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Clinical Implications of *KRAS* Mutations in Lung Cancer Patients Treated with Tyrosine Kinase Inhibitors: An Important Role for Mutations in Minor Clones[1]

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Abstract

Mutations inducing resistance to anti–epidermal growth factor receptor (EGFR) therapy may have a clinical impact even if present in minor cell clones which could expand during treatment. We tested this hypothesis in lung cancer patients treated with tyrosine kinase inhibitors (TKIs). Eighty-three patients with lung adenocarcinoma treated with erlotinib or gefitinib were included in this study. The mutational status of *KRAS* and *EGFR* was investigated by direct sequencing (DS). *KRAS* mutations were also assessed by mutant-enriched sequencing (ME-sequencing). DS detected *KRAS* mutations in 16 (19%) of 83 tumors; ME-sequencing identified all the mutations detected by DS but also mutations in minor clones of 14 additional tumors, for a total of 30 (36%) of 83. *KRAS* mutations assessed by DS and ME-sequencing significantly correlated with resistance to TKIs ($P = .04$ and $P = .004$, respectively) and significantly affected progression-free survival (PFS) and overall survival (OS). However, the predictive power of mutations assessed by ME-sequencing was higher than that obtained by DS (hazard ratio [HR] = 2.82, $P = .0001$ *vs* HR = 1.98, $P = .04$, respectively, for OS; HR = 2.52, $P = .0005$ *vs* HR = 2.21, $P = .007$, respectively, for PFS). Survival outcome of patients harboring *KRAS* mutations in minor clones, detected only by ME-sequencing, did not differ from that of patients with *KRAS* mutations detected by DS. Only *KRAS* mutations assessed by ME-sequencing remained an independent predictive factor at multivariate analysis. *KRAS* mutations in minor clones have an important impact on response and survival of patients with lung adenocarcinoma treated with EGFR-TKI. The use of sensitive detection methods could allow to more effectively identify treatment-resistant patients.

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Abbreviations: CR, complete response; DS, direct sequencing; DCR, disease control rate; EGFR, epidermal growth factor receptor; ME-sequencing, mutant-enriched sequencing; NSCLC, non–small cell lung cancer; OR, objective response; OS, overall survival; PR, partial response; PFS, progression-free survival; SD, stable disease; TKI, tyrosine kinase inhibitor; HR, hazard ratio

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Introduction

The development of small-molecule tyrosine kinase inhibitors (TKIs), such as gefitinib or erlotinib, which specifically inhibit signaling from the epidermal growth factor receptor (EGFR), has greatly influenced the treatment of non–small cell lung cancer (NSCLC) patients [1–3], allowing dramatic responses, even within a short time since the administration [4,5]. However, these remarkable responses are limited to a small subset of patients. A high sensitivity to gefitinib [4,5] or erlotinib [6] treatment has been reported in *EGFR*-mutated lung cancers. Unfortunately, drug resistance can occur because of additional mutations in the *EGFR* gene. The major lesion identified to date is an *EGFR* T790M mutation that blocks the binding of erlotinib or gefitinib to the kinase ATP binding pocket [7,8]. It has recently been reported that T790M mutations present in small fractions of tumor cells before therapy are crucial in response to treatment [9]. Indeed, neoplastic cells carrying this mutation are drug-selected until the tumor becomes widely resistant. Therefore, use of extremely sensitive analytic procedures to detect resistant-inducing mutations in minor clones has been suggested.

Other mechanisms of developing resistance to TKI include constitutive activation of downstream mediators. *KRAS*, a main downstream signaling molecule in the EGFR pathway, is frequently affected by somatic mutations in NSCLC, particularly in lung adenocarcinomas [10,11]. Most (>95%) *KRAS* mutations reported in NSCLC were found at codon 12 [11]. Constitutive activation of Ras proteins by somatic mutations may render tumor cells independent of EGFR signaling and thereby resistant to EGFR-TKI therapy. Pao et al. reported for the first time that lung adenocarcinoma patients with *KRAS* mutations are not responsive to gefitinib or erlotinib [12]. After this seminal article, numerous studies confirmed this observation [13–18]. In these studies, the mutational status of *KRAS* was investigated by direct sequencing (DS). Although DS is reliable for screening germ line or prevalent somatic mutations, sequencing of low-prevalence mutations is problematic. In fact, DNA sequencing is useful only when the fraction of mutated alleles is greater than 20% [19]. In recent years, a number of more sensitive techniques have been developed. Among them, one of the most sensitive is mutant-enriched sequencing (ME-sequencing) that can detect one copy of mutant allele among as many as 10^3 to 10^4 copies of wild-type alleles [20,21]. We have recently used this technique to investigate the mutational status of *KRAS* in a large series of colorectal adenocarcinomas. By means of the enriched procedure, we found mutations in minor clones, undetectable by DS, in 15% of the tumors examined [22]. The clinical meaning of *KRAS* mutations affecting minor clones is still unknown. In the present study, we compared the predictive power of *KRAS* mutations assessed by DS or by ME-sequencing on a series of patients affected by lung adenocarcinomas treated with TKI.

