Epidermal Growth Factor Receptor Intron-1 Polymorphism Predicts Gefitinib Outcome in Advanced Non-small Cell Lung Cancer

Marcello Tiseo, MD, Marzia Capelletti, PhD,* Giuseppe De Palma, MD,† Vittorio Franciosi, MD,* Andrea Cavazzoni, PhD,‡ Paola Mozzoni, PhD,§ Roberta R. Alfieri, PhD,‡ Matteo Goldoni, PhD,§ Maricla Galetti, PhD,‡ Beatrice Bortesi, PhD,* Cecilia Bozzetti, PhD,* Maura Loprevite, PhD,‖ Luca Boni, MD,¶ Roberta Camisa, PhD,* Guido Rindi, MD, PhD,# Pier Giorgio Petronini, PhD,‡ and Andrea Ardizzoni, MD**

Introduction: Epidermal growth factor receptor (EGFR) gene intron 1 contains a polymorphic single sequence dinucleotide repeat $(CA)_n$ whose length has been found to inversely correlate with transcriptional activity. This study was designed to assess the role of $(CA)_n$ polymorphism in predicting the outcome of gefitinib treatment in advanced non-small cell lung cancer (NSCLC).

Methods: Blood and tumor tissue from 58 patients with advanced NSCLC submitted to gefitinib were collected. EGFR intron 1 gene polymorphism, along with EGFR gene mutation, gene copy number and immunohistochemistry expression were determined. Moreover, a panel of lung cancer cell lines characterized for EGFR intron 1 polymorphism was also studied.

Results: EGFR intron 1 polymorphism showed a statistically significant correlation with the gefitinib response (response rate 25 versus 0%, for patients with a $(CA)_{16}$ and with a $(CA)_{else}$ genotype, respectively; $p = 0.044$). Patients with a $(CA)_{16}$ genotype had a longer survival compared with those with a $(CA)_{else}$ genotype (11.4 versus 4.8 months, respectively; $p = 0.037$). In addition, cell lines lacking the $(CA)_{16}$ allele showed a statistically significant higher IC_{50} compared with cell lines bearing at least one $(CA)_{16}$ allele ($p = 0.003$).

Conclusions: This study supports a potential role of EGFR intron 1 polymorphism in predicting the outcome of gefitinib treatment in advanced NSCLC.

*Division of Medical Oncology, University Hospital of Parma; †Department of Experimental and Applied Medicine, Occupational Medicine and Industrial Hygiene, Laboratory of Industrial Hygiene, University of Brescia; ‡Department of Experimental Medicine, University of Parma; §Department of Clinical Medicine, Nephrology and Health Science, Laboratory of Industrial Toxicology, University Hospital of Parma; ‖Division of Medical Oncology A, National Institute for Cancer Research, Genova; ¶Unit of Clinical Trials, National Institute for Cancer Research, Genova; and #Department of Pathology, University Hospital of Parma, Parma, Italy.

Disclosure: The authors declare no conflicts of interest.

Address for correspondence: Marcello Tiseo, Division of Medical Oncology, University Hospital of Parma, Via Gramsci 14, 43100 Parma, Italy. E-mail: mtiseo@ao.pr.it

ISSN: 1556-0864/08/0310-1104

Key Words: EGFR, Intron 1 polymorphism, Gefitinib, NSCLC.

(*J Thorac Oncol.* 2008;3: 1104–1111)

Gefitinib and erlotinib, small molecules reversibly binding the ATP pocket in the epidermal growth factor receptor (EGFR) intracellular tyrosine kinase domain, represent a new reality in the treatment of non-small cell lung cancer (NSCLC). Nevertheless, it appears that the greatest benefit obtained with these EGFR tyrosine kinase inhibitors (TKIs) is confined to some patient subgroups. Patients with the highest probability of response appear to be those of female sex, never-smokers, with adenocarcinoma histology and, particularly, with bronchiolo-alveolar carcinoma.[1]

Although clinical characteristics could be useful for the identification of candidate to EGFR TKIs, selection of patients should ideally rely on biologic tumor characteristics.[2] Specific mutations in the tyrosine kinase domain (exons 18–21) of the EGFR gene have been found to account for the increased sensitivity to gefitinib or erlotinib.[3] Recent trials have suggested that EGFR gene copy number and EGFR expression can be used to predict which patients are more likely to respond to EGFR TKIs.[4]

Moreover, Dziadziuszko et al.[5] demonstrated that EGFR mRNA expression in paraffin-embedded NSCLC specimens is also a predictive biomarker of gefitinib response. Transcription of the EGFR gene is regulated by two enhancer elements. In particular, the one located in intron 1, downstream of the transcription initiation site, has an important regulatory function.[6] It contains a characteristic simple sequence repeat of *CA* dinucleotides, whose length exhibits interethnic differences[7–9] and has been shown to correlate inversely with EGFR gene transcription, differentially modifying DNA conformation after binding of transcriptor factors.[10–12] This sequence contains a number of $(CA)_n$ repeats ranging from 9 to 23[13,14] and alleles with 16 *CA* repeats showed the highest frequency (43%) in Caucasians.[7]

