/T* (dbSNP-ID: rs712829) *EGFR* polymorphisms were studied as previously described (28, 32). The *EGFR −191C/A* (dbSNP-ID:rs712830) and *R497K* (*G/A*, dbSNP-ID:rs11543848), and the *AKT1-SNP3 C/T* (dbSNP-ID:rs3730350) and *SNP4 G/A* (dbSNP-ID:rs1130233) were studied with Taqman probe–based assays using the ABI PRISM-7500HT. Specific primers and probes for *EGFR R497K* and *AKT1* polymorphisms were obtained from Applied Biosystems (C__16170352_20, C__193157_10 and C__7489835_10, respectively), whereas primers and probes for *EGFR −191C/A* were designed using the FileBuilder-2.0 software (Applied Biosystems), on the basis of the gene sequence from Genbank, (F): 5′-CCCCGCACGGTGTGA-3′; (R): 5′-GGCTAGCTCGGGACTCC-3′; (P): 5′-CCTCGGCCGCG[G/T]CG-3′, for the FAM/VIC-fluorescent reporter, respectively. The PCRs were done using 20 ng of DNA diluted in 5.94 μL DNase-RNase–free water, with 6.25 μL of Taqman Universal PCR Master Mix and 0.31 μL of the mix, including the primers and probes, in a total volume of 12.5 μL. After thermal cycling, the 7500HT instrument determined the allelic content of each sample in the plate by reading the generated fluorescence using the SDS-2.0 software (40).

In vitro Studies

Previous studies evaluated the relationships of *EGFR* polymorphisms and expression with EGFR-TKI sensitivity (27), but no data are available on the correlation between *AKT1* polymorphisms and AKT1 expression or gefitinib sensitivity. Therefore, we evaluated *AKT1* genotypes and mRNA expression in 15 NSCLC cell lines, including cells already studied for determinants of gefitinib sensitivity (20). All the cells were obtained from the American Type Culture Collection within the last 5 y and tested within the last 6 mo by morphology check by microscope and growth curve analysis according to the Cell Line Verification Test Recommendations (American Type Culture Collection Technical Bulletin no. 8, 2008). Periodic assays were done to detect *Mycoplasma* contamination (the last test was done on March 2009), and the identity of the cells was further confirmed by sequence analysis of their specific mutational status in *EGFR* and *K-Ras*, as already described by the Cancer Cell Line Project of the Wellcome Trust Sanger Institute.[10] NCI-H23, NCI-H292, NCI-H322, NCI-H460, NCI-H522, NCI-H827, NCI-H1650, NCI-H1703, NCI-H1975, NCI-H2288, and NCI-H3255 were cultured in RPMI-1640, whereas A549, SW1573, Calu-1, and Calu-6 were cultured in DMEM. Cell viability was assessed after a 72-h exposure to gefitinib (0.001–100 μmol/L) by MTT, in at least duplicate experiments, which showed a dose-dependent inhibition of cell growth as previously described (20).

DNA extraction and genotyping were done as described above. RNA was extracted using Trizol (Invitrogen), and 500 ng were reverse transcribed using random hexamers from the DyNAmo-cDNA-Synthesis kit (Finnzymes-Oy). The *AKT1* mRNA expression was studied by Taqman-PCR with the Hs00920503_m1 kit (Applied Biosystems). The relative levels of *AKT1* mRNA were measured using a standard curves method and normalized to β-actin. The standard curves were derived from dilutions of cDNA from the Quantitative-PCR Human-Reference Total-RNA (Stratagene). All the samples were amplified in triplicate, with appropriate nontemplate controls, and the CV was <2%.

Statistical Analysis

Demographic and clinical information were compared across genotype using Pearson χ^2 two-sided test. Patients achieving complete response or PR were defined as "responders," whereas patients with SD or progressive disease (PD) were defined as "nonresponders." Additional analyses were done by grouping patients with complete response/PR and SD ("patients with clinical benefit") versus PD (40).

TTP was calculated from the date of the first dose of gefitinib to the date of clinical and/or radiological evidence of progression/death, whichever occurred first, whereas OS was calculated from the day of treatment start to the end point (death or censoring). The Kaplan-Meier method was used to plot TTP and OS, and the log-rank test was used to compare curves.

The univariate analysis included clinical characteristics, including baseline demographic characteristics, such as sex, performance status (PS; 0–1 versus 2–3), age (<median versus ≥median), clinical stage (IIIB versus IV), histology [adenocarcinoma versus other histology, and bronchiolo-alveolar carcinoma (BAC) versus

[9] Available from http://ctep.cancer.gov/reporting/ctc.html.

[10] http://www.sanger.ac.uk/genetics/CGP/CellLines/

Published OnlineFirst February 16, 2010; DOI: 10.1158/1535-7163.MCT-09-0665

other histology], smoking history [never, i.e., <100 cigarettes/lifetime versus former (quit smoking >12 mo before starting gefitinib) + current smokers] and prior chemotherapy (yes versus no), as well as clinical treatment effects, such as toxicity (grade 0–1 versus grade 2+), and response to gefitinib, categorized as described above. Regarding polymorphisms, statistical analyses were done grouping patients according to the three different genotypes for each polymorphism and to different allelic activity, as reported previously, i.e., *EGFR –191C/C* versus *C/A-A/A, EGFR –216G/G* versus *G/T-T/T, EGFR R497K G/G-G/A* versus *A/A, AKT1-SNP3 C/C-C/T* versus *T/T*, and *SNP4 G/G-G/A* versus *A/A* (30, 32, 36). For the *EGFR (CA)n* polymorphism, patients were classified as *S/S, L/L*, and *S/L* if the number of repeats was ≤16 on both alleles, >16 on both alleles and ≤16 in one allele, and >16 in the other (25, 32).

The significant prognostic variables in the univariate analysis were included in the multivariate analyses using Cox's proportional hazards model to identify factors of independent significance. A step-down procedure was used based on the likelihood ratio test, and hazard ratios were calculated to estimate the magnitude and the direction of the effect. According to the usual practice in genetic epidemiology, the multivariate statistical model treated the most common polymorphic variants as the baseline.

The Pearson χ^2 test was also used to determine the correlation between *EGFR* mutations and polymorphisms, and to evaluate whether the baseline demographic and biological characteristics were significantly different in the gefitinib-treated and gefitinib-nontreated patients.

The Student's *t* test, one-way ANOVA, and the nonparametric Kruskal-Wallis test were used when analyzing the correlation of *AKT1-SNP4* polymorphisms with *AKT1* mRNA expression and *in vitro* gefitinib sensitivity, as well as *EGFR* and *K-Ras* mutations and gefitinib sensitivity, whereas the Spearman correlation test was used to investigate the correlation between gene expression and gefitinib IC_{50}s.