Materials and Methods

Patients and Tissues

Eighty-three patients with histologic diagnosis of lung adenocarcinoma treated with either erlotinib (55 patients) or gefitinib (28 patients) monotherapy at three national referral centers (University of Chieti, Regina Elena National Cancer Institute, and Istituto Clinico Humanitas) between 2005 and 2007 were included in this study. Major inclusion criteria encompassed the following: age older than 18 years, advanced inoperable disease (stage III or IV), documented progressive disease after at least one previous line of chemotherapy for advanced disease or major contraindications to chemotherapy treatment, treatment was also allowed for patients who had failed a platinum-based chemotherapy regimen in either the neoadjuvant or adjuvant setting, and absence of major comorbidities contraindicating the treatment with an EGFR inhibitor. Informed consent was obtained from all patients. Treatment consisted of standard-dose EGFR-TKI (250 mg/die of gefitinib or 150 mg/die of erlotinib) and was administered as first (n = 14), second (n = 38), or third or greater (n = 31) line for advanced inoperable disease. Patients' characteristics are summarized in Table 1*A*. Response to treatment was evaluated by standard criteria from the World Health Organization [23] every 8 to 10 weeks; response was confirmed at 4 weeks or longer for patients in complete response (CR) or partial response (PR). Paraffin-embedded tumor specimens were obtained from all patients before any therapy. Samples were cut into 5-μm-thick sections, placed onto glass slides, and stained with hematoxylin-eosin. Neoplastic areas were manually microdissected to have at least 70% to 80% of neoplastic cells in the sample before DNA extraction.

Mutational Analysis of KRAS *and* EGFR *by DS*

Genomic DNA was isolated by standard procedures. Genetic analysis of the *EGFR* gene was performed as previously described [24].

Table 1. Clinicopathologic and Biologic Parameters and Clinical Outcome of Patients Treated with EGFR-TKI (n = 83).

	Patients	
	No.	%
(A) Clinicopathologic and biologic parameters		
Age (years)		
Median	65	
Range	43–80	
Sex		
Male	48	58
Female	35	42
Stage at treatment		
III	13	16
IV	70	84
Smoking history		
Never	29	35
Former	31	37
Current	23	28
KRAS mutations		
DS		
Present	16	19
Absent	67	81
Enriched sequencing		
Present	30	36
Absent	53	64
EGFR mutations		
Present	20	24
Absent	63	76
(B) Clinical outcome		
OR (WHO)		
CR	3	4
PR	8	10
SD	26	31
PD	46	55
Overall response (CR + PR)	11	13
DCR (CR + PR + SD)	37	45
PFS		
Median (95% CI)	5 (2.90–7.10)	
Progression-free at 1 year	30	36
Progression-free at 2 years	16	19
OS		
Median (95% CI)	14 (5.33–22.67)	
Alive at 1 year	41	49
Alive at 2 years	30	36

PD indicates progressive disease.

Genetic analysis of *KRAS* was performed by polymerase chain reaction (PCR) amplification of exon 2. The following primers were used for PCR amplification: 5′-ACTGAATATAAACTTGTGGTAGTTGGAGCT-3′; 5′-TAATATGCATATTAAAACAAGATTTACCTC-3′. The PCR products were purified and subjected to bidirectional sequencing using the same primers used for amplification. Samples harboring mutations were reamplified and resequenced using the same experimental conditions. Sequence chromatograms were analyzed by Mutation Surveior 3.0 (SoftGenetics, State College, PA), followed by manual review.

Mutational Analysis of KRAS *by ME-Sequencing*

The ME-sequencing assay was used to increase the detection sensitivity of mutations at codon 12. This technique involves a first PCR reaction, an enzymatic digestion, and a second PCR leading to enrichment of mutant allele before sequencing (Figure 1). The first PCR was performed using Primer A, 5′-ACTGAATATAAACTTGTGGTAGTTGGACCT-3′, and Primer B, 5′-TCAAAGAATGGTCCTGGACC-3′. Underlined bases represent mismatches from the *KRAS* DNA sequence (C to G and G to C, for primers A and B, respectively). The mismatches introduce a new CCTGG sequence. The restriction enzyme *Bst*NI (Takara Bio, Inc, Otsu, Shiga, Japan) was used to digest the CCTGG sequence in the amplicon of the wild type. In contrast, *KRAS* mutant alleles were not digested because of the mutations, resulting in the enrichment of mutant alleles. The first-round PCR amplification was done for 25 cycles (1 minute at 94°C, 40 seconds at 54°C, 40 seconds at 72°C) using 1× TaqMan buffer, 1.5 mM $MgCl_2$, 800 μM deoxynucleotide triphosphate, 300 nM each primer, 0.3 U of HotStarTaq DNA polymerase (Applied Biosystems, Inc, Foster City, CA), and 100 ng of genomic DNA. After digestion with *Bst*NI, a second PCR was performed to selectively reamplify the mutant species that were left uncut by *Bst*NI. The second-round PCR was done using Primers A and C, 5′-TAATATGTCGACTAAAACAAGATTTACCTC-3′, for 40 cycles (1 minute at 94°C, 40 seconds at 54°C, 40 seconds at 72°C). Underlined bases of primer C represent mismatches from the Kirsten *ras* DNA sequence (TCG-C to CAT-T). The product of the second PCR was examined by sequencing using the conditions reported above.