Preclinical studies in head and neck cancer cell lines suggested that EGFR gene intron 1 polymorphism may cor-

relate with response to EGFR TKIs.[15] To investigate this correlation in the clinic, we evaluated the allele status and length of the intron 1 polymorphic region in NSCLC patients treated with gefitinib within an Expanded Access Program. Since this region is not generally somatically mutated[15] and to overcome the limited availability of tumor specimens in the clinical practice, EGFR $(CA)_n$ status was characterized in DNA obtained from whole blood. When sufficient tumor tissue was available, the other known predictive factors (EGFR expression, gene mutations and gene copy number) were also investigated and correlated with response and survival.

To further investigate the correlation between the length of EGFR $(CA)_n$ repeats and gefitinib response, we studied in vitro growth inhibition in a panel of NSCLC cell lines with different EGFR intron 1 polymorphisms.

PATIENTS AND METHODS

Patients

Patients, enrolled prospectively in this study, were included in Iressa Expanded Access Program and in our previous trial,[16] conducted at Parma University Hospital and the National Institute for Cancer Research in Genova. This study was approved by the institutional ethical review board and written informed consent was obtained from each patient before enrollment, together with the Expanded Access Study consent form.

Eligibility criteria included histologically confirmed NSCLC with measurable, locally advanced or metastatic disease, progressing or relapsing after at least one line of standard chemotherapy. Patients had performance status ranging from grade 0 to 2, according to Eastern Cooperative Oncology Group. Patients received gefitinib (250 mg/d) and were evaluated for response according to the Response Evaluation Criteria in Solid Tumors criteria. Tumor response was assessed by computed tomography scan after 2 months of treatment. Patients obtaining complete response, partial response or stable disease remained on therapy until disease progression or excessive toxicity with tumor reassessment every 2 months. Toxicity, assessed monthly by clinical examination and blood counts and chemistry, was evaluated according to National Cancer Institute-Common Toxicity Criteria version 2.0. Survival was calculated from the date of therapy initiation to the date of death or to the date of last contact.

An additional cohort of advanced NSCLC patients, who did not receive gefitinib treatment but only standard chemotherapy and well balanced for each of the clinical characteristics (age, gender, histology, performance status and stage of disease) with the patients treated with gefitinib, was sought within the clinical data base and blood bank of the same institutions to serve as control group.

Genotype Characterization

Genomic DNA was isolated from whole venous blood samples and amplified as previously described.[17] Fluorescent-labeled polymerase chain reaction products were run on an automated capillary electrophoresis-based DNA sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and were analyzed using Genescan to determine the sizes of repeated length.

EGFR Gene Mutational Analysis

Genomic DNA was extracted from microdissected tumor cells of formalin-fixed paraffin-embedded tissue sections. Amplifications of exons 18 through 21 of EGFR were performed using nested primers as published.[18–20] Polymerase chain reaction fragments were sequenced and analyzed in both forward and reverse directions and mutations were verified by three independent amplifications.

EGFR Gene Copy Number by Fluorescence In Situ Hybridization

EGFR gene copy number per cell was investigated on formalin-fixed paraffin-embedded specimens using LSI EGFR Spectrum orange/CEP 7 Spectrum green probe set (Vysis, Downers Grove, IL). The fluorescence in situ hybridization (FISH) protocol and sample classification is described in details elsewhere.[21] The FISH analysis was performed independently by two authors (C.B. and G.R.) who were blinded to the patients' clinical characteristics and all other molecular variables.

EGFR, p-EGFR, p-MAPK and p-AKT Immunohistochemistry

Paraffin-embedded tissue sections from tumor specimens obtained at the time of primary diagnosis were analyzed for EGFR, p-EGFR, p-MAPK and p-AKT. Protein expression was evaluated by immunohistochemistry (IHC) using methods and assessment criteria described elsewhere.[16,22] The immunostaining for EGFR, p-EGFR, p-MAPK and p-AKT expression was classified into two categories: "negative" (including negative samples and samples with >10% positive cells, but with weak staining [1+]) and "positive" (if more than 10% of the tumor cells stained moderately [2+], and if more than 10% of the cells stained strongly [3+]).[16] IHC was assessed by two observers on all sections, blinded to clinical and other biologic results. If discrepancies occurred, a consensus score was made by the two readers after discussion of the slide. The IHC data and also the results of EGFR gene mutation and FISH analyses have been reported in a previous publication.[16]

Cell Cultures and MTT Assay

The human NSCLC cell lines A549, H460, H1650, H1299, Calu-3, SKMES-1, SKLU-1 were cultured as recommended. Cell viability was assessed after 3 days of gefitinib treatment by tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sigma, Dorset, UK] assay as previously described.[23] Representative results of at least three independent experiments were used for evaluation of dose-response curves, calculated from experimental points using single or double Hill functions (Origin 6.0 Software, Microcal).[24] Concentration that inhibits 50% (IC_{50}) (e.g., the point at which viability is 50%) was extrapolated from the dose-response curves.[23]