All the analyses of the samples were done in a blinded fashion relative to clinical outcome. Data were analyzed using the SPSS-16 software (SPSS, Inc.). *P* values between 0.05 and 0.1 were regarded as marginally suggestive of an association ("trend toward a significant correlation"), but only $P < 0.05$ were considered statistically significant. However, in the univariate analyses for clinical outcome according to polymorphisms, a Bonferroni correction required a $P < 0.05/6 = 0.008$ for statistical significance.

Results

Clinical Characteristics and Outcome

Table 1 summarizes the clinical characteristics and their accordance with the outcome. Response and TTP data were available from all, and OS data were available from 95 patients. There were 83 deaths (event rate, 86.5%), whereas 6 individuals were alive without PD at last contact.

Of the 96 patients evaluated, 17.7% had PR, whereas 38.6% and 43.7% had SD and PD, respectively. Female gender and never-smoker status were significantly associated with a better response. Female gender was also associated with clinical benefit (i.e., PR+ SD) as well as BAC histology.

Median TTP and OS were 3.2 and 7.7 months, respectively. BAC histology was associated with significantly longer TTP and OS, whereas negative smoker history was associated with longer TTP. Surprisingly, older age (≥64 years) was also associated with a longer TTP but not with OS. Finally, a significant association was detected between both TTP and OS, and response. Similarly, TTP and OS were associated with clinical benefit. Median OS of responders, patients with clinical benefit, and nonresponders were 18.8, 11.3, and 4.1 months, respectively.

Mutations and Outcome

Seven of the 62 patients with available tissues for mutation analysis harbored *EGFR* exon-19 deletions, whereas 2 had the L858R *EGFR* exon-21 and 15 carried *K-Ras* mutations. No tumor with *K-Ras* mutations harbored *EGFR* mutations. *EGFR*-activating mutations were significantly associated with response [67% of the patients harboring *EGFR* mutations achieved response versus 6% of the patients without mutations ($P < 0.01$)] and showed a trend toward an association with clinical benefit: 8 of the 9 patients with mutations achieved clinical benefit, i.e., 89%, versus 55% of the patients without mutations ($P = 0.07$). *EGFR* mutations were also significantly associated with longer TTP [median TTP, 9.0 months in patients with *EGFR* mutations versus 3.0 months in patients without mutations ($P < 0.01$)]. Similar results were reported for OS, with median OS of 18.0 versus 6.0 months in patients with and without mutations ($P < 0.01$), respectively. In contrast, no significant associations were reported between *K-Ras* mutations and TTP and OS, but none of the patients bearing *K-Ras* mutations achieved PR after gefitinib treatment ($P = 0.08$).

Polymorphisms and Outcome

Previous studies showed no differences in polymorphisms analyzed in tumors and normal tissues (41, 42). However, because high-level gene amplification of *EGFR* in NSCLC cells might result in homozygous genotypes in individuals who are heterozygous in the germline, we performed preliminary studies of *EGFR –191C/A* and *R497K* polymorphisms in 45 paired samples of germ line and lung cancer DNA, showing identical interindividual genotypes between normal and malignant tissues. Therefore, for the 96 patients enrolled in this study, genotyping was done in DNA extracted from the available 51 tumors and 45 blood samples. However, in 17 of the 96 patients enrolled, we obtained data from both the blood and the tumors.

Published OnlineFirst February 16, 2010; DOI: 10.1158/1535-7163.MCT-09-0665

Table 1. Clinical outcome according to clinical characteristics

Characteristic	Patients *n* (%)	Response *n* (%)*	*P*	TTP mo (95% CI)	*P*	OS mo (95% CI)	*P*
No. Patients	96	17 (17.7)		3.2 (2.6–3.8)		7.7 (6.8–8.6)	
Baseline demographic characteristics							
Age, median y	64.0						
<64	47 (49.0)	8 (17.0)	0.87	3.0 (2.6–3.3)	0.02	6.8 (4.1–9.5)	0.07
>64	49 (51.0)	9 (18.4)		5.7 (3.3–8.1)		8.0 (3.5–12.6)	
Sex							
Male	55 (57.3)	3 (5.5)	<0.01	3.0 (2.8–3.2)	0.08	7.0 (4.3–9.7)	0.19
Female	41 (42.7)	14 (34.1)		5.8 (3.7–7.9)		10.5 (5.7–15.3)	
Smoking history							
Smokers	66 (68.8)	4 (6.1)	<0.01	3.0 (2.7–3.3)	0.01	7.6 (6.6–8.7)	0.11
Never smokers†	29 (31.2)	13 (44.8)		5.7 (3.4–8.0)		8.0 (6.2–9.7)	
Clinical stage							
IIIB	9 (9.4)	0 (0.0)	0.35	3.0 (2.7–3.2)	0.09	7.0 (4.1–9.9)	0.54
IV	87 (90.6)	17 (19.5)		3.3 (2.3–4.3)		7.7 (6.8–8.6)	
ECOG PS							
0–1	76 (79.2)	12 (15.8)	0.34	3.3 (1.6–5.0)	0.84	8.0 (5.9–10.1)	0.37
2–3	20 (21.8)	5 (25.0)		2.0 (1.9–2.1)		2.2 (1.0–3.5)	
Histology							
Adenocarcinoma	54 (56.3)	10 (18.5)	0.81	4.5 (2.8–6.2)	0.78	15.0 (12.3–17.7)	0.62
Other histology	42 (43.7)	7 (16.7)		3.0 (2.8–3.2)		16.0 (0.0–32.8)	
BAC	13 (13.5)	4 (30.8)	0.18	8.0 (4.5–11.5)	0.02	14.0 (3.2–24.8)	0.03
Other histology	83 (86.5)	13 (15.7)		3.0 (2.7–3.2)		7.0 (5.3–8.7)	
Previous treament							
No	14 (14.6)	2 (14.3)	0.72	5.7 (2.3–9.1)	0.40	7.2 (6.8–8.6)	0.62
Yes	82 (85.4)	15 (18.3)		3.1 (2.5–3.7)		7.7 (6.8–19.2)	
Treatment effects							
Response							
PR	17 (17.7)	/	/	12.0 (8.0–16.0)	<0.01	18.8 (13.4–14.1)	<0.01
SD+PD	79 (82.3)	/	/	3.0 (2.8–3.2)		7.0 (5.2–8.8)	
Clinical benefit							
PR+SD	54 (56.3)	/	/	6.6 (5.7–7.5)	<0.01	11.3 (8.2–14.5)	<0.01
PD	42 (43.7)	/	/	2.0 (1.9–2.1)		4.1 (2.9–5.4)	
Skin rash (grade)							
0–1	65 (73.0)	11 (16.9)	0.18	3.1 (2.8–3.4)	0.83	7.7 (6.6–8.8)	0.60
2–3	24 (27.0)	5 (20.8)		4.0 (1.5–6.5)		7.4 (5.8–9.1)	
Diarrhea (grade)							
0–1	81 (93.1)	13 (16.0)	0.38	3.2 (2.5–3.8)	0.48	7.7 (6.8–8.6)	0.74
2–3	6 (6.9)	2 (33.0)		1.9 (0.7–3.1)		6.0 (1.0–11.)	