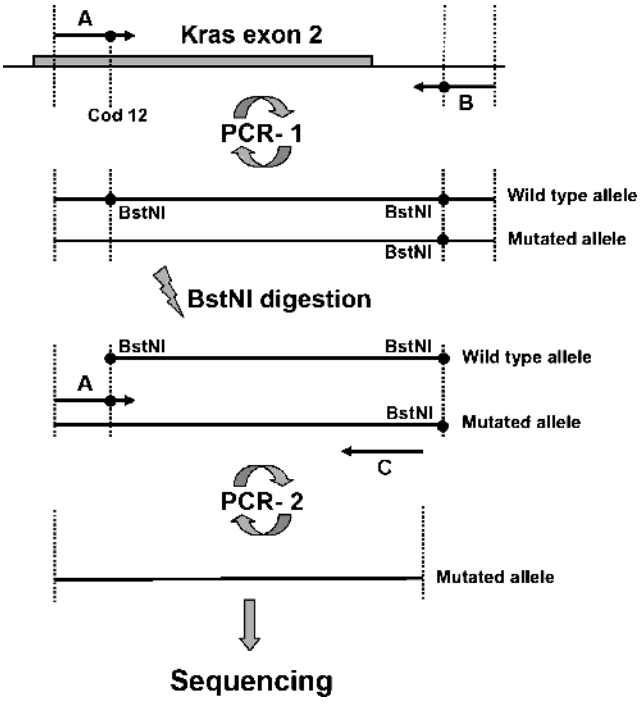

Figure 1. Schematic diagram showing the ME-sequencing method. See the Materials and Methods section for a detailed description. A, B, and C are the primers used for PCR amplification. The symbol (●) on the primers indicates a mismatch from the genomic normal KRAS sequence, which gives rise to a restriction site for the *Bst*NI enzyme.

The method described is a variant of a previously reported protocol [20,21] based on two enzymatic reactions and detection of mutations by agarose gel electrophoresis. In our protocol, we use a single enzymatic reaction, thus avoiding the possibility of false-positive results due to incomplete digestion, and the mutational status is investigated by sequencing that reveals the exact nature of the mutation. The technique described has been engineered to get the highest sensitivity and specificity by an accurate selection of reagents and several modifications to the original protocol. All mutations were confirmed by at least one second sequencing of independent PCR products. By serial dilution experiments, we found that this enriched assay could detect up to 0.1% of mutant DNA in wild-type DNA (data not shown). ME-sequencing could detect mutations at codon 13 with a sensitivity comparable to that of DS.

Statistical Methods

Associations between categorical variables were assessed using the χ^2 test or Fisher exact test as appropriate. Logistic regression analysis was applied to estimate the effect of covariates on disease control rate (DCR; objective response [OR] + PR + stable disease [SD] *vs* progressive disease). Progression-free survival (PFS) and overall survival (OS) were measured for each patient from the first day of treatment with gefitinib or erlotinib. Survival curves were estimated using the Kaplan-Meier method, and differences among them were evaluated by the log-rank test. Multivariate Cox proportional hazards regression models were used to assess the effect of covariates on PFS and OS. $P < .05$ was considered as significant.

Results

Clinical Variables

OR rate in the population analyzed was 13%, with 45% of patients achieving DCR (CR, PR, or SD). Approximately half of the patients went on to receive a subsequent line of chemotherapy treatment on progression, and all patients were followed up until death. Median PFS for the entire population was 5 months (95% confidence interval [CI] = 2.9–7.1 months), and median OS was 14 months (95% CI = 5.33–22.67 months). The percentage of patients alive at 1 and 2 years was 49.4% and 36.2%, respectively. The percentage of progression-free patients at 1 and 2 years was 36% and 19%, respectively (Table 1*B*). Females (42%) had a higher OR than males (23% *vs* 6%, $P = .046$) and a longer median survival (22 *vs* 6 months, $P = .003$). Never smokers (35%) had a nonsignificantly higher OR than current or former smokers (21% *vs* 9%, $P = .1$) and a significantly longer median survival (24 *vs* 6 months, $P = .002$).

Mutational Analysis of KRAS *and* EGFR *in Lung Adenocarcinomas*

KRAS mutations were analyzed by DS of PCR products and ME-sequencing. DS was able to detect *KRAS* mutations in 16 (19%) of