Statistical Analysis

The association between clinical and biologic characteristics was tested using the standard χ^2 test or the Fisher exact test. The impact of clinical and biologic characteristics on response rate was analyzed using multivariate logistic regression. Overall survival was defined as the time between study registration and death, whatever the cause. Estimates of overall survival were calculated according to the Kaplan-Meier method. Comparisons of estimated survival curves were performed by means of the log-rank test. Finally, to investigate the joint effect of the clinical and biologic characteristics on overall survival, a Cox proportional hazard model was fitted to the data.

To assess for possible evolutionary changes occurring in the EGFR $(CA)_n$ repeat polymorphism, Hardy-Weinberg analysis was performed by matching allele frequencies in the observed individuals to those expected under Hardy-Weinberg Equilibrium. Genotypes were dichotomized as EGFR $(CA)_{16}$, including at least one $(CA)_{16}$ allele, and EGFR $(CA)_{else}$, lacking the EGFR $(CA)_{16}$ allele, and compared using a χ^2 test goodness-of-fit test.

Differences between mean IC_{50} values in cell lines classified as bearing a $(CA)_{16}$ or a $(CA)_{else}$ EGFR intron 1 polymorphism were assessed by unpaired Student *t* test.

RESULTS

Patients Characteristics

A total of 58 previously treated patients with advanced NSCLC were prospectively enrolled in this study. The main characteristics of the patients are shown in Table 1.

For the entire group, the objective response rate was 15% (two complete and seven partial responses), the disease control rate (complete response + partial response + stable disease) was 38% and overall median survival was 6 months.

TABLE 1. Patient Characteristics

	No. of Patients (n = 58)	%
Gender		
Males	39	67
Females	19	33
Age, years		
Median (range)	67 (44–82)	
Histology		
Adenocarcinoma	43	74
Nonadenocarcinoma	15	26
Stage		
IV	58	100
PS ECOG		
0	14	24
1	36	62
2	8	14
Smoking status		
Current or former smokers	47	81
No smokers	11	19

Patients with an objective response had a median survival of 18.5 months versus 5.1 month of nonresponders ($p = 0.014$).

Female sex, never smoking status and adenocarcinoma histology were associated with a higher probability of response to gefitinib treatment (Table 2). During treatment, toxicity of any grade and type was noted in 29 patients (50%) and consisted mainly of skin rash and gastro-intestinal disorders; in particular, 24 patients (41%) developed an acne-like rash. We found a positive predictive role for general toxicity ($p = 0.025$), but not for skin toxicity ($p = 0.142$) (Table 2). In terms of disease control, both general toxicity ($p = 0.003$) and skin toxicity ($p = 0.013$) evidenced a statistically significant role in predicting gefitinib activity. Moreover, patients who developed toxicity during gefitinib treatment had a statistically significant improvement in survival ($p = 0.002$) (Table 2).

EGFR Intron 1 Polymorphism

EGFR intron 1 polymorphism could be characterized in 51 patients (for seven patients the blood sample were not available). Alleles ranging within 14 to 21 dinucleotides were found, with the most frequent being $(CA)_{16}$ and $(CA)_{18}$. Heterozygosity was observed in 64.7% (33/51) of patients and in 69.7% (23/33) of these the shorter allele consisted of $(CA)_{16}$ repeats. Seventeen different $(CA)_n$ genotypes were found and the most common were 16 to 16 (29.4%) and 16 to 18 (13.7%). Allele and genotype frequencies, reported in Table 3, were in agreement with other published Caucasians series.[7,8]

Among the 15 patients with a genotype lacking the $(CA)_{16}$ allele, 14 had at least one long allele ($\geq$17 dinucleotides). To evaluate the predictive value of EGFR intron 1 polymorphism, we divided patients into two subgroups: 36 (70.6%) bearing at least one $(CA)_{16}$ allele [$(CA)_{16}$ genotype] and 15 (29.4%) lacking the $(CA)_{16}$ allele [$(CA)_{else}$ genotype].

According to this dichotomy, genotype frequencies were not significantly different from what expected on the basis of the Hardy-Weinberg Equilibrium ($\chi^2 = 0.956$, $p = 0.620$).

Eighty percent of patients carrying the $(CA)_{16}$ allele were adenocarcinoma. All mutated patients showed at least one $(CA)_{16}$ allele. No correlation was found between intron 1 polymorphism and sex, smoking status, toxicity and other biologic factors (Table 4).