Abbreviation: 95% CI, 95% confidence interval; ECOG, Eastern Cooperative Oncology Group.
*% were calculated with respect to *n* of the correspondent characteristic.
†Never (<100 cigarettes/lifetime) vs former (quit smoking >12 mo before starting gefitinib) + current smokers.

All polymorphisms followed the Hardy-Weinberg's equilibrium (Table 2), as calculated with the SNP analyzer software,[11] and allelic frequencies for the *EGFR* polymorphisms were comparable with those reported in previous studies in Caucasian-predominant NSCLC patients (25, 28), whereas the frequencies of *AKT1-SNP3* and *SNP4* were similar to those observed in Caucasian populations (36, 37). No significant correlations were detected between genotype and baseline demographic characteristics. Similarly, no associations were found between *EGFR* polymorphisms and *EGFR* mutations, as reported previously (43).

Polymorphisms were not associated with response, although none of the patients harboring the *AKT1-SNP3 T/T* or the *SNP4 A/A* variants achieved response after gefitinib (Table 2). No significant differences in TTP and OS were detected with respect to *EGFR* and

[11] http://snp.istech21.com/snpanalyzer/2.0/

Published OnlineFirst February 16, 2010; DOI: 10.1158/1535-7163.MCT-09-0665

Table 2. Clinical outcome according to polymorphisms

Polymorphism	Patients *n* (%)	HWE *P*	Polymorphism	Patients *n* (%)	Response *n* (%)	*P*	TTP mo (95% CI)	*P*	OS mo (95% CI)	*P*
EGFR (CA)n			*EGFR (CA)n*							
S/S	30 (31.9)	0.87	*S/S*	30 (31.9)	3 (10)	0.25	3.2 (0.7–5.7)	0.90	7.9 (3.5–12.2)	0.13
S/L	44 (46.8)		*S/L-L/L*	64 (68.1)	14 (21.9)		3.1 (2.4–3.8)		11.6 (6.5–16.7)	
L/L	20 (21.3)									
EGFR –191C/A			*EGFR –191C/A*							
CC	78 (83.0)	0.06	*CC*	78 (83.0)	14 (17.9)	0.23	3.2 (2.5–3.9)	0.46	7.9 (7.0–8.7)	0.37
CA	13 (13.8)		*CA-AA*	16 (17.0)	3 (18.7)		3.2 (3.0–3.4)		6.0 (2.8–9.2)	
AA	3 (3.2)									
EGFR –216G/T			*EGFR -216G/T*							
GG	34 (36.2)	0.86	*GG*	34 (36.2)	5 (14.7)	0.52	3.2 (2.6–3.8)	0.28	8.0 (3.0–13.0)	0.18
GT	43 (45.7)		*GT-TT*	60 (63.8)	12 (20.0)		3.2 (0.8–5.7)		11.6 (5.7–17.5)	
TT	17 (18.1)									
EGFR R 497K			*EGFR R 497K*							
GG	47 (51.1)	0.45	*GG-GA*	81 (88.0)	13 (16.0)	0.36	3.3 (2.4–5.0)	0.32	7.4 (6.5–8.4)	0.55
GA	34 (37.0)		*AA*	11 (12.0)	3 (27.3)		3.1 (1.5–4.7)		8.0 (0.0–17.3)	
AA	11 (11.9)									
AKT1-SNP3			*AKT1-SNP3*							
CC	65 (69.1)	0.39	*CC-CT*	89 (94.7)	17 (19.1)	0.54	3.2 (2.2–4.1)	0.92	7.7 (6.8–8.6)	0.97
CT	24 (25.6)		*TT*	5 (5.3)	0 (0.0)		3.0 (2.3–3.7)		4.0 (0.2–7.8)	
TT	5 (5.3)									
AKT1-SNP4			*AKT1-SNP4*							
GG	50 (53.2)	0.94	*GG-GA*	88 (93.6)	17 (19.8)	0.23	3.2 (2.2–4.2)	0.04	8.0 (6.7–9.3)	<0.01
GA	38 (40.4)		*AA*	6 (6.4)	0 (0.0)		2.0 (1.1–2.9)		2.2 (0.0–5.7)	
AA	6 (6.4)									

NOTE: A total of 94 samples of the 96 patients were evaluable for *EGFR (CA)n*, *EGFR –191C/A*, *EGFR –216G/T*, *AKT1-SNP3*, and *SNP4*, and 92 of the 96 patients were evaluable for *EGFR R497K*. Two had no suitable DNA for all the analyses, and in other two subjects, *EGFR R497K* genotypes was not detectable.
Abbreviation: HWE, Hardy-Weinberg equilibrium.

AKT1-SNP3 genotypes. However, the *AKT1-SNP4-A/A* genotype was associated with shorter TTP and OS. In particular, the median OS between patients harboring the *AKT1-SNP4 A/A* variant was 2.2 months, whereas patients carrying the *G/A* and the *G/G* genotype had a median OS of 7.0 and 8.0 months, respectively (Fig. 1A). To keep at minimum the probability to find a statistically significant difference purely by chance, the usual nominal level ($P = 0.05$) has been lowered to 0.008 by Bonferroni adjustment for multiple comparisons. After the Bonferroni adjustment, no statistically significant differences were detected for TTP, whereas the *AKT1-SNP4* genotype was still significantly correlated to OS ($P = 0.007$).

None of the patients harboring the *AKT1-SNP4-A/A* genotype had *EGFR* or *K-Ras* mutations. The median age of these patients (four males and two females) was 67.5 y (range, 49–80). Regarding the histology, three had adenocarcinoma, two squamocellular carcinoma, and one large-cell carcinoma. Five of six of these patients were smokers, whereas four had a PS of 0, and two had a PS of 2.

Because the polymorphism results may reflect a generally worse prognosis with the presence of the *A/A* variant as opposed to a pharmacogenetic response, 127 gefitinib-nontreated NSCLC patients were also assessed for the *AKT1-SNP4*. These patients were enrolled in the NVALT-7 study and treated only with pemetrexed ($n = 62$) or carboplatin-pemetrexed ($n = 65$) regimen without receiving EGFR-TKIs as salvage therapy. In this study, the patients who received carboplatin-pemetrexed had a significantly longer TTP than patients receiving pemetrexed alone, but no significant differences were detected for OS (39).

