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Detection of *EGFR* mutations in plasma DNA from lung cancer patients by mass spectrometry genotyping is predictive of tumor *EGFR* status and response to *EGFR* inhibitors

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Abstract

Aims—*EGFR* mutations now guide the clinical use of *EGFR*-targeted therapy in lung cancer. However, standard *EGFR* mutation analysis requires a minimum amount of tumor tissue, which may not be available in certain situations. In this study, we combined a mass spectrometry genotyping assay (Sequenom) with a mutant-enriched PCR (ME-PCR) to detect *EGFR* mutations in free plasma DNA from patients with lung cancer.

Method—DNAs were extracted from 31 plasma samples from 31 patients and analyzed by both methods for *EGFR* exon 19 deletion and *EGFR* L858R mutation. Results in plasma DNA samples were compared with *EGFR* mutation status obtained in tumor DNA (18/31 *EGFR* mutant). The relationship of *EGFR* mutation status in tumor and/or plasma samples to overall survival was assessed.

Results—The *EGFR* mutation status in plasma DNA was identical to the primary tumor in 61% of patients (19/31). By mass spectrometry genotyping, the plasma samples contained mutant DNA corresponding to 5/14 *EGFR* exon 19 deletions and 3/4 *EGFR* L858R mutations previously diagnosed in the matched tumors. Two samples were positive in plasma DNA but negative in primary tumor tissue. Results were similar for ME-PCR. For patients treated with erlotinib, overall survival was correlated with the presence of *EGFR* mutation in plasma and/or tumor tissue ($p=0.002$), with the two patients positive only in plasma DNA showing responses and favorable outcomes.

Conclusion—The detection of *EGFR* mutations in plasma DNA samples by mass spectrometry genotyping and ME-PCR is feasible. A positive *EGFR* result in plasma DNA has a high predictive value for tumor *EGFR* status and for favorable clinical course on *EGFR*-targeted therapy and could therefore be useful in guiding clinical decisions in patients with insufficient or unavailable tumor specimens.

Keywords

Lung cancer; *EGFR*; Plasma; Mass spectrometry; Mutant-enriched PCR; Adenocarcinoma

Introduction

The detection of *EGFR* mutations in lung adenocarcinomas has become a routine molecular test with important implications for patient prognosis and selection of therapy. The presence of an activating mutation predicts response to the *EGFR* tyrosine kinase inhibitors (TKI) erlotinib or gefitinib, and is prognostically favorable regardless of therapy (1). Unfortunately, in some cases, tumor tissue is either inadequate for molecular testing because of its small quantity or very low tumor content or is not readily available. Therefore, there is a need to develop new techniques for detecting clinically significant *EGFR* mutations in patients with little or no available tumor DNA.

Plasma samples from patients with lung cancer contain much higher levels of DNA than plasma from cancer-free patients. Most of this excess circulating DNA is believed to be released from the dying lung cancer cells at primary or metastatic sites (2). As such, plasma DNA may therefore provide a noninvasive source of genotypic information which could be used as a substitute for tumor tissue for detecting cancer-specific molecular markers that could be used to predict response and prognosis. Several groups have detected *EGFR* mutations in DNA isolated from plasma (3–7) or serum samples (8, 9) and show some correlation between mutation status in plasma and tumor tissue (3, 4, 6, 8, 9, 10). Furthermore, *EGFR* mutation detected in plasma or serum may, by itself, be predictive of response to *EGFR* TKI (3, 5, 6, 7, 9).

In this study, we report the detection of *EGFR* L858R mutations and *EGFR* exon 19 deletions in plasma samples from patients with NSCLC using a novel, mass spectrometry assay. The detection of these mutations in plasma samples is correlated with better survival when patients are treated with TKIs.

Material and Methods

Patients characteristics

We studied 31 patients with a biopsy-proven diagnosis of stage III or IV NSCLC and available plasma and tumor tissue. All patients gave informed consent, and the collection and analysis of their health information was approved by the Memorial Sloan-Kettering Cancer Center (MSKCC) Institutional Review Board. The patients were followed for tumor responses and survival outcomes.

Analysis of *EGFR* mutations in tumors tissues

***EGFR* Exon 19 deletion assay**—Detection of the small in-frame deletions in exon 19 of *EGFR* was performed by fragment analysis of fluorescently labeled PCR products as previously described (11). Briefly, a 207-bp genomic DNA fragment encompassing the entire exon 19 was amplified using the primers A1 and A2 (Table 1). PCR products were subjected to capillary electrophoresis on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). This assay can detect an *EGFR* exon 19 deletion in as little as 5–10% of tumor cells in a given sample (11).