EGFR intron 1 polymorphism showed a statistically significant correlation with gefitinib activity; in fact, 25% of patients with a $(CA)_{16}$ genotype had an objective response, as opposed to none of the patients with a $(CA)_{else}$ genotype (odds ratio = 10.7, 95% confidence interval 1.21–1415; $p = 0.044$) (Table 2). The effect of the $(CA)_{16}$ genotype was confirmed in the multivariate model including gender, smoking status, EGFR intron 1 genotype and development of toxicity during treatment (odds ratio = 10.1, 95% confidence interval 0.88–1475; $p = 0.07$).

It was of note that patients bearing a $(CA)_{16}$ genotype showed a longer survival compared with those with a $(CA)_{else}$ genotype (11.4 versus 4.8 months, respectively; hazard ratio [HR] = 0.49, 95% confidence interval 0.25–0.97; $p = 0.037$) (Figure 1). When gender, smoking status, histology, EGFR intron 1 genotype and development of toxicity during treat-

TABLE 2. General Characteristics, EGFR Intron 1 Polymorphism, EGFR Gene Mutations, EGFR Gene Copy Number and Immunohistochemistry Protein Expression in Gefitinib Responder and Nonresponder Patients

Characteristics	CR + PR *n* (%)	SD + PD *n* (%)	*p*	DC *n* (%)	PD *n* (%)	*p*	Median Survival (Months)	*p*
Sex			0.047			0.151		0.245
Female	6 (32)	13 (68)		10 (53)	9 (47)		8.4	
Male	3 (8)	36 (92)		12 (31)	27 (69)		5.3	
Smoking status			0.056			0.302		0.064
No smokers	4 (36)	7 (64)		6 (55)	5 (45)		12.7	
Current or former	5 (11)	42 (89)		16 (34)	31 (66)		5.3	
Histology			0.422			0.128		<0.001
Adenocarcinoma	8 (19)	35 (81)		19 (44)	24 (56)		8.4	
Nonadenocarcinoma	1 (7)	14 (93)		3 (20)	12 (80)		2.8	
Toxicity			0.025			0.003		0.002
Yes	8 (28)	21 (72)		17 (59)	12 (41)		12	
No	1 (3)	28 (97)		5 (17)	24 (83)		2.8	
Skin toxicity			0.142			0.013		0.026
Yes	6 (25)	18 (75)		14 (58)	10 (42)		11.4	
No	3 (9)	31 (91)		8 (23)	26 (77)		4.8	
EGFR intron 1 polymorphism			0.044			0.004		0.037
$(CA)_{16}$ genotype	9 (25)	27 (75)		19 (53)	17 (47)		11.4	
$(CA)_{else}$ genotype	0 (0)	15 (100)		1 (7)	14 (93)		4.8	
EGFR gene mutations			<0.001			0.035		0.544
Mutated	5 (100)	0 (0)		5 (100)	0 (0)		18.5	
Wild-type	1 (6)	15 (94)		6 (37)	10 (63)		8.8	
EGFR FISH			0.326			0.630		0.179
Positive	3 (43)	4 (57)		4 (57)	3 (43)		8.8	
Negative	2 (18)	9 (82)		4 (36)	7 (64)		18.5	
EGFR IHC			0.530			0.582		0.436
Positive	5 (31)	11 (69)		7 (44)	9 (56)		5.7	
Negative	0 (0)	3 (100)		2 (67)	1 (33)		17.3	
p-EGFR IHC			0.278			0.314		0.221
Positive	0 (0)	5 (100)		1 (20)	4 (80)		5.1	
Negative	4 (31)	9 (69)		7 (54)	6 (46)		18.5	
p-MAPK IHC			0.608			0.188		0.892
Positive	3 (37)	5 (63)		6 (75)	2 (25)		8.8	
Negative	2 (20)	8 (80)		4 (40)	6 (60)		5.1	
p-AKT IHC			0.548			0.624		0.863
Positive	2 (40)	3 (60)		3 (60)	2 (40)		12.9	
Negative	3 (18)	13 (82)		6 (41)	10 (59)		8.8	

Percentage along each row.

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; DC, disease Control, CR + PR + SD; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

ment were introduced in the multivariate model the effect of the $(CA)_{16}$ genotype on OS did not change (HR = 0.55, 95% confidence interval 0.27–1.15; $p = 0.111$).

To confirm the predictive effect of EGFR intron 1 polymorphism we examined the outcomes of an additional control cohort of NSCLC patients well balanced for each of the clinical characteristics who did not receive gefitinib treatment but only standard chemotherapy. In this control group of 31 NSCLC patients no statistically significant differences were seen in response rate and in overall survival of patients classified according $(CA)_n$ genotype. In fact, 29.2% of patients (17/24) with a $(CA)_{16}$ genotype and 28.6% of patients (2/7) with a $(CA)_{else}$ genotype had an objective response ($p = 1$); moreover, no survival difference was observed between these groups (12.4 versus 9.3 months, respectively; $p = 0.558$).