Although the purpose of this analysis was to make a preliminary evaluation whether the polymorphic genotypes associated with OS were predictive of drug activity or prognostic factors, and not to perform a case-control study, we analyzed whether the baseline demographic characteristics were significantly different in the gefitinib-treated and gefitinib-nontreated patients. No data on BAC histology and smoking status in patients of the NVALT-7 study were available, but no significant differences were measured for age (≤65 versus >65, $P = 0.47$),

Published OnlineFirst February 16, 2010; DOI: 10.1158/1535-7163.MCT-09-0665

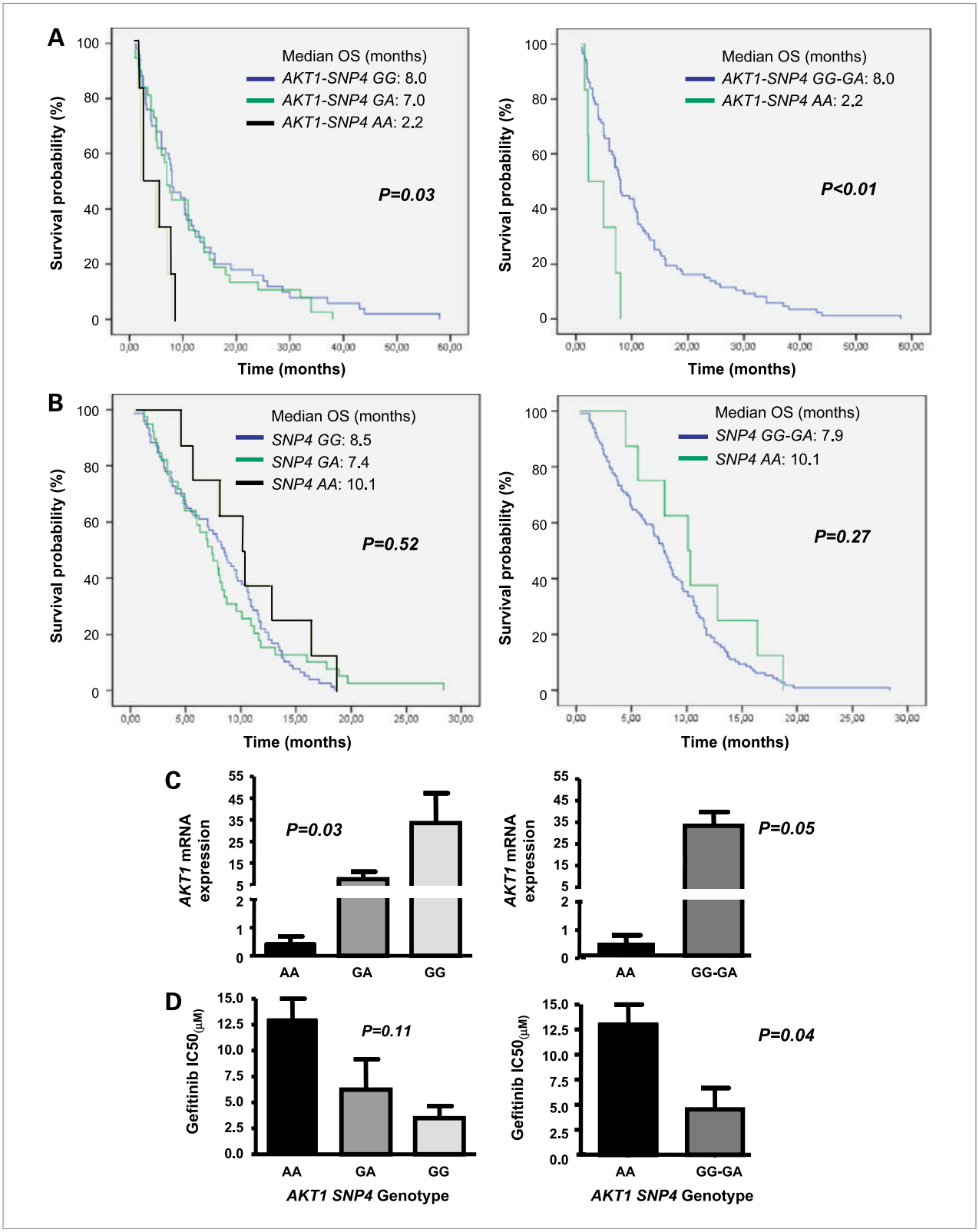

Figure 1. Kaplan-Meier curves of OS according to *AKT1-SNP4* polymorphisms in the gefitinib-treated (A) and gefitinib-nontreated patients (B) and analyses of the association between *AKT1-SNP4* genotypes with *AKT1* mRNA expression, calculated as ratio with β-actin with respect to standard curves (C), and gefitinib IC_{50}s (D) in 15 NSCLC cell lines.

Published OnlineFirst February 16, 2010; DOI: 10.1158/1535-7163.MCT-09-0665

sex ($P = 0.70$), PS (0-1 versus 2-3, $P = 0.10$), and histology (adenocarcinoma versus others, $P = 0.06$) in this additional series of NSCLC patients who did not receive EGFR-TKI treatment with respect to the NSCLC patients treated with gefitinib.

AKT1-SNP4 genotypes were detectable from 124 of 127 patients. The allelic frequencies of *G/G*, *G/A*, and *A/A SNP4* genotypes were similar to those in gefitinib-treated patients (62.1, 31.4, and 6.5%, respectively, $P = 0.80$ in the Pearson χ^2 test of the G/G-G/A and A/A groups with respect to the gefitinib-treated patients). No significant differences were reported in OS in patients with the *AKT1-SNP4-A/A* and patients with the *AKT1-SNP4-G/G-G/A* genotypes (Fig. 1B).

Multivariate Analysis

To evaluate the risk of disease progression and death, we carried out two Cox regression analyses entering variables significantly associated with TTP and OS in the univariate model. Among biological characteristics (Table 3), the *AKT1-SNP4 A/A* variant showed a trend toward a significant association with risk of progression, in the analysis including the *EGFR* mutation covariate. However, in the regression model done on all the patients evaluable for the polymorphisms analyses ($n = 94$), excluding the *EGFR* mutational status, the *AKT1-SNP4-A/A* genotype showed a significantly higher risk of progression ($P = 0.04$). Regarding the risk of death, *AKT1-SNP4-A/A* was an independent prognostic factor. In particular, the *A/A* genotype was significantly associated with increased risk of death, in the model of 62 and of 94 patients, including and excluding the mutational status, respectively.

Considering all the significant variables in the univariate analysis (i.e., response, age, and smoking status for TTP and response for OS), we found that response was always associated with both TTP and OS, whereas age and smoking status were not independent predictors of TTP and OS in the model including and excluding the *EGFR* mutational status. In this analysis, the *AKT1-SNP4-A/A* genotype showed a trend toward a significant association with TTP in the model of 94 patients ($P = 0.09$), and it was still a significant predictor of OS in both models.

Toxicity

Of the 89 patients evaluable for skin toxicity, 24 patients (27%) developed grade 1 skin rash, whereas 21% and 6% had grade 2 and 3 skin rash, respectively. Regarding the 87 patients evaluable for gastrointestinal toxicity, 29 patients (33%) had grade 1 diarrhea and 7% developed grade >1 diarrhea. According to previous analyses (32), patients were grouped in patients with toxicity versus patients without and patients with grade 0 to 1 versus patients with "high" toxicity (grade 2–3). No significant correlations were observed between toxicities and most patients' characteristics, TTP, or OS. However, patients affected by adenocarcinomas experienced higher skin toxicity ($P = 0.04$) than patients with other tumor types.