***EGFR* Exon 21 L858R assay**—This mutation was detected by a PCR-restriction fragment length polymorphism assay (PCRRFLP), based on a *Sau*96I restriction site created by the mutation 2573T>G as previously described (11). Briefly, a 222-bp genomic fragment encompassing the entire exon 21 was amplified using primers B1 and B2 (Table 1). Digestion of the mutant PCR product with *Sau*96I enzyme (New England BioLabs) generates a shorter 87 bp fragment. The digested, fluorescently labeled PCR products were analyzed by capillary electrophoresis on an ABI 3730 Genetic analyzer (Applied

Biosystems, Foster City, CA). The detection sensitivity of this assay for the *EGFR* exon 21 L858R mutation is also 5–10% of tumor cells (11).

Blood sample collection and processing

The majority (n=23) of blood samples were collected prior to initiation of therapy, with the rest (n=8) drawn after initiation of therapy (TKI or cytotoxic chemotherapy). Blood samples were collected in tubes containing EDTA, and centrifuged 20 min to separate the plasma. Aliquots of plasma were stored at –80°C until DNA extraction. DNA extraction was performed from 800µL of plasma using the Qiagen DNA Virus extraction kit (without RNase step, which induces some background in mass spectrometry assays).

Whole genome amplification

For whole genome amplification, plasma DNA was processed by a blunt-end ligation method as described (12, 13). Whole genome amplification was carried out using GenomiPhi V2 DNA amplification kit (GE Healthcare).

Analysis of *EGFR* mutations by mass spectrometry

Plasma DNA was analyzed by mass spectrometry-based genotyping (Sequenom Inc, San Diego, CA), as described previously (14). Briefly, tumor DNA was subject to a first PCR amplification [total volume: 5µL, 1.25x buffer, 1.625mM MgCl₂, 500µM dNTP, 100nM from each primer, 0.5 U HotStar Taq DNA Polymerase (Qiagen)] using primers C1 and C2 (*EGFR* Exon 19 deletion assay), D1 and D2 (*EGFR* L858R mutation assay) (Table 1). To increase the sensitivity of the *EGFR* L858R PCR, we introduced a locked nucleic acid (LNA) oligonucleotide in the PCR mix at the concentration of 0.6 µM (D4, Table 1). Alkaline Phosphatase treatment was performed in a total volume of 2µL (final volume: 7µL), incubated at 37°C for 40min and 85°C for 5 min. A single allele base extension reaction was performed using the indicated primers (Table S1 for *EGFR* Exon 19 deletions; D3 (Table 1) for *EGFR* L858R). Two microliters of a solution containing 0.74 µL ddH₂O, 0.2 µL Buffer (Sequenom Inc.), 0.1µL Termination Mix (Sequenom Inc.), 0.94 µL of 7–13.67 µM extension primer (depending of the mass) and 0.02 µL of Thermo Sequenase (Sequenom Inc.) were added to the 7 µL SAP (Shrimp Alkaline Phosphatase) treated PCR products. Thermal cycling was performed following the recommendations of Sequenom. A different well was used for each single allele base extension primer. The detection sensitivity of unmodified Sequenom assays for mutant alleles is estimated to be 5% tumor cells (range 1–10%) (15). Using the LNA at 0.6 µM, the sensitivity of the Sequenom *EGFR* L858R assay increases to 0.1 % (Supplementary figure S1).

Analysis of *EGFR* mutations by mutant-enriched PCR

EGFR Exon 19 deletion assay—Cases showing discordant results between tumor mutation status and plasma mutation status were further studied using a more sensitive mutant-enriched PCR assay (16). Briefly, a 138-bp genomic fragment of exon 19 was amplified with the primers E1 and E2 (Table 1) for 17 cycles at 60°C. After purification (QIAquick Purification kit, Qiagen), PCR products were subjected to restriction enzyme digestion by the MseI enzyme (New England BioLabs) for 4 hours at 37°C. On an *EGFR* allele with an exon 19 deletion, the restriction site is absent yielding an undigested PCR product. During a second round of amplification (primers E1 and E3 (Table 1), 40 cycles at 60 °C) only the mutant and the incompletely digested wild type fragments are amplified. The PCR products were analyzed by fragment analysis on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). This assay has been reported to detect an *EGFR* exon 19 deletion at a level of 0.05% (16).

EGFR Exon 21 L858R assay—A similar method, described by Asano, et al, was used for the *EGFR* Exon 21 assay (16). In this assay, the first PCR amplification results in a 130-bp genomic fragment (primers F1 and F2 (Table 1), 17 cycles at 60 °C). After purification (QIAquick Purification kit, Qiagen), the *MscI* restriction enzyme (New England BioLabs) was used to cut at the TGGCCA sequence of the wild-type allele (37 °C, 4 hours). A second amplification was done using the primers F3 and F4 (Table 1) (40 cycles at 60 °C). After purification, the PCR products were digested with the *Sau961* (GGNCC) (New England BioLabs) (2 hours at 37 °C) and analysed by fragment analysis on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). A positive result corresponds to the presence of a 99-bp fragment. This assay has been reported to detect an *EGFR* L858R mutation at a level of 0.05% (16).