EGFR Gene Mutations, Gene Copy Number and IHC Expression

EGFR gene mutational analysis was performed on 21 patients for whom paraffin embedded blocks were available. Five heterozygous mutations were observed; all these mutations have previously been described [four exon 19 deletions (two 2235del15, one 2240del12 and one 2240del18) and one

TABLE 3. Genotype and Allele Frequencies of EGFR Intron 1 Polymorphism in 51 Patients

Genotypes $(CA)_n$ Repeats	*n* (%)	Alleles $(CA)_n$ Repeats	*n* (%)
14–15	1 (2)	14	2 (2)
14–21	1 (2)	15	4 (3.9)
15–17	1 (2)	16	51 (50.0)
15–20	1 (2)	17	8 (7.8)
15–21	1 (2)	18	15 (14.7)
16–16	15 (29.4)	19	3 (2.9)
16–17	5 (9.8)	20	14 (13.7)
16–18	7 (13.7)	21	5 (4.9)
16–19	1 (2)		
16–20	6 (11.8)		
16–21	2 (3.9)		
17–18	2 (3.9)		
18–18	1 (2)		
18–19	1 (2)		
18–20	3 (5.9)		
19–21	1 (2)		
20–20	2 (3.9)		

exon 21 missense mutation (L858R)].[18–20] All EGFR mutated patients had an objective response to gefitinib. Among responders 5/6 (83%) showed EGFR gene mutation, while none with stable or progressive disease did ($p < 0.001$). EGFR mutated patients evidenced a median survival of 18.5 months compared with 8.8 months of EGFR wild type ones ($p = 0.544$) (Table 2).

EGFR FISH analysis was performed on 18 tumors specimens and was positive in seven patients (38.9%). FISH analysis demonstrated polysomy or amplification of EGFR gene in 60% of responding patients. The differences between responders and nonresponders were not statistically significant and, moreover, no difference was observed in median survival according FISH status (Table 2).

The IHC expression of EGFR, p-EGFR, p-MAPK and p-AKT was assessed in the tumor specimens of 21 patients. EGFR was positive in 84%, p-EGFR in 28%, p-MAPK in 44% and p-AKT in 23% of patients. Clinical response to gefitinib and survival did not differ according to protein expression of any of the biomarkers assessed (Table 2).

Sensitivity of NSCLC Cell Lines to Gefitinib According to EGFR Intron 1 Polymorphism

After characterization of EGFR $(CA)_n$ polymorphism, five NSCLC cell lines were classified as bearing a $(CA)_{16}$ genotype [$(CA)_{16–16}$: A549, SKMES-1 and H460; $(CA)_{16–17}$: H1299; $(CA)_{16–18}$: Calu-3], whereas two as carrying a $(CA)_{else}$ genotype [$(CA)_{18–21}$: H1650; $(CA)_{20–20}$: SKLU-1].

Figure 2 shows that after exposure to gefitinib, cell lines with a $(CA)_{else}$ genotype displayed a reduced growth inhibition compared with those with a $(CA)_{16}$ genotype (on average, IC_{50} values of 19.20 ± 2.26 μM versus 5.85 ± 3.22 μM, respectively; $p = 0.003$). We considered resistant cell lines showing $IC_{50} > 10$ μM accordingly to other previously published papers.[15,25]

TABLE 4. Baseline Characteristics According to EGFR Intron 1 Polymorphism [$(CA)_{16}$ Genotype Versus $(CA)_{else}$ Genotype]

	$(CA)_{16}$ Genotype *n* (%)	$(CA)_{else}$ Genotype *n* (%)	*p*
Gender			0.060
Males	21 (62)	13 (38)	
Females	15 (88)	2 (12)	
Smoking status			0.472
Current or former smokers	27 (67)	13 (33)	
No smokers	9 (82)	2 (18)	
Histology			0.083
Adenocarcinoma	29 (78)	8 (22)	
Nonadenocarcinoma	7 (50)	7 (50)	
Toxicity			0.759
Yes	20 (74)	7 (26)	
No	16 (67)	8 (33)	
Skin toxicity			0.554
Yes	18 (75)	6 (25)	
No	18 (67)	9 (33)	
EGFR gene mutations			0.260
Mutated	5 (100)	0 (0)	
Wild-type	9 (60)	6 (40)	
EGFR FISH			1
Positive	8 (67)	4 (33)	
Negative	3 (60)	2 (40)	
EGFR IHC			0.515
Positive	9 (60)	6 (40)	
Negative	2 (100)	0 (0)	
p-EGFR IHC			0.117
Positive	2 (40)	3 (60)	
Negative	10 (83)	2 (17)	
p-MAPK IHC			0.308
Positive	6 (85)	1 (15)	
Negative	5 (56)	4 (44)	
p-AKT IHC			0.260
Positive	5 (100)	0 (0)	
Negative	9 (60)	6 (40)	

Percentage along each row.
FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

DISCUSSION

The purpose of this study was to assess the possible relationship between EGFR intron 1 polymorphism and the outcome of gefitinib treatment in patients with advanced NSCLC.