No correlations were observed grouping patients according to *EGFR* or *K-Ras* mutational status.

Polymorphisms and Toxicity

The correlations between polymorphisms and toxicity are described in Table 4. *EGFR –191C/A* polymorphism were associated with grade >1 diarrhea ($P < 0.001$). In

Table 3. Multivariate analysis of biological parameters for TTP and OS

Covariates for TTP	HR (95% CI)	Wald *P*
Model including *EGFR* mutational status ($n = 62$)		
Histology: others vs BACs	1.4 (0.6–3.2)	0.47
EGFR mut: *EGFR* Wt vs Mut	2.1 (1.5–3.9)	0.01
AKT1-SNP4: AA vs GG+GA	1.7 (1.0–2.6)	0.06
Model excluding *EGFR* mutational status ($n = 94$)		
Histology: others vs BACs	1.3 (0.7–3.4)	0.09
AKT1-SNP4: AA vs GG+GA	3.4 (2.2–5.9)	0.04
Covariates for OS	**HR (95% CI)**	**Wald *P***
Model including *EGFR* mutational status ($n = 62$)		
Histology: others vs BACs	1.4 (0.6–3.2)	0.47
EGFR mut: EGFR Wt vs Mut	2.1 (1.0–4.3)	0.05
AKT1-SNP4: AA vs GG+GA	2.3 (1.2–2.9)	0.04
Model excluding *EGFR* mutational status ($n = 94$)		
Histology: others vs BACs	1.8 (1.0–3.4)	0.06
AKT1-SNP4: AA vsGG+GA	4.8 (1.2–6.3)	0.02

Abbreviation: HR, hazard ratio.

Published OnlineFirst February 16, 2010; DOI: 10.1158/1535-7163.MCT-09-0665

Table 4. Toxicity according to polymorphisms (number of patients)

Polymorphism	Skin rash (0 vs 1+)	*P*	Skin rash (0–1 vs 2+)	*P*	Diarrhea (0 vs 1+)	*P*	Diarrhea (0–1 vs 2–3)	*P*
EGFR (CA)n								
SS	9 vs 18	0.11	17 vs 10	0.11	13 vs 13	0.99	24 vs 2	1.00
SS-LL	32 vs 28		48 vs 12		39 vs 20		55 vs 4	
EGFR –191 C/A								
CC	36 vs 36	0.27	54 vs 18	0.99	44 vs 26	0.56	69 vs 1	<0.01
CA-AA	5 vs 10		11 vs 4		8 vs 7		10 vs 5	
EGFR –216 G/T								
GG	11 vs 19	0.18	21 vs 10	0.31	14 vs 16	0.06	24 vs 6	<0.01
GT-TT	30 vs 27		44 vs 12		38 vs 17		55 vs 0	
EGFR R497K								
GG-GA	37 vs 38	0.83	56 vs 18	0.99	48 vs 26	0.17	71 vs 3	0.02
AA	4 vs 7		8 vs 3		4 vs 6		7 vs 3	
AKT1-SNP3								
CC-CT	40 vs 42	0.36	62 vs 20	0.60	48 vs 32	0.64	74 vs 6	0.99
TT	1 vs 4		3 vs 2		4 vs 1		5 vs 0	
AKT1-SNP4								
GG-GA	36 vs 44	0.41	60 vs 20	0.99	47 vs 32	0.40	73 vs 6	0.99
AA	4 vs 2		5 vs 1		5 vs 1		6 vs 0	

NOTE: Because no DNA was available for SNPs evaluation in 2 of the patients with data on toxicity, the statistical analyses were done in a total number of 87 and 85 patients for skin and gastrointestinal toxicity, respectively. The worst toxicity grade for each patient was reported.

particular, five of the six patients who experienced severe diarrhea carried the *EGFR –191C/A* variants *C/A* and *A/A* (i.e., one third and 50% of the *C/A* and *A/A* patients had grade 2–3, whereas only 1 of 70 *C/C* patients had grade >1 diarrhea). Indeed, the Fisher's exact test for 3 × 2 table showed a strong association ($P < 0.0001$). The *EGFR –216G/T* polymorphism was also associated with severe diarrhea: all the patients who experienced grade >1 diarrhea carried the *G/G* variant ($P < 0.01$). Furthermore, the *G/G* variant showed a trend toward a significant association with any grade of diarrhea ($P = 0.06$). Similar results were found for the *EGFR R497K* polymorphism, showing high toxicity in 4.6%, 3.2%, and 30% of the patients carrying the genotype *G/A*, *G/G*, and *A/A*, respectively ($P = 0.01$ in the 3 × 2 table). Furthermore, the frequency of the *A/A* genotype was significantly higher in patients with grade >1 (50%) than in patients with grade 0 to 1 diarrhea (10%).

No significant association was reported between *EGFR* polymorphisms and skin toxicity, as well as between *EGFR (CA)n* and *AKT1-SNPs*, and both skin and gastrointestinal toxicity.

In vitro Studies

The significant association between *AKT1-SNP4* and clinical outcome prompted us to perform an *in vitro* study on a panel NSCLC cells already characterized for *EGFR/K-Ras* mutations and gefitinib sensitivity (Table 5). *AKT1-SNP4* genotypes were associated with *AKT1* mRNA expression, and when combining the *AKT1-SNP4 G/G* and *G/A* versus *A/A*, there was a significant association with both *AKT1* mRNA and gefitinib IC_{50}s (Fig. 1C and D). Finally, there was a significant inverse correlation between *AKT1* mRNA levels and gefitinib IC_{50}s (Spearman $r = -0.54$; $P = 0.04$). Gefitinib sensitivity was also significantly associated with the occurrence of *EGFR*-activating mutations ($P = 0.04$), while there was a trend toward significant association with reduced sensitivity in the cells harboring *K-Ras* mutations ($P = 0.07$).

Discussion

This study evaluated the effect of selected functional polymorphisms on outcome in advanced NSCLC patients and, to our knowledge, is the first to show the effect of a polymorphism in *AKT1* on the TTP and OS of gefitinib-treated patients. Furthermore, we reported a significant association between *EGFR* polymorphisms with gastrointestinal toxicity after gefitinib treatment, as reported previously in erlotinib-treated patients (32).

A growing body of studies is trying to identify the molecular predictors of response and toxicity to EGFR-TKIs

Published OnlineFirst February 16, 2010; DOI: 10.1158/1535-7163.MCT-09-0665

in NSCLC. This includes tumor-related molecular markers, such as *EGFR* mutations, copy number, and protein expression, but also germ line markers such as polymorphisms in *EGFR* or EGFR-related genes (44).