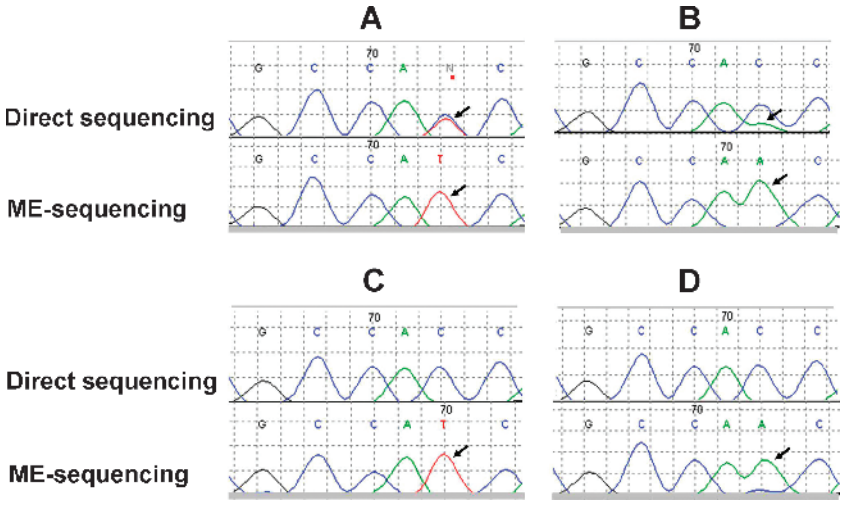

Figure 2. Representative examples of matched DS and ME-sequencing chromatograms for *KRAS* analysis of lung adenocarcinoma samples. (A and B) Tumors in which *KRAS* mutations (arrows) were visible with both DS and ME-sequencing. (C and D) cases in which the mutations were detectable only by ME-sequencing (arrows). The enriched procedure is so effective that only the mutated allele is evident.

83 tumor samples; ME-sequencing correctly identified all of the mutations detected by DS but detected *KRAS* mutations in 14 additional tumor samples for a total of 30 (36%) of 83 (Table 1*A* and Figure 2). All mutations were located at codon 12, with the exception of a single patient in which *KRAS* was mutated at codon 13 (Table 2). The 83 tumors were also investigated for mutations in the tyrosine kinase domain of the *EGFR* gene. Twenty-one *EGFR* mutations were present in 20 (24%) of 83 patients (Table 2). Interestingly, three patients harbored mutations at both the *EGFR* (exon 19 deletion, exon 21 point mutation, and double exon 19 and exon 21 mutations, respectively) and the *KRAS* gene; in such cases, *KRAS* mutations could be detected only by ME-sequencing.

Gene Mutations and Response to Treatment

KRAS mutations detected by either method were significantly associated with resistance to EGFR-TKI treatment ($P = .04$ and $P = .004$ for *KRAS* mutations assessed by DS and ME-sequencing, respectively; Table 3). In particular, all patients found to be positive by either assay were nonresponders, indicating that both methods had 100% specificity. Conversely, the sensitivity was significantly higher with the mutant-enriched assay. The direct method allowed to identify 16 (22%) of 72 nonresponders, whereas the enriched technique revealed the presence of 30 (42%) of 72 nonresponders ($P = .019$). As opposed to *KRAS* mutations, *EGFR* mutations were significantly correlated with a higher likelihood of response ($P = .0001$; Table 3). In the three patients that harbored both *EGFR* and *KRAS* mutations, detected only with the enriched method, the response to TKI treatment was SD in one patient and PD in two patients. Owing to the complete lack of events in responders, a multivariate logistic regression analysis was conducted by using DCR instead of OR as the dependent variable. *KRAS* status assessed by ME-sequencing was the only independent predictor of disease control ($P = .019$), whereas *EGFR* mutations were of borderline significance ($P = .07$; Table 4*A*). When *KRAS* mutations were assessed by DS, *KRAS* status was not retained in the multivariate model, and *EGFR* mutations became the only significant factor influencing DCR (data not shown).

Table 2. Gene Mutations in *KRAS* and *EGFR* in Lung Adenocarcinomas.

Gene and Type of Mutations	No. of Mutations Detected by DS	No. of Mutations Detected by ME-Sequencing
KRAS		
Codon 12		
G34T (G12C)	8	14
G35T (G12V)	3	6
G35A (G12D)	3	6
G34A (G12S)	0	1
G35C (G12A)	1	1
GG34-35TT (G12F)	0	1
Codon 13		
G38T (G13V)	1	1
EGFR		
Exon 19		
2235-2249del (E746-A750del)	5	—
2236-2250del (E746-A750del)	3	—
2237-2254del (E746_T751del)	1	—
2240-2257del (L747_P753del)	1	—
Exon 21		
T2573G (L858R)	10	—
T2582A (L861Q)	1	—

Gene Mutations and Survival

Kaplan-Meier analysis of survival curves demonstrated that patients with *KRAS* mutations, detected by either method, experienced

Table 3. Gene Mutations and Response to Treatment with TKIs.

Gene Mutations	Response to Treatment				
	CR	PR	SD	PD	*P*
KRAS mutations (detected by ME-sequencing)					
Mutated	0 (0%)	0 (0%)	6 (20%)	24 (80%)	.004
Wild-type	3 (6%)	8 (15%)	20 (38%)	22 (41%)	
KRAS mutations (detected by DS)					
Mutated	0 (0%)	0 (0%)	2 (12.5%)	14 (87.5%)	
Wild-type	3 (4.5%)	8 (11.9%)	24 (35.8%)	32 (47.8%)	.04
EGFR mutations					
Mutated	3 (15%)	6 (30%)	6 (30%)	5 (25%)	
Wild-type	0 (0%)	2 (3.2%)	20 (31.7%)	41 (65.1%)	.0001

Table 4. Multivariate Analysis of Predictive Variables for DCR, PFS, and OS.