In this specific intron of the EGFR gene, a highly polymorphic $(CA)_n$ repeat was identified. Recent in vitro and in vivo studies indicate that transcriptional activity of EGFR gene is related to the number of *CA* repeats in intron 1.[9,15,26,27] As potential consequence, the response to EGFR TKIs may differ between patients as a function of their genotype. This finding is in line with the observation that skin toxicity during gefitinib or erlotinib therapy is related to antitumor response[28] supporting the hypothesis that sensitivity to EGFR TKI treatment could be influenced not only by different levels of target

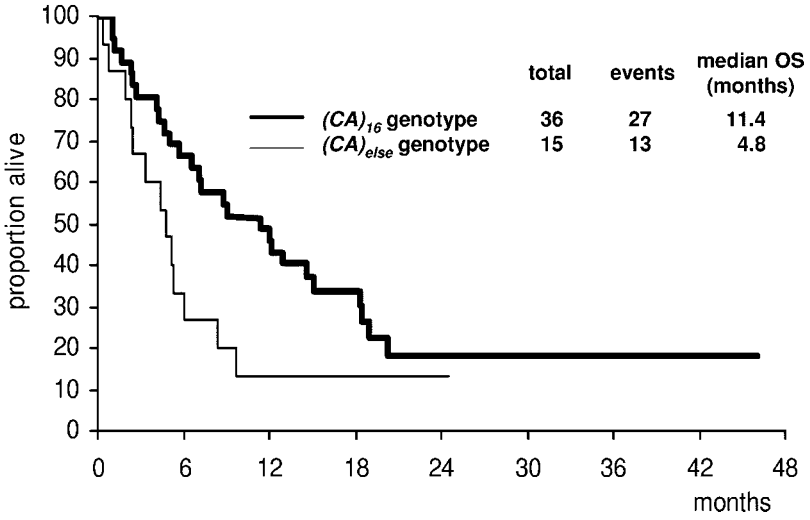

FIGURE 1. Kaplan-Meier plot of survival time according to EGFR intron 1 polymorphism. [$(CA)_{16}$ genotype versus $(CA)_{else}$ genotype; HR = 0.49, 95% confidence interval 0.25–0.97; $p = 0.037$].

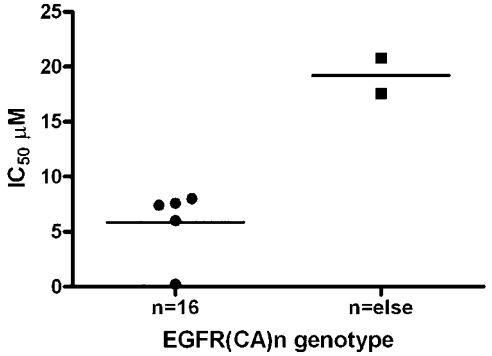

FIGURE 2. Distribution of IC_{50} values among NSCLC cell lines classified for the EGFR intron 1 polymorphism. Cells were treated with gefitinib concentrations ranging from 0.1 to 50 μM for 72 hours and then cell viability was determined by MTT assay as described in section "Patients and Methods." Representative results of at least 3 independent experiments. [$(CA)_{16}$ genotype versus $(CA)_{else}$ genotype, on average, IC_{50} values of 5.85 ± 3.22 μM versus 19.20 ± 2.26 μM, respectively; $p = 0.003$]. n = number of *CA* repeats.

expression in the tumor, but also by some inherited genetic factor in the host.

Our study evidence a potential role of EGFR intron 1 polymorphism in predicting the outcome of gefitinib treatment in advanced NSCLC, confirming the previous observation performed recently.[29–31] Indeed, we observed that patients lacking the $(CA)_{16}$ allele almost universally experience rapid disease progression despite gefitinib treatment, suggesting a possible negative predictive value of $(CA)_{16}$ allele absence in the response to EGFR TKI therapy. In addition, patients with at least one $(CA)_{16}$ allele [i.e., with $(CA)_{16}$ genotype] had a longer survival than those lacking the $(CA)_{16}$ allele [i.e., with $(CA)_{else}$ genotype].

It could be argued that $(CA)_{16}$ genotype is just another prognostic factor with the ability to confer a better prognosis regardless of treatment. However, the only study (Eastern Cooperative Oncology Group 3590) where the pure prognostic value of this polymorphism was assessed came to the opposite conclusion.[32] In fact, this trial, a randomized prospective study of adjuvant therapy following resection of stage II–IIIA NSCLC, evidenced a prognostic significance of EGFR intron 1 polymorphism, demonstrating that patients with more than 35 $(CA)_n$ repeats, calculated as sum of the two allele lengths, had a significantly longer overall survival compared with patients with 35 or fewer $(CA)_n$ repeats. Therefore, in this study longer EGFR intron 1 simple sequence repeats, evaluated in surgical specimens of patients not treated with EGFR TKIs, are associated with improved survival.