Statistical analyses—The relationship between *EGFR* mutation and factors such as sex, age, stage, histologic type and smoking was examined by χ^2 test or Fisher's exact test using GraphPad software (La Jolla, CA). Time from the date of lung cancer diagnosis to death was used to compare overall survival (OS) between the different groups. Survival curves were estimated by the Kaplan-Meier method with log-rank test using MedCalc Software (Mariakerke, Belgium).

Results

Patients and tumor characteristics

Patient characteristics are summarized in Table 2. There were 16 women and 15 men, 30 with lung adenocarcinoma and one patient with poorly differentiated squamous cell carcinoma. Seventeen patients were former or current-smokers and 14 were never smokers. At time of blood collection, all patients were stage IV except one with stage III disease. By the mutation-specific PCR assays used in routine practice at MSKCC to establish *EGFR* mutation status in tumor tissue (10), 14 tumors had *EGFR* Exon 19 deletion (15-bp deletion in 12 cases, 18-bp deletion in one case and 9-bp deletion in one case), and 4 tumors had the *EGFR* L858R mutation. The remaining 13 patients did not have any mutation in the tumor at the time of diagnosis (Table 2).

Among the 31 patients, 25 patients received the *EGFR* tyrosine kinase inhibitor (TKI), erlotinib. Among the 18 patients with *EGFR* mutant tumors, 6 patients had received erlotinib between 10 days and 22 months before the first blood collection while the remaining 12 patients were erlotinib-naïve. Finally, 24 patients received chemotherapy in first line or second line therapy.

EGFR mutations in plasma DNA

Plasma samples were collected before treatment or between 10 days and 22 months after treatment initiation. Plasma samples were first analyzed by Sequenom mass spectrometry assays. The Sequenom *EGFR* Exon 19 assay was positive in 5/14 plasma samples from patients who had *EGFR* Exon 19 deletion detected in their tumor (36%). All 5 positive patients showed concordance for the specific subtype (size) of exon 19 deletion detected in the tumor and plasma DNA (Figure 1). The *EGFR* Exon 19 deletion assay was also positive in 2 patients (WT-1, WT-2) who did not have an *EGFR* mutation detected in their tumor tissue (Table 3).

The Sequenom *EGFR* L858R assay showed the L858R mutation in 3/4 patients with L858R detected in the tumor (75%, Table 3). The *EGFR* L858R assay was also positive for one patient for which an *EGFR* Exon 19 deletion, but not a L858R mutation, had been detected in the tumor tissue (patient Ex19-1, Table 3) (Figure 2).

In parallel, all plasma samples were analyzed by ME-PCR for *EGFR* Exon 19 and Exon 21 mutations. All *EGFR* Exon 19 deletion positive cases observed in plasma DNA by Sequenom assays were confirmed by ME-PCR (Table 3). The *EGFR* Exon 19 ME-PCR assay did not detect additional plasma DNA-positive cases among patients with *EGFR* Exon 19 deletion in their tumor. The ME-PCR technique confirmed the positivity of the two positive plasma samples among patients with tumors thought to be *EGFR* wild type (WT-1, WT-2) (Table 3). Concerning the *EGFR* L858R mutation, ME-PCR confirmed the presence of a L858R mutation in the plasma of 3/5 patients (two patients with L858R mutation diagnosed in the tumor and one patient with *EGFR* L858R mutation diagnosed only in plasma samples) (Table 3).

To verify the discordant *EGFR* mutations between plasma DNA and primary tumor, we reanalyzed the tumor DNA of one of the 3 cases with discordant positive results between the original analysis of tumor DNA and the Sequenom/ME-PCR assays on plasma DNA (patient WT-1, Table 3). Both Sequenom genotyping and ME-PCR confirmed the absence of *EGFR* Exon 19 deletion in the tumor of this patient. Tumor DNA was not available for the other 2 patients with discordant positive plasma DNA results (WT-2, Ex19-1) (Table 3). Finally, *EGFR* status was highly concordant between mutations detected in plasma DNA and tumor DNA, matching in 61% with only 2 patients with *EGFR* mutation detected only in plasma DNA (positive predictive value = 80%).

Correlation between *EGFR* mutations in tumor and/or plasma and clinicopathological characteristics

We examined correlations between overall survival (OS), age, sex, and smoking history. Patients with *EGFR* mutation in the tumor who received erlotinib had a better OS than patients who received erlotinib in the absence of *EGFR* mutation ($p=0.01$) (Figure 3A). The correlation was even stronger when patients with *EGFR* mutation in plasma and/or tumor DNA were compared with patients with *EGFR* mutations in neither ($p=0.002$) (Figure 3B). Among the 20 patients treated with erlotinib and presenting *EGFR* mutations in tumor and/or plasma DNA, the detection of an *EGFR* mutation in plasma DNA by itself was not a predictor of survival ($p=0.4$, $n=10$ in each group). There was no significant correlation between *EGFR* mutations in plasma DNA and age ($p=0.3$), sex ($p=1$), smoking history ($p=0.6$), or treatment before the blood collection ($p=0.3$) but the numbers for most of these comparisons were small.

