

Assessment of Epidermal Growth Factor Receptor and K-Ras Mutation Status in Cytological Stained Smears of Non-Small Cell Lung Cancer Patients: Correlation with Clinical Outcomes

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ABSTRACT

Objective. Epidermal growth factor receptor (*EGFR*) and *K-ras* mutations guide treatment selection in non-small cell lung cancer (NSCLC) patients. Although mutation status is routinely assessed in biopsies, cytological specimens are frequently the only samples available. We determined *EGFR* and *K-ras* mutations in cytological samples.

Methods. DNA was extracted from 150 consecutive samples, including 120 Papanicolaou smears (80%), 10 cell blocks (7%), nine fresh samples (6%), six ThinPrep[®] tests (4%), and five body cavity fluids (3.3%). Papanicolaou smears were analyzed when they had >50% malignant cells. Polymerase chain reaction and direct sequencing of exons 18–21 of *EGFR* and exon 2 of *K-ras* were performed. *EGFR* mutations were simultaneously determined in biopsies and cytological samples from 20 patients. Activity of *EGFR* tyrosine kinase inhibitors (TKIs) was assessed.

Results. The cytological diagnosis was adenocarcinoma in 110 samples (73%) and nonadenocarcinoma in 40 (27%) samples. *EGFR* mutations were identified in 26 samples (17%) and *K-ras* mutations were identified in 18 (12%) samples. *EGFR* and *K-ras* mutations were mutually exclusive. In *EGFR*-mutated cases, DNA was obtained from stained smears in 24 cases (92%), pleural fluid in one case (4%), and cell block in one case (4%). The response rate to *EGFR* TKIs in patients harboring mutations was 75%. The mutation status was identical in patients who had both biopsies and cytological samples analyzed.

Conclusion. Assessment of *EGFR* and *K-ras* mutations in cytological samples is feasible and comparable with biopsy results, making individualized treatment selection possible for NSCLC patients from whom tumor biopsies are not available. *The Oncologist* 2011;16:877–885

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INTRODUCTION

Mutations in the tyrosine kinase (TK) domain of the epidermal growth factor receptor (*EGFR*) gene in non-small cell lung cancer (NSCLC) patients are associated with clinical response to the TK inhibitors (TKIs) gefitinib and erlotinib [1–3]. These mutations are somatic and are more common in patients with clinical features known to be associated with sensitivity to TKIs, such as female gender, adenocarcinoma, Asian ethnicity, and lack of smoking history. The discovery of these mutations and their clinical significance has become a major step forward in the development of targeted therapies with EGFR TKIs such as gefitinib and erlotinib [4–6].

Activating mutations within the TK domain of *EGFR* are found in approximately 10%–20% of NSCLC patients and are associated with response to EGFR TKIs [5, 6]. Gefitinib showed markedly better efficacy than standard chemotherapy in this subset of patients [7, 8]. Deletions in exon 19 and the single L858R point mutation in exon 21 account for 90% of all *EGFR* mutations [1–3, 6]. These mutations mediate oncogenic effects by altering downstream signaling and antiapoptotic mechanisms [9] and are associated with clinical response and survival following TKI therapy. Other genetic alterations described in NSCLC, such as the T790M point mutation or insertion mutations in exon 20 of *EGFR*, amplification of *MET*, and mutations in *K-ras*, are associated with a lack of activity of TKIs [10–12]. As a result, assessment of mutation status has become part of the standard management of NSCLC patients [6, 10].

Unfortunately, molecular testing is often hampered by the inability to obtain appropriate tumor samples. Currently, advanced lung cancer is frequently diagnosed by fine-needle aspiration (FNA) performed via transbronchial needle aspiration (TBNA), endoscopic ultrasound (EUS), or percutaneously, guided by computerized tomography (CT) or echography [13–15]. The samples obtained are often considered inadequate to yield sufficient material to perform a complete molecular analysis [16, 17]. This is particularly relevant in patients with advanced NSCLC, in whom cytological specimens, often stained smears, are frequently the only diagnostic material available, raising the question of whether or not more invasive procedures should be performed [16]. We report our institution's experience assessing *EGFR* and *K-ras* mutations in tumor DNA extracted from cytological samples, in particular from Papanicolau-stained slides. Clinical outcomes of patients harboring mutations and their response to TKI therapy are also discussed.