In addition, the lack of either predictive or prognostic effect of $(CA)_{16}$ genotype in the control gefitinib-untreated group of our study provides further evidence that improved survival in patients with $(CA)_{16}$ genotype is due to improved efficacy of gefitinib in this group. Therefore, the improved survival found in our study for patients bearing a shorter allele length was attributed to a higher efficacy of gefitinib in this group of patients.

Our in vitro studies were consistent with this hypothesis. In fact, NSCLC cell lines lacking the $(CA)_{16}$ allele demonstrated a statistically significant higher IC_{50} compared with lines bearing at least one $(CA)_{16}$ allele.

The most obvious mechanism by which this polymorphism is associated with gefitinib response is by affecting the levels of EGFR, with an inverse correlation between the number of intron 1 *CA* dinucleotides and protein expression, which is known to influence efficacy of EGFR TKIs.[21,33] This inverse correlation has been noted in preclinical models,[15] although in clinical studies, including our own, results are less clear-cut.

It should be borne in mind that tumors may have other somatic genetic alterations, such as the EGFR gene mutations, EGFR gene amplification and also amplifications in intron 1 region,[9,34] which can compensate for the deficient transcriptional activity of long alleles or suppress those of short alleles. In this study, we confirmed significant clinical benefit of gefitinib in patients with EGFR mutations, but not

the predictive significance of EGFR gene copy number, probably due to small number of cases. In our series, all EGFR gene mutations were found in patients with $(CA)_{16}$ allele as opposed to none in patients with $(CA)_{else}$ genotype, although this difference was not statistically significant. A similar lack of statistically significant correlation between EGFR mutations and intron 1 polymorphism was found in the only other reported study on this subject.[35]

In line with the hypothesis of a genetic predisposition affecting a differential sensitivity to EGFR TKIs, we found a predictive role for general toxicity and a survival advantage for patients who developed adverse events, particularly skin toxicity. We did not observe any correlation between $(CA)_n$ repeat polymorphism and toxicity and the only other study assessing this issue came to the same conclusions.[36] Other genetic factors, such as ABCG2 and ABCB1 polymorphisms,[36] could account for the correlation between toxicity and response to EGFR TKIs.

From a practical perspective, assessment of intron 1 polymorphism as a predictor for clinical outcome is attractive. It can be easily measured in normal tissues, is a constant feature and is technically simple, objective and quantitative in both fresh-frozen and formalin-fixed, paraffin-embedded tissue.

Despite the small sample size and the lack histologic material for correlative studies in most of them, our study constitutes a starting point for elucidating the molecular basis of genotypic differences in response to EGFR inhibitors. It should also be emphasized that other polymorphisms in linkage disequilibrium with the $(CA)_n$ repeat may also contribute to variability in EGFR expression and to treatment response. In conclusion, in addition to known gefitinib predictive factors, our data suggest a potential role of EGFR intron 1 polymorphism in predicting response to gefitinib in advanced NSCLC. Further prospective and larger studies in the field are warranted.

ACKNOWLEDGMENTS

Supported, partially, by Italian Ministry of Health and CariParma Foundation grants. This investigation was also supported by grants from the Associazione Davide Rodella, Montichiari and the A.VO.PRO.RI.T., Parma.