Discussion

The detection of *EGFR* mutations in tumor tissue has important prognostic and predictive value, and can be used to select therapy for the treatment of lung adenocarcinoma. Many patients with stage IV adenocarcinoma are diagnosed with small biopsies, or by fine needle aspiration of tumors which often yields insufficient DNA for molecular testing. Detection of *EGFR* mutations in plasma samples would be of value for patients in whom sufficient tumor tissue is not available.

In the present study, we found that Sequenom mass spectrometry genotyping assays on plasma DNA samples show concordance with tumor genotyping in 61% of the cases (19/31). In plasma DNA, we detected 36% (5/14) of *EGFR* Exon 19 deletion cases, and 75% (3/4) of *EGFR* L858R mutation cases, compared with the matched primary tumors. Table 4 provides details on the different methods used in previous reports to detect *EGFR* and *KRAS* mutations in plasma samples (3–10, 17, 18). Depending of the technique, the concordance between *EGFR* status in tumor and plasma/serum samples varies from 66 to 100% with the highest correlation index being reported for the denaturing high-performance chromatography (3) and the mutant-enriched PCR (6). It is important to note that cases in

which plasma DNA is negative for the mutation found in the tumor cannot be considered as conventional “false-negatives” as the possibility that there is little or tumor DNA being shed in the blood in a given case cannot be excluded.

In the study by Bai et al. (3), 89.6% of the patients received prior therapy before blood collection. Timing of chemotherapy was not reported by He et al (6). In the present study, *EGFR* mutations were more often detectable in plasma samples from patients who received treatment before the blood collection, 67% (4/6) versus 33% (4/12), although the difference was not statistically significant. That could be explained by an increasing amount of circulating DNA due to tumor cell death in response to treatment. Similar results were observed by Kimura et al (8) who observed the *EGFR* E746-A750del more frequently in post-treatment samples. In contrast, using a microfluidic digital PCR method, Yung et al described a decreased concentration of the mutant sequences in the plasma after treatment compared with pre-treatment plasma samples (n=5) (4).

In the present study, two mutations detected in plasma samples were not detected in the corresponding tumor samples. One of these 2 cases was a poorly differentiated squamous cell carcinoma. This phenomenon has also been observed in previous studies (3, 5, 6, 8, 9) and could be explained by tumor heterogeneity, or by the presence of a second primary tumor harboring the mutation detected in the plasma DNA. The clinical observation that these 2 patients showed a good survival with *EGFR* TKIs supports the notion that these plasma DNA mutations were representative of underlying disease rather than being technical false positives.

More surprising was the detection of both *EGFR* Exon 21 and *EGFR* Exon 19 mutations in the same plasma DNA sample, while only *EGFR* Exon 19 deletion was observed in the primary matched tumor. We propose that it is more probable that this patient harbored a second primary tumor rather than both mutations being present in the same clonal tumor. Separate primary tumors with discordant *EGFR* or *KRAS* mutations are well described (19).

Mutant-enriched PCR (ME-PCR) has been previously used to detect *EGFR* L858R mutation and *EGFR* Exon 19 deletions in plasma samples (6). In the present study, ME-PCR did not detect more *EGFR* mutations in plasma DNA samples than the mass spectrometry genotyping assays. The Sequenom mass spectrometry platform has the advantage of being able to detect multiple mutations in the same assay, meaning that less sample and less time is needed to screen for different mutations in plasma DNA. For instance, testing for *EGFR* mutations could be run in parallel with testing for *KRAS* mutations. Indeed, detection of *KRAS* mutations in plasma DNA has already been reported (17, 18) using other methods and the detection of *KRAS* mutations in tumors by Sequenom is also feasible (15).

As observed in previous studies (3, 5, 6, 7, 9), erlotinib was more effective in improving survival in patients with *EGFR* mutation in the tumor and/or plasma DNA, with the correlation with outcome being enhanced by using either source of DNA to determine the *EGFR* status.

In conclusion, we show that mass spectrometry genotyping assays on the Sequenom platform can detect *EGFR* L858R mutations and *EGFR* Exon 19 deletions in plasma DNA samples from patients with lung cancer. The *EGFR* mutation status is identical to the primary matched tumor in 61% of the samples but, given the high positive predictive value of the technique (8/10) and the favorable outcome of patients with mutations detected only in plasma DNA, it is likely that most or all patients with a positive result in plasma DNA are correctly assigned to the *EGFR* mutant group. Thus, patients with TKI-sensitive *EGFR* mutations detected in plasma DNA could begin *EGFR* TKI treatment without the need for re-biopsy or delays caused by difficult or unsuccessful retrieval of archival tumor tissue. The