	HR (95% CI)	*P*
(A) DCR		
KRAS mutations (ME-sequencing) (yes *vs* no)	3.78 (1.24–11.51)	.019
EGFR mutations (no *vs* yes)	2.94 (0.84–10.29)	.07 (NS)
Smoking history (never *vs* former/current)	1.94 (0.060–6.31)	.27 (NS)
Sex (female *vs* male)	1.28 (0.41–3.99)	.67 (NS)
(B) PFS		
KRAS mutations (ME-sequencing) (yes *vs* no)	1.87 (1.08–3.24)	.02
EGFR mutations (no *vs* yes)	2.49 (1.15–5.38)	.02
Smoking history (never *vs* former/current)	1.21 (0.87–1.68)	.26 (NS)
Sex (female *vs* male)	1.37 (0.75–2.48)	.29 (NS)
(C) OS		
KRAS mutations (ME-sequencing) (yes *vs* no)	2.29 (1.23–4.25)	.01
EGFR mutations (no *vs* yes)	2.80 (1.01–7.76)	.04
Smoking history (never *vs* former/current)	1.21 (0.81–1.72)	.4 (NS)
Sex (female *vs* male)	1.79 (0.89–3.64)	.1 (NS)

NS indicates not significant.

a significantly shorter OS (Figures 3*A* and 4*A*). However, differences in OS were more pronounced when *KRAS* status was assessed by ME-sequencing (HR = 2.82, 95% CI = 1.98–8.27, $P = .0001$ *vs* HR = 1.98, 95% CI = 1.04–6.75, $P = .04$, with ME-sequencing and DS, respectively). To address the question of whether *KRAS* mutations detected only by ME-sequencing carried the same clinical consequences as those detected by DS, we divided our study population into three different groups: *KRAS*–wild-type patients, patients with *KRAS* mutations detected by both DS and ME-sequencing, and patients with *KRAS* mutations detected only by ME-sequencing. *KRAS* mutations detected only by ME-sequencing had the same clinical consequences in OS as those detected by means of DS (Figure 3*B*). Indeed, OS curves for *KRAS*-mutated patients completely overlapped, regardless of the detection method, and were significantly different from that of *KRAS*–wild-type patients ($P = .001$). Similar results were obtained for PFS analysis. *KRAS* status, as assessed by either method, significantly