REFERENCES

1. Giaccone G. Epidermal growth factor receptor inhibitors in the treatment of non-small cell lung cancer. *J Clin Oncol* 2005;23:3235–3242.
2. Bunn PA, Jr., Dziadziuszko R, Varella-Garcia M, et al. Biological markers for non-small cell lung cancer patient selection for epidermal growth factor receptor tyrosine kinase inhibitor therapy. *Clin Cancer Res* 2006;12:3652–3656.
3. Janne PA, Engelman JA, Johnson BE. Epidermal growth factor receptor mutations in non-small cell lung cancer: implications for treatment and tumor biology. *J Clin Oncol* 2005;23:3227–3234.
4. Dziadziuszko R, Hirsch FR, Varella-Garcia M, Bunn PA Jr. Selecting lung cancer patients for treatment with epidermal growth factor receptor tyrosine kinase inhibitors by immunohistochemistry and fluorescence in situ hybridization—why, when, and how? *Clin Cancer Res* 2006; 12**(Suppl 14)**:4409s–4415s.
5. Dziadziuszko R, Witta SE, Cappuzzo F, et al. Epidermal growth factor receptor messenger RNA expression, gene dosage, and gefitinib sensitivity in non-small cell lung cancer. *Clin Cancer Res* 2006;12:3078–3084.
6. McInerney JM, Wilson MA, Strand KJ, Chrysogelos SA. A strong intronic enhancer element of the EGFR gene is preferentially active in high EGFR expressing breast cancer cells. *J Cell Biochem* 2001;80:538–549.
7. Liu W, Innocenti F, Chen P, Das S, Cook EH, Jr., Ratain MJ. Interethnic difference in the allelic distribution of human epidermal growth factor receptor intron 1 polymorphism. *Clin Cancer Res* 2003;9:1009–1012.
8. Buerger H, Packeisen J, Boecker A. 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;203:545–550.
9. 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;4:715–727.
10. Gebhardt F, Zanker KS, Brandt B. Modulation of epidermal growth factor receptor gene transcription by a polymorphic dinucleotide repeat in intron 1. *J Biol Chem* 1999;274:13176–13180.
11. Buerger H, Gebhardt F, Schmidt H, et al. Length and loss of heterozygosity of an intron 1 polymorphic sequence of EGFR is related to cytogenetic alterations and epithelial growth factor. *Cancer Res* 2000; 60:854–857.
12. Gebhardt F, Buerger H, Brandt B. Modulation of EGFR gene transcription by secondary structures, a polymorphic repetitive sequence and mutations-a link between genetics and epigenetics. *Histol Histopathol* 2000;15:929–936.
13. Zhang W, Park DJ, Lu B, et al. Epidermal growth factor receptor gene polymorphisms predict pelvic recurrence in patients with rectal cancer treated with chemoradiation. *Clin Cancer Res* 2005;11:600–605.
14. Etienne-Grimaldi MC, Pereira S, Magné N, et al. Analysis of the dinucleotide repeat polymorphism in the epidermal growth factor receptor (EGFR) gene in head and neck cancer patients. *Ann Oncol* 2005;16: 934–941.
15. Amador ML, Oppenheimer D, Perea S, et al. An epidermal growth factor receptor intron 1 polymorphism mediates response to epidermal growth factor receptor inhibitors. *Cancer Res* 2004;64:9139–9143.
16. Loprevite M, Tiseo M, Chiaramondia M, et al. Buccal mucosa cells as "in vivo" model to evaluate gefitinib activity in patients with advanced non-small cell lung cancer. *Clin Cancer Res* 2007;13:6518–6526.
17. McKay JA, Murray LJ, Curran S, et al. Evaluation of the epidermal growth factor receptor (EGFR) in colorectal tumors and lymph node metastases. *Eur J Cancer* 2002;8:2258–2264.
18. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–2139.
19. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304: 1497–1500.
20. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* 2004;101:13306–13311.
21. 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;97:643–655.
22. Grossi F, Loprevite M, Chiaramondia M, et al. Prognostic significance of K-ras, p53, bcl-2, PCNA, CD34 in radically resected non-small cell lung cancers. *Eur J Cancer* 2003;39:1242–1250.
23. Cavazzoni A, Petronini PG, Galetti M, et al. Dose-dependent effect of FHIT-inducible expression in Calu-1 lung cancer cell line. *Oncogene* 2004;23:8439–8446.
24. Goldoni M, Vettori MV, Alinovi R, Caglieri A, Ceccatelli S, Mutti A. Models of neurotoxicity: extrapolation of benchmark doses in vitro. *Risk Anal* 2003;23:505–514.
25. Noro R, Gemma A, Kosaihira S, et al. Gefitinib (IRESSA) sensitive lung cancer cell lines show phosphorylation of Akt without ligand stimulation. *BMC Cancer* 2006;6:277–289.
26. Brandt B, Mayer-Staeckling S, Schmidt H, Agelopoulos K, Buerger H. Mechanism of egfr gene transcription modulation: relationship to cancer risk and therapy response. *Clin Cancer Res* 2006;12:7252–7259.
27. Araujo A, Ribeiro R, Azevedo I, et al. Genetic polymorphisms of the epidermal growth factor and related receptor in non-small-cell lung cancer—a review of the literature. *Oncologist* 2007;12:201–210.
28. Perez-Soler R, Saltz L. Cutaneous adverse effects with HER1/EGFR-

targeted agents: is there a silver lining? *J Clin Oncol* 2005;23:5235–5246.
29. 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;8:129–133.
30. Han SW, Jeon YK, 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.
31. Ichihara S, Toyooka S, Fujiwara Y, et al. The impact of epidermal growth factor receptor gene status on gefitinib-treated Japanese patients with non-small-cell lung cancer. *Int J Cancer* 2007;120:1239–1247.
32. Dubey S, Stephenson P, Levy DE, et al. EGFR dinucleotide repeat polymorphism as a prognostic indicator in Non-small Cell Lung Cancer. *J Thorac Oncol* 2006;1:406–412.
33. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* 2006;353:123–132.
34. Tidow N, Boecker A, Schmidt H, et al. Distinct amplification of an untranslated regulatory sequence in the egfr gene contributes to early steps in breast cancer development. *Cancer Res* 2003;63:1172–1178.
35. Taron M, Ichinose Y, Rosell R, et al. Activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor are associated with improved survival in gefitinib-treated chemorefractory lung adenocarcinomas. *Clin Cancer Res* 2005;11:5878–5885.
36. Cusatis G, Gregorc V, Li J, et al. Pharmacogenetics of ABCG2 and adverse reactions to gefitinib. *J Natl Cancer Inst* 2006;98:1739–1742.