recent development and validation of two mutation-specific monoclonal antibodies for the detection of *EGFR* mutations by immunohistochemistry, generated against the L858R mutant and the exon 19 mutant with the common 15bp/5AA deletion, suggests clinical scenarios in which these could be combined with plasma DNA testing to enhance clinical confidence, for instance in cases where cytologic diagnosis is based on small clusters of cells insufficient for DNA extraction but sufficient for immunohistochemistry (20, 21). Finally, it is important to note that, although we and others have demonstrated that the molecular diagnosis of *EGFR* status based on plasma DNA samples is feasible, analysis for *EGFR* mutations in tumor DNA remains the gold standard.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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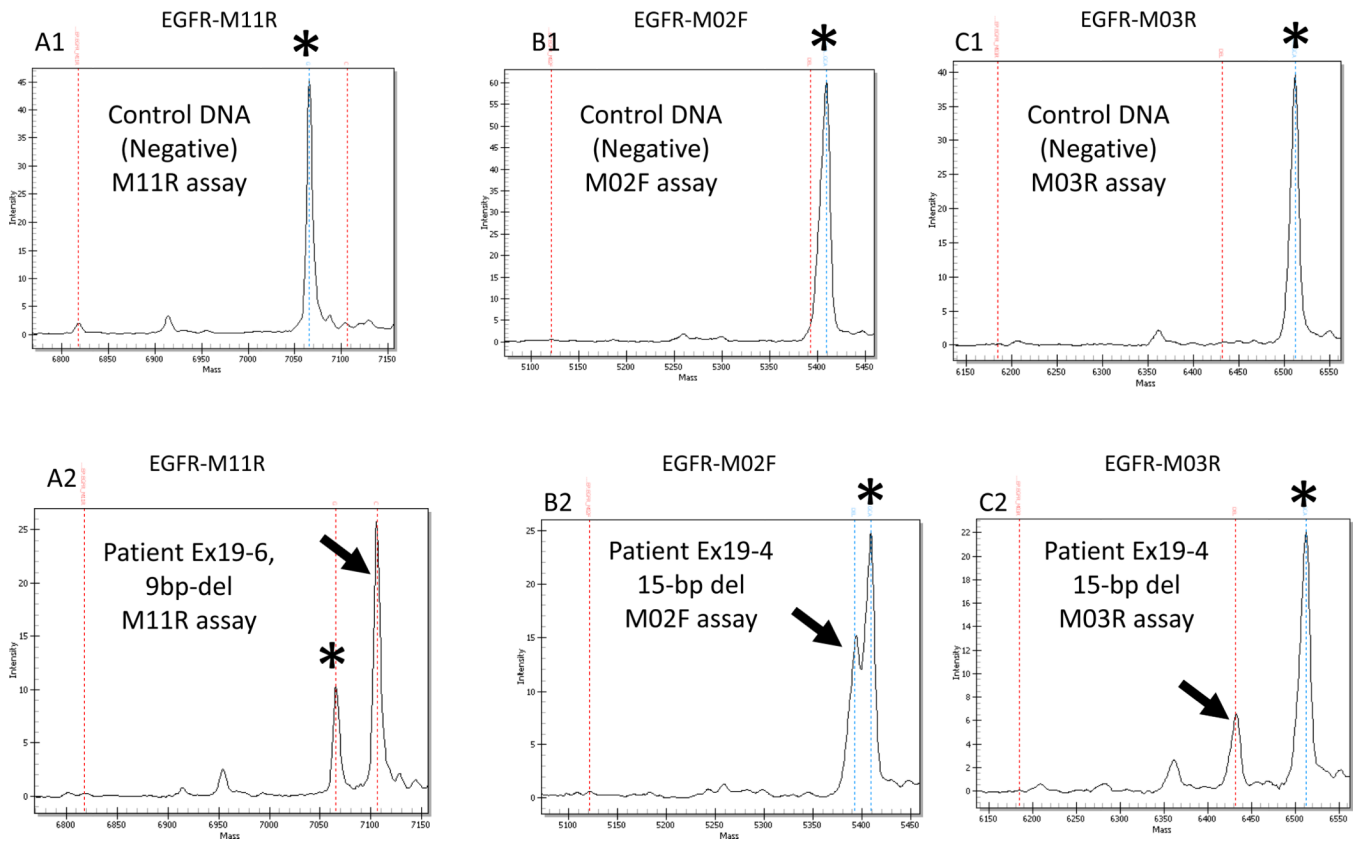


Figure 1.

Sequenom assay results for *EGFR* exon 19 deletion in two plasma samples (patients Ex19-6 and Ex19-4) with the *EGFR*-M11R (**A1** and **A2**), *EGFR*-M02F (**B1** and **B2**) and *EGFR*-M03R (**C1** and **C2**) assays. The control DNA provides a negative control for each assay and only the germline call is visible, indicated by an asterisk (**A1**, **B1**, **C1**). The mutant call is indicated by an arrow. The mass of the product is specific for each assay and is listed in table S1 (Supplementary file). Sample Ex19-6 shows a mutant call for the *EGFR*-M11R assay (**A2**) indicating a 9-bp deletion. Sample Ex19-4 shows a mutant call for the *EGFR*-M02F (**B2**) and the *EGFR*-M03R assays (**B3**) indicating a 15-bp deletion size.