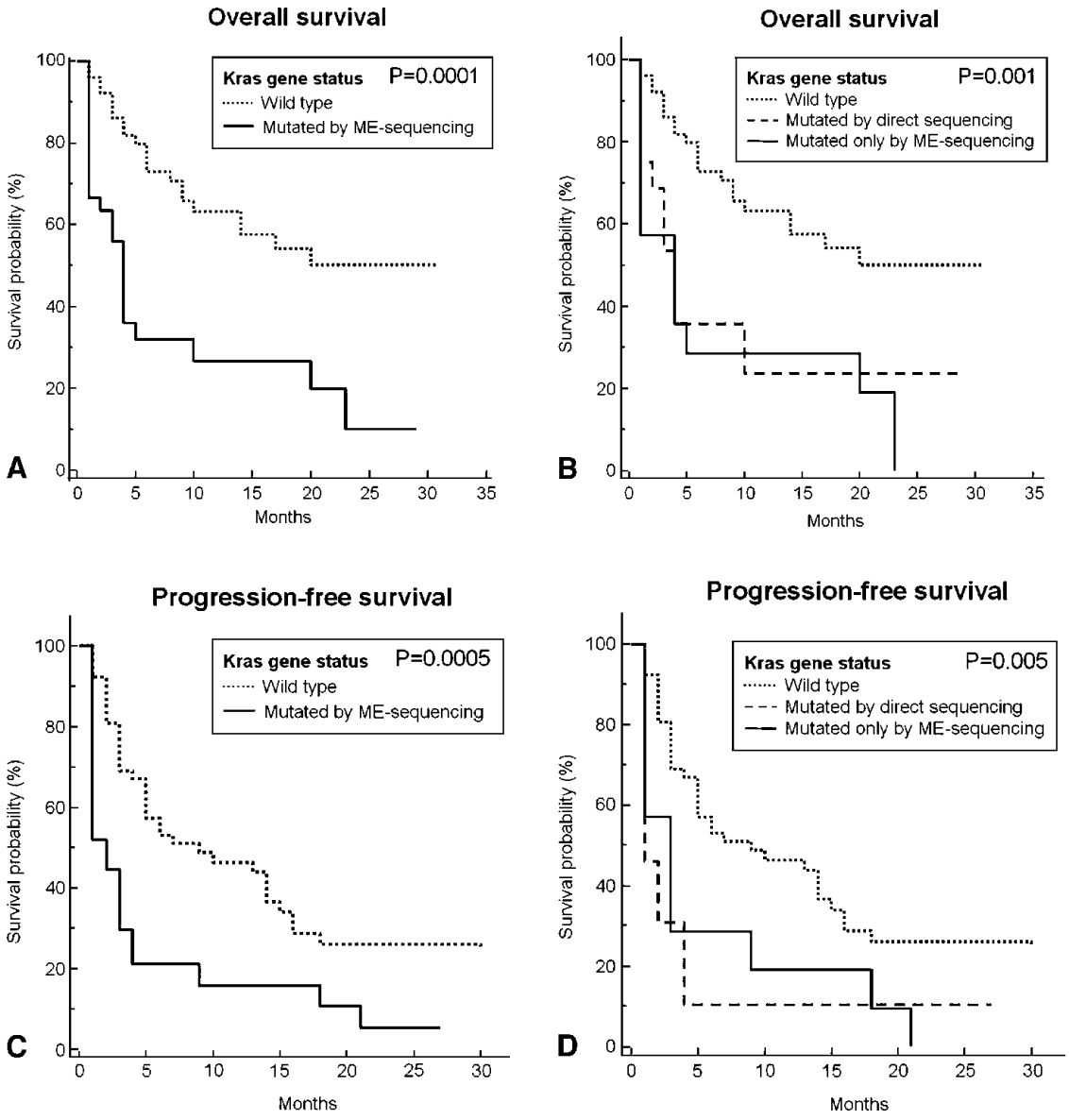

Figure 3. Overall (A and B) and progression-free (C and D) survival curves in lung adenocarcinoma patients according to the mutational status of *KRAS* assessed by ME-sequencing (A and C), by DS or only by ME-sequencing (B and D), see test for details. Curve differences are statistically significant. All *P* values refer to log-rank tests. Months indicate months from the beginning of treatment.

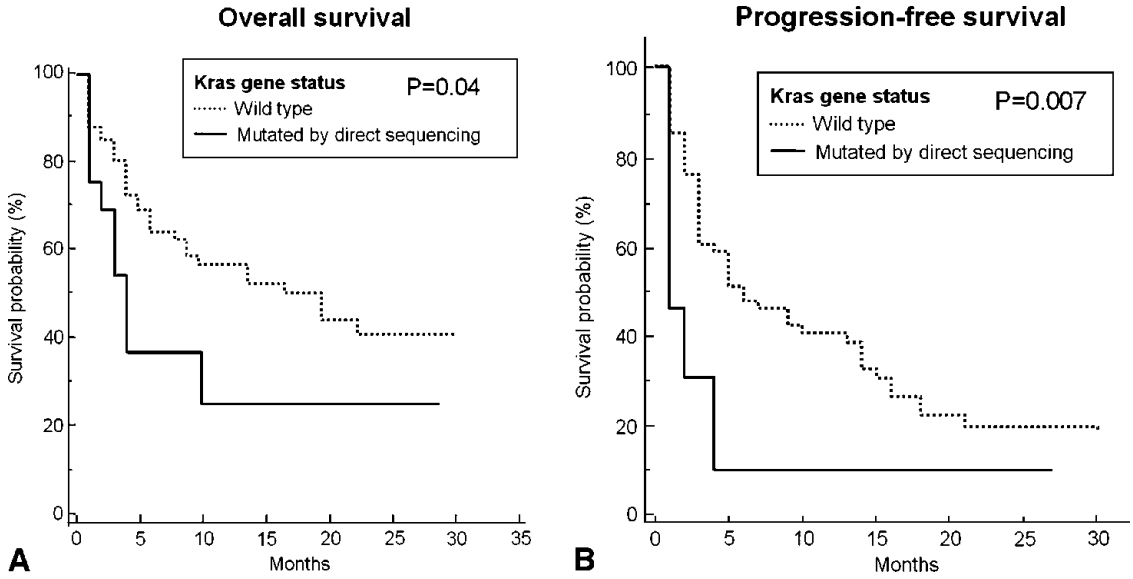

Figure 4. Overall (A) and progression-free (B) survival curves in lung adenocarcinoma patients according to the mutational status of *KRAS* assessed by DS. Curve differences are statistically significant. All *P* values refer to log-rank tests. Months indicate months from the beginning of treatment.

impacted on PFS (Figures 3*C* and 4*B*), but differences were more pronounced when *KRAS* status was assessed by ME-sequencing (HR = 2.52, 95% CI = 1.68–6.36, *P* = .0005 *vs* HR = 2.21, 95% CI = 1.45–10.09, *P* = .007, with ME-sequencing and DS, respectively). As highlighted above for OS, PFS curves for *KRAS*-mutated patients almost completely overlapped, regardless of the detection method, and were significantly different from that of *KRAS*–wild-type patients (*P* = .005; Figure 3*D*).

Kaplan-Meier analysis of survival curves also demonstrated a significantly longer PFS and OS for patients whose tumors harbored *EGFR* mutations (*P* = .0002 and *P* = .0004, respectively; Figure 5). Interestingly, among patients with *EGFR* mutations (*n* = 20), the three patients who also harbored *KRAS* mutations (detectable only by the enriched method) fared significantly worse in OS (*P* = .003; Figure 6).

Multivariate analysis using stepwise Cox regression confirmed that *EGFR* and *KRAS* mutations detected by ME-sequencing were the only two independent predictors of both PFS and OS (Table 4, *B* and *C*). Conversely, when *KRAS* mutations were assessed by DS, *KRAS* status was not retained in the multivariate model, and *EGFR*