MATERIALS AND METHODS

Patients

Cytological samples from patients with suspected lung cancer were obtained consecutively at our institution by TBNA, EUS, CT, ultrasound-guided FNA, or blind percutaneous FNA. Rapid onsite evaluation was performed by a pathologist for all FNA procedures in order to guarantee that samples were adequate. Stained smears received from other hospitals for consultation and cytological samples obtained from body cavity fluids were also analyzed. When paraffin-embedded tumor biopsies were available, molecular analysis was performed and results were compared with those obtained from cytological samples. The following categories were used to define smoking status: smoker, >100 cigarettes per lifetime; nonsmoker, <100 cigarettes per lifetime. An institutional review board–approved protocol allows biopsy specimens to be used for research purposes. All patients signed informed consent before the procedure.

DNA Extraction

A pathologist reviewed the Papanicolau-stained slides in order to select the best slides for molecular analysis. The criterion to select adequate slides was that they showed $\geq 50\%$ malignant cells. Only one slide was used for DNA extraction in each case. Prior to DNA extraction, Papanicolau-stained smears were first rinsed in alcohol and scraped into Eppendorf tubes. Slides were not destained prior to DNA extraction. DNA was extracted using Nucleospin® Tissue (catalogue no., 740952.50; Macherey-Nagel GmbH & Co. KG, Düren, Germany). DNA concentration was measured using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE).

EGFR and *K-ras* Mutation Analysis

Polymerase chain reaction (PCR) and direct sequencing of exons 18–21 of *EGFR* and exon 2 of *K-ras* were performed using an ABI Prism™ 310XL DNA sequencer (Applied Biosystems, Madrid, Spain). The primer sequences used, cycling conditions, and annealing temperatures of touchdown PCR for *EGFR* analysis are shown in Table 1. The presence of an appropriate PCR product was confirmed by resolving the PCR products on a 2% agarose gel. PCR products were purified using the GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare Bio-Sciences AB, Björkgatan, Sweden) following the manufacturer's instructions. Fragments were sequenced and analyzed in both the sense and antisense directions. DNA templates were processed for the DNA sequencing reaction using ABI

Table 1. Primer sequences and cycling conditions for touchdown PCR

Exon	Primers	AT	PCR conditions
EGFR 18	Forward, 5'-CAAGTGCCGTGTCCTGGCACCCAAGC-3'	AT ₁ , 64°C	Step 1: 95°C, 10 minutes
	Reverse, 5'-CCAAACACTCAGTCAAACAAAGAG-3'	AT ₂ , 57°C	Step 2: 95°C, 30 seconds
EGFR 19	Forward, 5'-GTGCATCGCTGGTAACATCC-3'	AT ₁ , 64°C	Step 3: AT ₁ decreases 0.5°C per cycle, 30 seconds
	Reverse, 5'-TGTGGAGATGAGCAGGGTCT-3'	AT ₂ , 57°C	Step 4: 72°C, 30.0 seconds
EGFR 20	Forward, 5'-ATCGCATTTCATGCGTCTTCA-3'	AT ₁ , 60.2°C	Step 5: Repeat steps 2–4 14 more times
	Reverse, 5'-ATCCCATGGCAAACCTCTTG-3'	AT ₂ , 53.2°C	Step 6: 95°C, 30 seconds
EGFR 21	Forward, 5'-GCTCAGAGCCTGGCATGAA-3'	AT ₁ , 64°C	Step 7: AT ₂ , 30 seconds
	Reverse, 5'-CATCCTCCCCTGCATGTGT-3'	AT ₂ , 57°C	Step 8: 72°C, 30.0 seconds
K-ras 2	Forward, 5'-GGTGGAGTATTTGATAGTGTA-3'	AT ₁ , 57°C	Step 9: Repeat steps 6–8 19 more times
	Reverse, 5'-GGTCCCTGCACCAGTAATATGC-3'	AT ₂ , 50°C	Step 10: 72°C, 10 minutes
			Step 11: 4°C, forever

Abbreviations: AT, annealing temperature; EGFR, epidermal growth factor receptor; PCR, polymerase chain reaction.

Prism™ BigDye Terminator, version 3.1 (Applied Biosystems).