Most previous studies focused on polymorphisms affecting EGFR expression, and reported controversial results on their effect on EGFR-TKIs activity (23, 24, 28, 29). More recently, two linked promoter polymorphisms (*−216G/T* and *−191C/A*) and the *R497K* polymorphism have been associated with the modulation of EGFR expression and activity, and showed some correlation with response to erlotinib and 11 other EGFR-TKIs in the NCI60 panel (27, 30, 31). Furthermore, Rudin et al. (32) showed a significant association between the polymorphisms in the *EGFR* promoter and diarrhea, independent from erlotinib plasma concentration, in 80 NSCLC, head and neck, and ovarian cancer patients. We observed similar results in our uniform population of 96 NSCLC patients treated with gefitinib, with severe diarrhea occurring significantly more frequently in patients harboring the *EGFR −191C/A* and *A/A*, the *EGFR -216G/G*, and the *EGFR R497K A/A* variants. The pathophysiology of anti-EGFR–induced diarrhea is thought to result from excessive chloride secretion, inducing a secretory diarrhea (45). Therefore, the diarrhea might result from the higher EGFR expression in the intestinal lumen associated with the *EGFR* promoter polymorphisms variants as suggested previously (32). In contrast, the *A* allele in the *R497K* polymorphism is associated with alterations in EGFR ligand binding, and studies in colorectal cancer tissues showed a decreased phosphorylation of EGFR, whereas no differences were detected for *EGFR* mRNA expression (42). However, the *R497K* polymorphism was also associated with the decreased activation of c-Myc (42), whose activity is also downregulated by the *Escherichia coli* heat-stable enterotoxin STa, a major causative agent of secretory diarrhea (46).

In the present study, no associations were detected with skin toxicity, which was correlated with erlotinib exposure levels in the study by Rudin et al. (32). Data from multiple studies with erlotinib showed a consistent relationship between rash and response, as well as between rash and survival (2, 47). The relationship between rash and outcome is currently less consistent for gefitinib, and no significant association was detected in our study. These results may be due to the lower plasma concentration of gefitinib compared with erlotinib when administered at the recommended doses of 250 and 150 mg/d, respectively. Furthermore, the 250 mg/d is well below the maximum tolerated dose and the incidence of skin toxicity is significantly lower than with 500 mg/d gefitinib (47).

Female gender, never-smoker status, and BAC histology were associated with response and improved TTP or OS. These results corroborate the findings of several published studies, showing that our population is similar to other Caucasian populations treated with gefitinib. *EGFR*-activating mutations were also predictive for response, progression, and OS, as reported previously (6, 44), but not for toxicity. However, the detection of mutations requires tumor material, whose handling can

Table 5. NSCLC cell lines characteristics

NSCLC cells	Histology	*EGFR* status	*K-Ras* status	*AKT1 SNP4*	*AKT1 mRNA* expression*	Gefitinib IC_{50} (μmol/L)
A549	Adenocarcinoma	*Wt*	*Mut (G12S)*	*GA*	16.86 ± 1.55	5.40 ± 1.55
Calu-1	Epidermoid carcinoma	*Wt*	*Mut (G12C)*	*GG*	36.75 ± 2.73	3.50 ± 1.55
Calu-6	Anaplastic carcinoma	*Wt*	*Mut (Q61K)*	*AA*	0.58 ± 0.05	15.32 ± 2.03
H1650	BAC	*DelE746A750*	*Wt*	*GA*	14.68 ± 1.97	1.01 ± 0.18
H1703	Squamous cell carcinoma	*Wt*	*Wt*	*GG*	4.47 ± 0.34	8.23 ± 0.47
H1975	Adenocarcinoma	*L858R, T790M*	*Wt*	*GG*	0.63 ± 0.09	8.12 ± 0.75
H2228	Adenocarcinoma	*Wt*	*Wt*	*GG*	39.64 ± 2.13	3.25 ± 1.55
H23	Adenocarcinoma	*Wt*	*Mut (G12C)*	*AA*	0.27 ± 0.05	11.20 ± 1.35
H292	Mucoepidermoid carcinoma	*Wt*	*Wt*	*GA*	4.70 ± 0.61	0.10 ± 0.02
H322	BAC	*Wt*	*Wt*	*GG*	123.12 ± 4.78	0.25 ± 0.02
H3255	Adenocarcinoma	*L858R*	*Wt*	*GG*	31.11 ± 3.04	0.02 ± 0.01
H460	Large cell	*Wt*	*Mut (Q61H)*	*GA*	0.61 ± 0.08	12.94 ± 1.09
H522	Adenocarcinoma	*Wt*	*Wt*	*GA*	0.54 ± 0.03	13.67 ± 2.31
HCC-827	Adenocarcinoma	*DelE722-726*	*Wt*	*GG*	21.82 ± 3.17	0.01 ± 0.01
SW1573	BAC	*Wt*	*Mut (G12C)*	*GG*	10.56 ± 1.38	5.21 ± 0.41

NOTE: Data on determinants of gefitinib sensitivity in several of these NSCLC cell lines have been partly published by Zucali et al. (20).
Abbreviations: Mut, Mutation; Wt, wild-type.
*Relative *AKT1* mRNA expression levels were expressed in arbitrary units and normalized to β-actin, using a method with standard curves derived from serial dilutions of cDNA from a reference cDNA obtained Quantitative-PCR Human-Reference Total-RNA.

Published OnlineFirst February 16, 2010; DOI: 10.1158/1535-7163.MCT-09-0665

be problematic in the advanced cancer setting in which diagnosis is usually done from small needle biopsy samples. From an analysis standpoint, polymorphisms are convenient also because there are no differences in tumor and normal tissues as reported previously (41, 42).

Several polymorphisms of determinants of drug activity/metabolism have already been shown to play a critical role in predicting toxicity and response of anticancer treatment (48). Previous studies identified functional polymorphisms affecting the expression and activity of Akt (36, 37), and this is the first study aimed at evaluating their possible effects in gefitinib-treated patients. Our data showed that the *AKT1-SNP4 A/A* genotype was associated with shorter OS. Given the small number ($n = 6$) of patients harboring the *AKT1-SNP4-A/A* genotype, to evaluate whether other poor prognostic factors could potentially explain their short survival, we carefully checked their baseline demographic and biological characteristics, which were similar to the average of the studied population. Furthermore, at multivariate analysis, the *AKT1-SNP4* polymorphism remained an independent predictive parameter of progression and death risk.