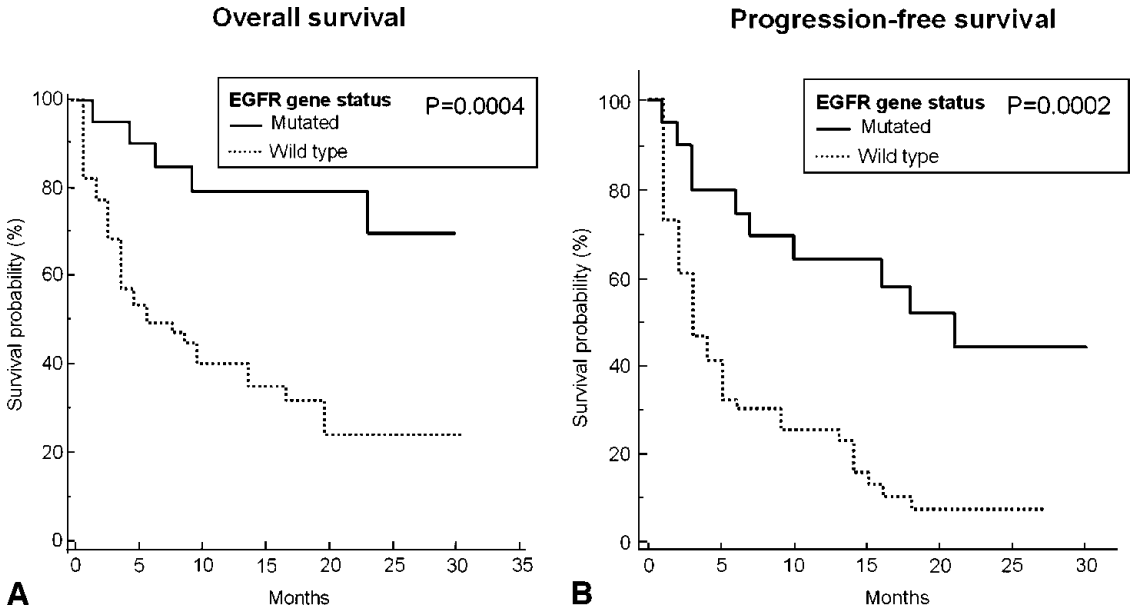

Figure 5. Overall (A) and progression-free (B) survival curves in lung adenocarcinoma patients according to the mutational status of *EGFR*. Curve differences are statistically significant. All *P* values refer to log-rank tests. Months indicate months from the beginning of treatment.

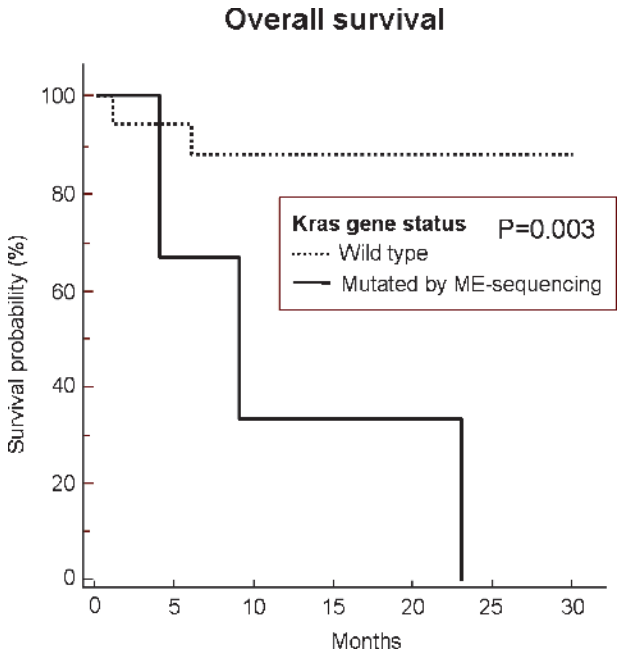

Figure 6. Overall curves in patients with lung adenocarcinoma harboring *EGFR* mutations according to the mutational status of *KRAS* assessed by ME-sequencing. Curve differences are statistically significant. The *P* value refers to log-rank test. Months indicate months from the beginning of treatment.

mutations became the only significant factor influencing survival (data not shown).

Discussion

We investigated the predictive role of *KRAS* mutations, detected with two different methods, in a series of patients affected by lung adenocarcinoma undergoing TKI treatment of advanced disease. We compared DS, a widely diffused method for mutational analysis, with ME-sequencing, an extremely sensitive technique developed to detect low-prevalence mutations. By DS, mutations were seen in 19% of patients. The prevalence of *KRAS* mutations in NSCLC, previously reported in 17 studies investigating the role of *KRAS* as a marker of resistance to TKI, has recently been reviewed [25]. Fifteen of these studies were conducted by DS, for a total of 818 cases examined. The reported mean frequency of *KRAS* mutation was 16%, close to the frequency we observed by DS in our series of cases. The ME-sequencing method used in the present study was endowed with a much higher detection power, allowing to reveal *KRAS* mutations in 36% of lung adenocarcinomas. Thus, in approximately 17% of the tumors examined, *KRAS* mutations were present but undetectable by DS. This can be ascribed to the fact that cells carrying *KRAS* mutations in these samples represented only a portion of the whole cell population. Considering that all samples were microdissected, our results indicate that, in approximately 17% of lung adenocarcinomas, *KRAS* mutations affect subpopulations of tumor cells. Mutant-enriched PCR methods have mainly been applied to detect neoplastic cells in body fluids or excretions for early molecular diagnosis of human cancer [21,26,27]. These very sensitive techniques are generally not used to detect mutations in primary tumor samples. However, in some circumstances, that is, the detection of mutations conferring drug resistance in small subpopulations of tumor cells, they can be extremely useful. A selective proliferation of small subclones with mutations inducing drug resistance has been reported in lung carcinomas treated with gefitinib [9] and in chronic myelogenous leukemias treated with imatinib [28]. These results indicate that targeted therapies may cause the selection of mutant cells, and even a small fraction of positive tumor cells at the beginning of treatment could lead to clinical resistance. Therefore, a very sensitive assay, rather than DS, is essential to investigate the presence of mutations conferring drug resistance. Conversely, when sensitive techniques are applied to the screening of mutations associated with drug sensitivity (i.e., *EGFR* mutations), they carry the risk of classifying as positive, cases in which the mutation is present in small subclones, prompting specific treatment even if most of the tumor cells are indeed insensitive. We believe that our suggestions should be taken into consideration in future trials.