Following sequencing reactions, DNA was purified using Performa® DTR Gel Filtration Cartridges (EdgeBio, Gaithersburg, MD). Sequence data were generated with the ABI Prism 310 DNA analyzer (Applied Biosystems). Mutations were identified by visual analysis of the sequenced chromatograms using SeqScape (Applied Biosystems). Sequence data were analyzed by two investigators and compared with the archived human sequence of *EGFR* (GenBank accession no., NG_007726.1; *K-ras* GenBank accession no., NG_007524.1).

Clinical Assessment and Statistical Analysis

The response rate of patients treated with TKIs was assessed using Response Evaluation Criteria in Solid Tumors [18]. The progression-free survival (PFS) interval was calculated from the date when treatment with the TKI was started to the date of progression or death from any cause. Descriptive statistics are used to present patient characteristics and results of the molecular analysis. Statistical comparisons were performed using Fisher's exact test. Survival data were analyzed using the Kaplan–Meier method [19]. Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL). Agreement between the mutation status assessed in cytological samples and in biopsies was assessed using Cohen's κ coefficient [20]. The relationship between the κ coefficient and strength of between-method agreement may be interpreted in the following manner: 0.81–1.00, almost perfect; 0.61–0.80, substantial; 0.41–0.60, moderate; 0.21–0.40, fair; 0–0.20, slight; and <0, poor [21].

RESULTS

Patients and Tumor Samples

Between January 2007 and December 2009, 150 cytological samples from NSCLC patients were consecutively obtained and analyzed. Patient characteristics, tumor histology, and DNA source are shown in Table 2. Samples were obtained from FNA of primary lung tumors in 68 cases (45.3%), metastatic lymph nodes in 70 cases (46.6%), bone metastases in three cases (2%), a left adrenal metastasis in one case (0.7%), pleural fluid in six cases (4%), pericardial fluid in one case (0.7%), and bronchoalveolar lavage in one case (0.7%). TBNA was the diagnostic procedure in 104 patients, whereas EUS-FNA was used in five patients, CT-guided percutaneous FNA was used in 10 patients, and ultrasound-guided percutaneous FNA was used in three patients. In 11 patients, the pathologist performed an FNA directly on superficial lymph nodes. In eight patients, the samples were stained smears received from other hospitals for consultation, obtained by FNA from lymph nodes (six mediastinal, through TBNA, and two superficial, by direct FNA). Six samples originated from body cavity fluids, and three resulted from bronchial biopsy touch preps. DNA was extracted from 120 Papanicolaou-stained smears (80%), 10 cell blocks (6.7%), nine fresh samples (6%), six ThinPrep® tests (4%), and five body cavity fluids (3.3%). DNA was successfully extracted from all samples studied. We obtained an average of 24.6 ng/ μ l (standard deviation, 15.3) of DNA.

The majority of the lesions were diagnosed as adenocarcinoma ($n = 110$; 73.3%) Twenty-seven patients (18%) had

Table 2. Patient characteristics, frequency of mutations, type of tumor, and source of DNA for mutational analysis

Characteristic	All cases	EGFR mutations	K-ras mutations
	n = 150 (%)	n = 26 (%)	n = 18 (%)
Gender			
Female	62 (41.3)	20 (76.9)	6 (33.3)
Male	88 (58.7)	6 (23.1)	12 (66.7)
Smoking			
Yes	110 (73.3)	9 (34.6)	18 (100)
No	40 (26.7)	17 (65.4)	0
Tumor type			
Adenocarcinoma	110 (73.3)	26 (100)	13 (72.2)
Squamous	27 (18)	0	1 (5.5)
Large cell	4 (2.7)	0	2 (11.1)
Poorly differentiated NSCLC	9 (6)	0	2 (11.1)
DNA source			
Stained slides	120 (80)	24 (92.4)	16 (88.9)
Fresh/liquid	14 (9.3)	1 (liquid) (3.8)	0
Cell block	10 (6.7)	1 (3.8)	0
ThinPrep®	6 (4)	0	2 (11.1)

Abbreviations: EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer.

squamous cell carcinoma, four (2.7%) had large cell carcinoma, and nine (6%) had poorly differentiated NSCLC.