Possible factors involved in resistance to anti-EGFR therapies include the occurrence of mutations in the target or in the downstream pathways, such as the *K-Ras* and the *EGFR* exon-20 *T790M* mutations, which are mainly associated with primary and secondary resistance to EGFR-TKIs, respectively (5, 18). Gene amplification, such as cMET amplification, involved in 20% of acquired resistance cases in NSCLC patients treated with EGFR-TKIs, and modulation of other pathways also contribute to resistance to EGFR-TKIs in NSCLC (19–21). Previous studies showed that SRC activation induced gefitinib resistance by modulation of both the extracellular signal-regulated kinase and Akt signaling pathways in human gallbladder adenocarcinoma cells (49). Oncogenic SRC protein also induced the epithelial-mesenchymal transition, which is associated with the loss of E-cadherin complexes at cell-cell adhesions and, consequently, with an increased invasive/metastatic potential and worse NSCLC prognosis (50). Previous immunohistochemical analyses for SRC, E-cadherin, pAkt, pERK, and p-cMET performed in tumor tissues from 51 patients enrolled in this study showed that cMET[pY1003] immunoreactivity was associated with shorter TTP (20). Interestingly, the majority of NSCLC patients with cMET[pY1003]-positive tumors had positive SRC expression and negative E-cadherin, consistent with the role of cMET in epithelial-mesenchymal transition (20). However, the identification of other biomarkers of gefitinib resistance may help in optimal patient selection, and the analysis of polymorphisms can be easily done in blood and is easier to adopt in the routine clinical setting than tumor expression arrays.

To evaluate whether the *AKT1-SNP4* polymorphism is a candidate biomarker predictive of drug activity or a prognostic factor, we used a population of gefitinib-nontreated advanced NSCLC enrolled in the NVALT-7 study. These patients were treated only with the pemetrexed ($n = 62$) or carboplatin-pemetrexed ($n = 65$) regimen, without receiving EGFR-TKIs as salvage therapy. In this study, the patients who received carboplatin-pemetrexed had a significantly longer TTP, but no significant differences were detected for OS (39). The lack of correlation between the *AKT1-SNP4-A/A* genotype and survival in these gefitinib-nontreated patients suggests that it is not a prognostic factor, whereas it might be a predictive factor of gefitinib activity. These results have to be validated in a larger cohort of patients, in prospective multicenter trials, as well as in case-control studies (48).

To gain further insight into the mechanisms behind our findings, we performed *in vitro* studies showing a significant association with both *AKT1* mRNA expression and gefitinib IC_{50}s, in agreement with the clinical results. Because sensitivity to gefitinib was correlated with the occurrence of *EGFR*-activating mutations as previously described (15), whereas the only 2 of the 15 available NSCLC cell lines harboring the *A/A* genotype have also *K-Ras* mutations, we cannot draw major conclusions from these results. However, there was a significant association considering the three different genotypes and *AKT1* mRNA levels, which were clearly correlated with sensitivity to gefitinib. Previous studies reported controversial relationship between Akt and EGFR expression and EGFR-TKI activity both in the preclinical and clinical setting (15, 34), and more genotype-phenotype correlation studies and functional analyses of the critical genes involved in the EGFR pathway are warranted.

In conclusion, the present study describes for the first time a correlation of the *AKT1-SNP4* polymorphism with TTP and OS in advanced NSCLC patients treated with gefitinib. Furthermore, *EGFR –191C/A, -216G/T*, and *R497K* polymorphisms were associated with gastrointestinal toxicity after gefitinib, as reported previously in erlotinib-treated patients (32). Validation of the role of these polymorphisms in further clinical trials will ultimately offer new tools for treatment optimization of currently available EGFR-TKIs in selected NSCLC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Francesco Crea, M.D., and Dr. Davide Torti, M.D., Scuola Superiore di Studi Universitari e Perfezionamento Sant'Anna, Pisa, Italy for their initial contributions and Dr. Marielle I Gallegos-Ruiz, Ph.D., VUmc, Amsterdam, the Netherlands, for sequencing analysis and Fondazione A.R.C.O. for grant support.

Grant Support

Fondazione A.R.C.O. (C. Tibaldi).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 07/21/2009; revised 11/06/2009; accepted 12/23/2009; published OnlineFirst 02/16/2010.