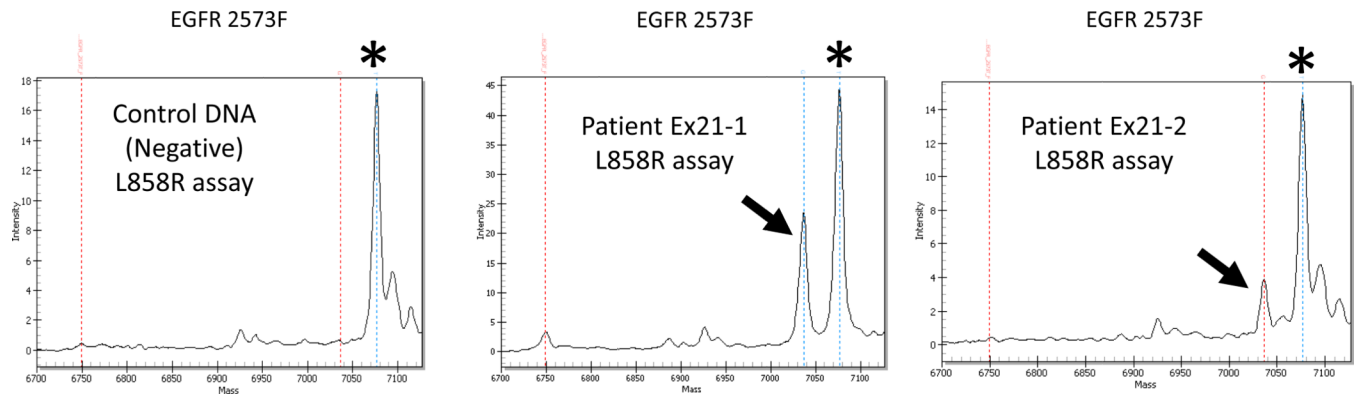


Figure 2.

Sequenom assay results for *EGFR* L858R mutations in two plasma samples (patients Ex21-1 and Ex21-2) with the *EGFR*-2573 assay. The control DNA provides a negative control and only the germline call is visible, indicated by an asterisk (a T call at 5402.6 Da). The mutant calls are visible at 5378.5 Da (G call) and are indicated by an arrow.