EGFR Mutation Status

We identified 26 *EGFR* mutations in the 150 samples analyzed. The frequency of *EGFR* mutations, patient characteristics, tumor type, and source of DNA for mutational analysis are shown in Table 2. All 26 mutations were described in adenocarcinomas. From the 26 mutations detected, 24 were identified in DNA from Papanicolaou-stained smears. Mutations were found in 20 of 62 women (32.3%) and in six of 88 men (6.8%). *EGFR* mutations were identified in 17 of 40 nonsmokers (42.5%), but in only nine of 110 smokers (8.2%) ($p < .001$) (Table 2). A representative case is presented in Figure 1.

Table 3 summarizes all *EGFR* mutations detected as well as the characteristics of the patients and the sampling technique used. We found 18 mutations (69.2%) in exon 19, six mutations (23%) in exon 21, one mutation (3.8%) in exon 20, and one mutation (3.8%) in exon 18. In exon 19 we found 11 deletions (42.3%), one missense mutation (3.8%), five deletion plus missense mutations (19.2%), and one deletion plus insertion (3.8%). Exon 20 had an insertion, and in exon 21, all six alterations were missense mutations.

Paraffin-embedded tissue was available from 20 patients. Analysis of *EGFR* mutation status in those samples revealed agreement with the results obtained in cytological

samples in every case (1.0 or perfect agreement, as assessed by the κ coefficient).

K-ras Mutation Status

K-ras mutation status was assessed in 111 cases. Table 4 shows *K-ras* mutation types, diagnosis, and patient characteristics. We found *K-ras* mutations in 18 patients (12%). *K-ras* and *EGFR* mutations were mutually exclusive. Mutations in *K-ras* were observed in DNA extracted from Papanicolaou-stained smears in 16 cases (89%), and from ThinPrep® tests in two cases (11%).

Clinical Activity of TKIs

Clinical follow-up was available for 16 of the 26 patients with *EGFR* mutations who presented with stage IV disease. Based on the identification of such mutations, these patients received subsequent therapy with a TKI, including erlotinib [14] and gefitinib [2]. *EGFR* TKIs were administered as first-line therapy in eight patients (50%), as second-line therapy in seven patients (44%), and as fifth-line therapy in one patient (6%). We observed one complete response and 11 partial responses (overall response rate, 75%). In addition, one patient showed a minor response and two had ongoing stable disease at 15 and 20 months. One patient presented rapid progression at the first evaluation of disease. The median PFS duration of patients with *EGFR* mu-