Previous studies, performed by DS, have shown that *KRAS* mutations identify a subgroup of NSCLC patients that have an extremely limited probability of responding to EGFR-targeted treatments. A recent review and meta-analysis of these data reveals that the test is highly specific (pooled estimate = 0.94) indicating that CR or PR to EGFR-TKI are highly unlikely in the presence of a *KRAS* mutation [25]. However, the sensitivity of the assay conducted by conventional methods is quite low (pooled estimate = 0.21), indicating that resistance to EGFR-TKI also occurs in a substantial number of patients with wild-type *KRAS*. In our series of patients, *KRAS* mutations detected by either method were significantly related to lower response rates. No patient whose tumor harbored a *KRAS* mutation showed a CR or PR. This indicates that both methods are highly specific. In particular, we want to point out that no false-positive results were observed by enriched sequencing, despite the higher number of *KRAS* mutations detected. Nevertheless, the sensitivity of the enriched assay was significantly higher than that obtained by conventional sequencing (42% *vs* 22%). Our data indicate that the ME-sequencing could allow to avoid an inefficient treatment in approximately 20% of patients found to be negative for *KRAS* mutations by conventional methods. Although the enriched assay consistently increases the sensitivity of *KRAS* as a predictive marker, resistance still occurs in many patients with wild-type *KRAS*, suggesting that other mechanisms including alterations that activate the mitogen-activated protein kinase pathway, such as *BRAF* and *PIK3CA* mutations, might hinder the effectiveness of anti-EGFR therapy. This has recently been shown in metastatic colorectal cancer patients treated with anti-EGFR monoclonal antibodies (cetuximab and panitumumab) [29,30]. Because it has been reported that DCR may be a more powerful predictor of survival than tumor shrinkage, we decided to perform a multivariate analysis using DCR as the dependent variable. Only the *KRAS* status assessed by ME-sequencing was found to be an independent predictor of disease control.

Kaplan-Meier survival analysis showed that *KRAS* mutations detected by either method significantly impacted on PFS and OS. However, the predictive power of the enriched procedure was by far superior in both HR and *P* value. When the study population was divided according to the *KRAS* status investigated with the two techniques, it clearly emerged that *KRAS* mutations detected only by ME-sequencing had similar consequences on both OS and PFS as those detected by DS. This suggests that subpopulations of tumor cells carrying *KRAS* mutations may be drug-selected and rapidly expand, making the tumor resistant to TKI. A multivariate analysis showed that only *KRAS* mutations detected by ME-sequencing and *EGFR* mutations were independent predictors for TKI treatment in both PFS and OS. The impact of *KRAS* mutations with survival in TKI-treated lung cancer patients has

been investigated in previous studies conducted by DS. At univariate analysis, *KRAS* mutations were found to be associated with significantly decreased PFS and/or OS [14–18]. At multivariate analysis, *KRAS* mutations were found to be associated with PFS in the study of Massarelli et al. [16], whereas Miller et al. did not report any significant correlation [17]. Our results are in keeping with most of these data and suggest that the use of a very sensitive method can improve the prognostic/predictive power of the *KRAS* marker.

We previously reported for the first time that *KRAS* and *EGFR* mutations are mutually exclusive events in lung adenocarcinomas [24,31]. In these studies, *KRAS* mutations were investigated by allele-specific oligoprobe hybridization, which, in our hands, has sensitivity similar to that of DS. In the present study, when *KRAS* status was assessed by ME-sequencing, we found three cases with concomitant *KRAS* and *EGFR* mutations. Because *KRAS* mutations in these cases were not detectable by DS, we speculate that they were present in subpopulations of these tumors. This observation may be clinically relevant, in that patients carrying both mutations were resistant to TKI treatment and showed an OS significantly shorter than that of patients with only *EGFR* mutations. Our results suggest that the presence of *KRAS* mutations in minor clones can overcome the effect of *EGFR* mutations, inducing tumor resistance.

Because mutations in the *KRAS* gene were found to be a marker of response to anti-EGFR antibodies (cetuximab, panitumumab) in colorectal cancer patients, we recently decided to evaluate the prevalence of *KRAS* mutations detected by DS and ME-sequencing on a series of 90 colorectal carcinomas [22]. The frequency of *KRAS* mutations was 39% by using the direct method and 54% by means of the enriched assay. These data indicate that also in approximately 15% of colorectal adenocarcinomas, *KRAS* mutations in minor clones are present and may hamper the effect of anti-EGFR treatments.

In conclusion, our data suggest that in future prospective studies, the use of more sensitive methods, such as ME-sequencing, could allow to more effectively identify patients resistant to anti-EGFR therapy.

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