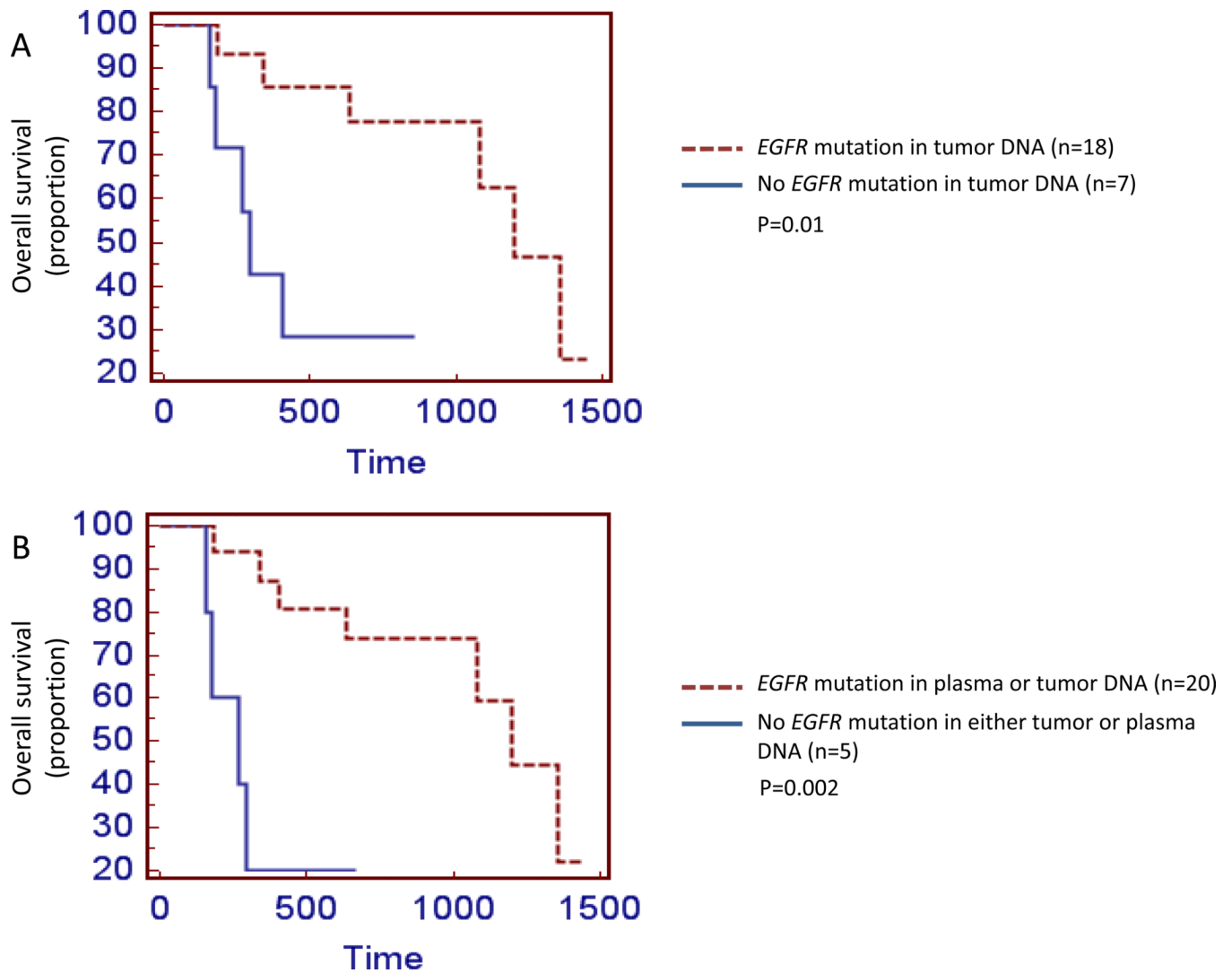


Figure 3. Overall survival curves for the 25 patients treated with erlotinib. Survival curves were evaluated by the Kaplan-Meier method and log-rank test. **3A** “Patients with *EGFR* mutation in tumor DNA (n=18)” versus “Patients without *EGFR* mutation in tumor DNA (n=7)”, p=0.01. **3B** “Patients with *EGFR* mutation in plasma or tumor DNA (n=20)” versus “Patients without *EGFR* mutation in either tumor or plasma DNA (n=5)”, p=0.002.

Table 1

Primers listed by assay

Fragment analysis	
A1	<i>EGFR</i> -Ex19-FW: 5'GCACCATCTCACAAATTGCCAGTTA 3'
A2	<i>EGFR</i> -Ex19-REV: 5' Fam-AAAAGGTGGGCCTGAGGTTCA 3'
B1	<i>EGFR</i> -Ex21-FW: 5'CCTCACAGCAGGGTCTTCTCTGT 3'
B2	<i>EGFR</i> -Ex21-REV: 5' Fam-TCAGGAAAATGCTGGCTGACCTA 3'
Sequenom Mass Spectrometry assay	
C1	<i>EGFR</i> -Ex19-FW 5' ACGTTGGATGGATCCCAGAAGGTGAGAAAAG 3'
C2	<i>EGFR</i> -Ex19-REV 5' ACGTTGGATGAGCAGAACTCACATCGAGG 3'
D1	<i>EGFR</i> -2573-FW 5' ACGTTGGATGACGTACTGGTAAAAACCCG 3'
D2	<i>EGFR</i> -2573-REV 5' ACGTTGGATGTCTTCTCTCCGCACCCAG 3'
D3	<i>EGFR</i> -2573 extension primer-REV: 5' CACCCAGCAGTTTGGCC 3'
	Mass of extension primer: 5131.3 Da
	Mass of extension product Call T: 5402.6 Da
	Call G: 5378.5 Da
D4	LNA- <i>EGFR</i> -Ex21-FW: 5'-GGCTGGCCAaactgc/3InvdT/-3'
Mutant-enriched PCR	
E1	<i>EGFR</i> -Ex19-FW: 5'ATCCCAGAAGGTGAGAAAAGATAAAAATTC 3'
E2	<i>EGFR</i> -Ex19-REV1: 5'CCTGAGGTTGAGAGCCATGGA 3'
E3	<i>EGFR</i> -Ex19-REV2: 5' Fam-CCTGAGGTTGAGAGCCATGGA 3'
F1	<i>EGFR</i> -Ex21-FW1: 5'CAGCCAGGAACGTACTGGTGA 3'
F2	<i>EGFR</i> -Ex21-REV1: 5'TCCCTGGTGTCAGGAAAATGCT 3'
F3	<i>EGFR</i> -Ex21-FW2: 5'CGCAGCATGTCAAGATCACAGAT 3'
F4	<i>EGFR</i> -Ex21-REV2: 5' Fam-TCCCTGGTGTCAGGAAAATGCT 3'

Table 2

Patient characteristics.