Published OnlineFirst February 16, 2010; DOI: 10.1158/1535-7163.MCT-09-0665

References

1. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial). J Clin Oncol 2003;21:2237–46.
2. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA 2003;290:2149–58.
3. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 2005;353:123–32.
4. Thatcher N, Chang A, Parikh P, et al. Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). Lancet 2005;366:527–37.
5. Pao W, Wang TY, Riely GJ, et al. KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. PLoS Med 2005;2:e17.
6. Eberhard DA, Giaccone G, Johnson BE, et al. Biomarkers of response to epidermal growth factor receptor inhibitors in Non-Small-Cell Lung Cancer Working Group: standardization for use in the clinical trial setting. J Clin Oncol 2008;26:983–94.
7. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
8. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
9. Sequist LV, Martins RG, Spigel D, et al. First-line gefitinib in patients with advanced non-small-cell lung cancer harboring somatic EGFR mutations. J Clin Oncol 2008;26:2442–9.
10. Saijo N, Takeuchi M, Kunitoh H. Reasons for response differences seen in the V15–32, INTEREST and IPASS trials. Nat Rev Clin Oncol 2009;6:287–94.
11. Tsao MS, Sakurada A, Cutz JC, et al. Erlotinib in lung cancer—molecular and clinical predictors of outcome. N Engl J Med 2005;353:133–44.
12. Zhu CQ, da Cunha Santos G, Ding K, et al. Role of KRAS and EGFR as biomarkers of response to erlotinib in National Cancer Institute of Canada Clinical Trials Group Study BR.21. J Clin Oncol 2008;26:4268–75.
13. Achille M, Gallegos-Ruiz M, Giaccone G, Soria JC. Response to erlotinib in first-line treatment of non-small-cell lung cancer in a white male smoker with squamous-cell histology. Clin Lung Cancer 2006;8:214–6.
14. Cho BC, Im CK, Park MS, et al. Phase II study of erlotinib in advanced non-small-cell lung cancer after failure of gefitinib. J Clin Oncol 2007;25:2528–33.
15. Helfrich BA, Raben D, Varella-Garcia M, et al. Antitumor activity of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib (ZD1839, Iressa) in non-small cell lung cancer cell lines correlates with gene copy number and EGFR mutations but not EGFR protein levels. Clin Cancer Res 2006;12:7117–25.
16. Cappuzzo F, Hirsch FR, Rossi E, et al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst 2005;97:643–55.
17. Hirsch FR, Varella-Garcia M, Bunn PA, Jr., et al. Molecular predictors of outcome with gefitinib in a phase III placebo-controlled study in advanced non-small-cell lung cancer. J Clin Oncol 2006;24:5034–42.
18. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PloSMed 2005;2:e73.
19. Witta SE, Gemmill RM, Hirsch FR, et al. Restoring E-cadherin expression increases sensitivity to epidermal growth factor receptor inhibitors in lung cancer cell lines. Cancer Res 2006;66:944–50.
20. Zucali PA, Ruiz MG, Giovannetti E, et al. Role of cMET expression in non-small-cell lung cancer patients treated with EGFR tyrosine kinase inhibitors. Ann Oncol 2008;19:1605–12.
21. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007;316:1039–43.
22. Nie Q, Wang Z, Zhang GC, et al. The epidermal growth factor receptor intron1 (CA)n microsatellite polymorphism is a potential predictor of treatment outcome in patients with advanced lung cancer treated with gefitinib. Eur J Pharmacol 2007;7:177–93.
23. Han SW, Lee KH, et al. Intron 1 CA dinucleotide repeat polymorphism and mutations of epidermal growth factor receptor and gefitinib responsiveness in non-small-cell lung cancer. Pharmacogenet Genomics 2007;17:313–319.
24. Tiseo M, Capelletti M, De Palma G, et al. Epidermal growth factor receptor intron-1 polymorphism predicts gefitinib outcome in advanced non-small cell lung cancer. J Thorac Oncol 2008;3:1104–11.
25. Liu G, Gurubhagavatula S, Zhou W, et al. Epidermal growth factor receptor polymorphisms and clinical outcomes in non-small-cell lung cancer patients treated with gefitinib. Pharmacogenomics J 2008;8:129–38.
26. Ma F, Sun T, Shi Y, et al. Polymorphisms of EGFR predict clinical outcome in advanced non-small-cell lung cancer patients treated with Gefitinib. Lung Cancer 2009;66:114–9.
27. Liu W, Wu X, Zhang W, et al. Relationship of EGFR mutations, expression, amplification, and polymorphisms to epidermal growth factor receptor inhibitors in the NCI60 cell lines. Clin Cancer Res 2007;13:6788–95.
28. Gregorc V, Hidalgo M, Spreafico A, et al. Germline polymorphisms in EGFR and survival in patients with lung cancer receiving gefitinib. Clin Pharmacol Ther 2008;83:477–84.
29. Buerger H, Packeisen J, Boecker A, et al. Allelic length of a CA dinucleotide repeat in the egfr gene correlates with the frequency of amplifications of this sequence—first results of an inter-ethnic breast cancer study. J Pathol 2004;203:545–50.
30. Liu W, Innocenti F, Wu MH, et al. A functional common polymorphism in a Sp1 recognition site of the epidermal growth factor receptor gene promoter. Cancer Res 2005;65:46–53.
31. Moriai T, Kobrin MS, Hope C, et al. A variant epidermal growth factor receptor exhibits altered type a transforming growth factor binding and transmembrane signaling. Proc Natl Acad Sci U S A 1994;91:10217–1021.
32. Rudin CM, Liu W, Desai A, et al. Pharmacogenomic and pharmacokinetic determinants of erlotinib toxicity. J Clin Oncol 2008;26:1119–27.
33. Cappuzzo F, Magrini E, Ceresoli GL, et al. Akt phosphorylation and gefitinib efficacy in patients with advanced non-small-cell lung cancer. J Natl Cancer Inst 2004;96:1133–41.
34. Cappuzzo F, Ligorio C, Jänne PA, et al. Prospective study of gefitinib in epidermal growth factor receptor fluorescence *in situ* hybridization-positive/phospho-Akt-positive or never smoker patients with advanced non-small-cell lung cancer: the ONCOBELL trial. J Clin Oncol 2007;25:2248–55.
35. Kim MS, Jeong EG, Yoo NJ, et al. Mutational analysis of oncogenic AKT E17K mutation in common solid cancers and acute leukaemias. Br J Cancer 2008;98:1533–5.
36. Harris SL, Gil G, Robins H, et al. Detection of functional single-nucleotide polymorphisms that affect apoptosis. Proc Natl Acad Sci U S A 2005;102:16297–302.
37. Emamian ES, Hall D, Birnbaum MJ, et al. Convergent evidence for impaired AKT1—3β signaling in schizophrenia. Nat Genet 2004;36:131–7.
38. Hildebrandt MAT, Yang H, Hung MC, et al. Genetic variations in the PI3K/PTEN/AKT/mTOR pathway are associated with clinical outcome in esophageal cancer patients treated with chemotherapy. J Clin Oncol 2009;27:857–71.
39. Smit EF, Burgers SA, Biesma B, et al. Randomized phase II and pharmacogenetic study of pemetrexed compared with pemetrexed plus carboplatin in pretreated patients with advanced non-small-cell lung cancer. J Clin Oncol 2009;27:2038–45.
40. Tibaldi C, Giovannetti E, Vasile E, et al. Correlation of CDA, ERCC1, and XPD polymorphisms with response and survival in gemcitabine/

Published OnlineFirst February 16, 2010; DOI: 10.1158/1535-7163.MCT-09-0665

cisplatin-treated advanced non-small cell lung cancer patients. Clin Cancer Res 2008;14:1797–803.

41. Marsh S, Mallon MA, Goodfellow P, McLeod HL. Concordance of pharmacogenetic markers in germline and colorectal tumour DNA. Pharmacogenomics 2005;6:873–7.
42. Wang WS, Chen PM, Chiou TJ, et al. Epidermal growth factor receptor R497K polymorphism is a favourable prognostic factor for patients with colorectal carcinoma. Clin Cancer Res 2007;13:3597–604.
43. Nomura M, Shigematsu H, Li L, et al. Polymorphisms, mutations, and amplification of the EGFR gene in non-small cell lung cancers. PLoS Med 2007;4:e125.
44. Heist RS, Christiani D. EGFR-targeted therapies in lung cancer: predictors of response and toxicity. Pharmacogenomics 2009;10:59–68.
45. Uribe JM, Gelbmann CM, Traynor-Kaplan AE, Barrett KE. Epidermal growth factor inhibits Ca2+-dependent Cl– transport in T84 human colonic epithelial cells. Am J Physiol 1996;271:C914–922.
46. Pitari GM, Zingman LV, Hodgson DM, et al. Bacterial enterotoxins are associated with resistance to colon cancer. Proc Natl Acad Sci U S A 2003;100:2695–9.
47. Perez-Soler R, Saltz L. Cutaneous adverse effects with HER1/EGFR-targeted agents: Is there a silver lining? J Clin Oncol 2005;23:5235–46.
48. Maitland ML, Ratain MJ, Cox NJ. Interpreting P values in pharmacogenetic studies: a call for process and perspective. J Clin Oncol 2007;25:4513–5.
49. Qin B, Ariyama H, Baba E, Tanaka R, et al. Activated Src and Ras induce gefitinib resistance by activation of signaling pathways downstream of epidermal growth factor receptor in human gallbladder adenocarcinoma cells. Cancer Chemother Pharmacol 2006;58:577–84.
50. Soltermann A, Tischler V, Arbogast S, et al. Prognostic significance of epithelial-mesenchymal and mesenchymal-epithelial transition protein expression in non-small cell lung cancer. Clin Cancer Res 2008;14:7430–7.

AACR American Association for Cancer Research

Molecular Cancer Therapeutics

Association of Polymorphisms in *AKT1* and *EGFR* with Clinical Outcome and Toxicity in Non–Small Cell Lung Cancer Patients Treated with Gefitinib

Elisa Giovannetti, Paolo A. Zucali, Godefridus J. Peters, et al.

Mol Cancer Ther 2010;9:581-593. Published OnlineFirst February 16, 2010.