Variable	N	%	EGFR mutation	EGFR WT	
Age, years	31		18	13	41.9%
Mean	62.2		65.22	57.85	
Range	38–86		41–86	38–73	
≥65 years	16	52%	12	4	31% p=0.07
<65 years	15	48%	6	9	69%
Sex					
Male	15	48%	9	6	46% p=1
Female	16	52%	9	7	54%
Smoking history					
Smoker	17	55%	8	9	69% p=0.2
Never smoker	14	45%	10	4	31%
Histologic subtype					
ADC	30	97%	18	12	92% p=0.4
SCC	1	3%	0	1	8%
Disease stage at blood collection					
III	1	3%	0	1	8% p=0.4
IV	30	97%	18	12	92%
Therapy					
Chemotherapy	24	77%	11	13	100% p=0.08
Erlotinib	25	81%	18	7	54%
Prior treatment					
Yes	8	26%	6	2	15% p=0.4
No	23	74%	12	11	85%

Table 3

Summary of mutations detected in Tumor and Plasma DNA samples.

Patient	Tumor-RFLP	Plasma-Sequenom	Plasma-ME-PCR	Tumor-Sequenom	Tumor-ME-PCR
Ex21-1	L858R	L858R	L858R	NP	NP
Ex21-2	L858R	L858R	-	NP	NP
Ex21-3	L858R	-	-	NP	NP
Ex21-4	L858R	L858R	L858R	NP	NP
Ex19-1	Ex19 del-18bp	Ex19 del-18bp/L858R	Ex19 del-18bp/L858R	unavailable	unavailable
Ex19-2	Ex19 del-15bp	-	-	NP	NP
Ex19-3	Ex19 del-15bp	Ex19 del-15bp	Ex19 del-15bp	NP	NP
Ex19-4	Ex19 del-15bp	Ex19 del-15bp	Ex19 del-15bp	NP	NP
Ex19-5	Ex19 del-15bp	Ex19 del-15bp	Ex19 del-15bp	NP	NP
Ex19-6	Ex19 del-9bp	Ex19 del-9bp	Ex19 del-9bp	NP	NP
Ex19-7	Ex19 del-15bp	-	-	NP	NP
Ex19-8	Ex19 del-15bp	-	-	NP	NP
Ex19-9	Ex19 del-15bp	-	-	NP	NP
Ex19-10	Ex19 del-15bp	-	-	NP	NP
Ex19-11	Ex19 del-15bp	-	-	NP	NP
Ex19-12	Ex19 del-15bp	-	-	NP	NP
Ex19-13	Ex19 del-15bp	-	-	NP	NP
Ex19-14	Ex19 del-15bp	-	-	NP	NP
WT-1	-	Ex19 del-15bp	Ex19 del-15bp	-	-
WT-2	-	Ex19 del-15bp	Ex19 del-15bp	unavailable	unavailable
WT-3	-	-	-	NP	NP
WT-4	-	-	-	NP	NP
WT-5	-	-	-	NP	NP
WT-6	-	-	-	NP	NP
WT-7	-	-	-	NP	NP
WT-8	-	-	-	NP	NP
WT-9	-	-	-	NP	NP
WT-10	-	-	-	NP	NP
WT-11	-	-	-	NP	NP

Patient	Tumor-RFLP	Plasma-Sequenom	Plasma-ME-PCR	Tumor-Sequenom	Tumor-ME-PCR
WT-12	-	-	-	NP	NP
WT-13	-	-	-	NP	NP

RFLP= Restriction Fragment Length Polymorphism. ME-PCR = Mutant-Enriched PCR. NP=Not performed.

Table 4
Different methods used in selected previous reports to detect *EGFR* and *KRAS* mutations in plasma and serum samples of lung cancer patients.

	Assay design	Gene	n	Sensitivity	Specificity	Sample
Kimura et al 2006 (8)	PCR + Direct Sequencing	EGFR	12	66%	63/71 % *	Serum
Kimura et al 2007 (9)	Scorpion-amplification refractory mutation system	EGFR	42	85%	94%	Serum
Bai et al 2009 (3)	Denaturing High-Performance liquid chromatography	EGFR	230	82%	90%	Plasma
He C et al 2009 (6)	Mutant-enriched PCR	EGFR	18	100%	89%	Plasma
Mack et al 2009 (5)	Scorpion-amplification refractory mutation system	EGFR	49	n.a.	n.a.	Plasma
Yung 2009 (4)	Microfluidics digital PCR	EGFR	35	92%	100%	Plasma
Jian 2009 (7)	Lightcycler PCR with Taqman-MGB probes	EGFR	88	n.a.	n.a.	Plasma
Kuang 2009 (10)	Scorpion-amplification refractory mutation system	EGFR	30	70%	85%	Plasma
Wang 2010 (17)	PCR-restriction fragment length polymorphism	KRAS	273	77%	95%	Plasma
Tsao 2009 (18)	Colorimetric membrane array method	KRAS	209	83%	96%	Peripheral blood
Tsao 2009 (18)	Weighted chemiluminescent membrane array	KRAS	209	93%	94%	Peripheral blood

* before and after treatment.

n.a. : Sensitivity and specificity are not available because of a lack of correlation with primary matched tumors.