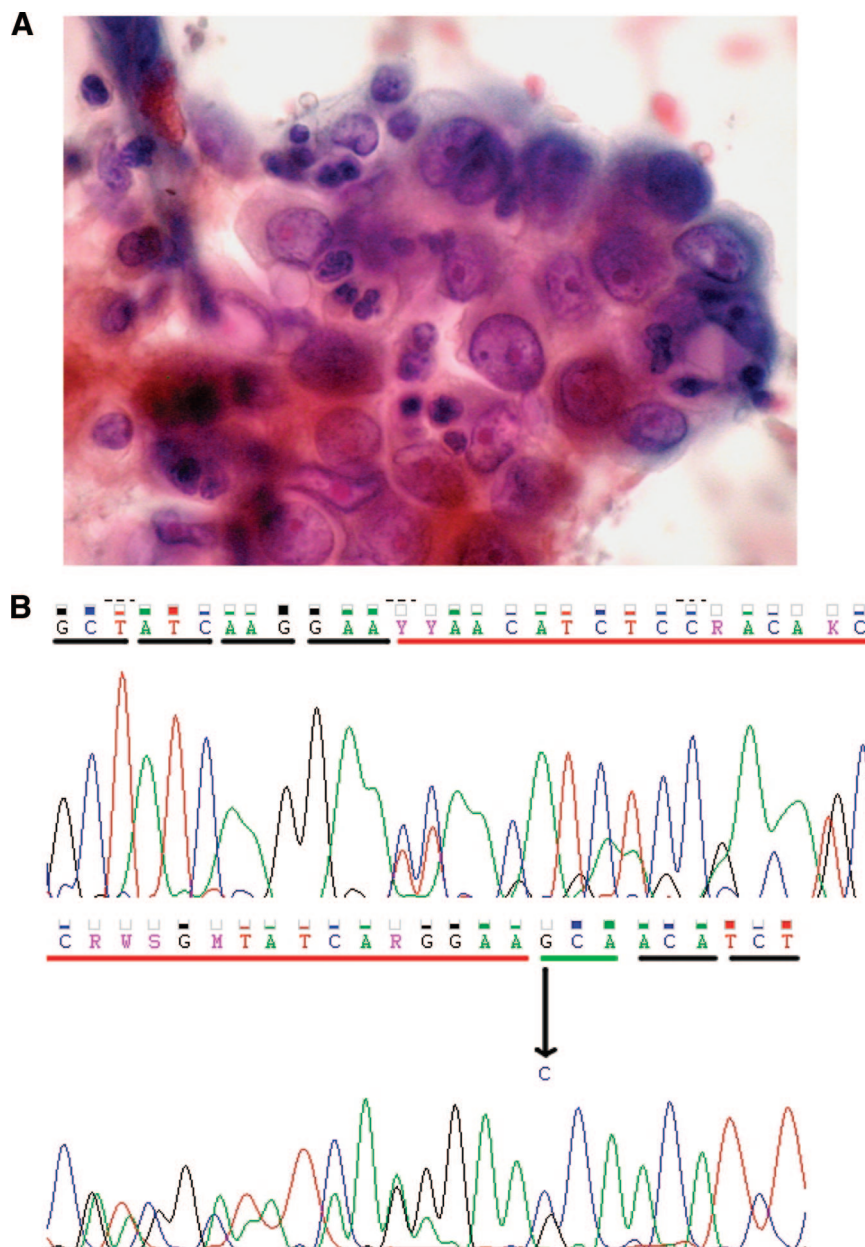


Figure 1. Representative example of a case included in our series. **(A):** Papanicolaou-stained smear showing lung adenocarcinoma cells obtained by fine-needle aspiration from a supraclavicular lymph node. **(B):** Direct sequencing of exon 19 showing a deletion and a point mutation (del L747–E749, A750P).

tations who were treated with erlotinib or gefitinib was 12.3 months (Fig. 2).

DISCUSSION

Molecular profiling is now considered the standard of care for patients with NSCLC [5, 6, 22]. The development of targeted therapies against the EGFR TK domain has led to the discovery of mutations and other genomic alterations that predict the efficacy of these agents. Paraffin-embedded and frozen tissue have been considered optimal samples for molecular studies. Consequently, most reports published on

EGFR analyses were performed using biopsies. However, biopsies are not always available, because the diagnosis of lung cancer sometimes relies on cytological samples obtained by FNA through minimally invasive procedures in order to avoid more invasive techniques [13–15]. Therefore, the possibility of performing molecular analyses on samples obtained using minimally invasive procedures is appealing [23]. To our knowledge, our study is the first large series to show that Papanicolaou-stained cytological samples are adequate for EGFR and K-ras mutation analysis. We believe this technique could be introduced into clinical practice.

Table 3. Characteristics of patients with *EGFR* mutations

Patient	<i>EGFR</i> mutation	Exon	Sex	Age	Sampling technique	Diagnosis	Smoking	DNA source
1	E709K, G719A	18	F	83	BC-FNA	AC (BAC)	No	SS
2	del E746-A750	19	F	53	BC-FNA	AC	No	SS
3	del E746-A750	19	F	60	Thoracocentesis	AC	Yes	Pleural fluid
4	del E746-A750	19	F	64	BC-FNA	AC	Yes	SS
5	del E746-A750	19	F	65	BC-FNA	AC	No	SS
6	del E746-A750	19	F	74	CT-FNA	AC	No	SS
7	del E746-A750	19	M	47	Direct FNA	AC	Yes	SS
8	del E746-A750	19	M	65	BC-FNA	AC	Yes	SS
9	del E746-A750	19	M	70	BC-FNA	AC	No	SS
10	del E746-A750	19	M	72	BC-FNA	AC	No	SS
11	del E746-A750	19	M	75	BC-FNA	AC	No	SS
12	del E746-A750	19	M	76	Consultation ^a	AC	No	SS
13	delL747-T751, K754E	19	F	47	BC-FNA	AC	Yes	SS
14	delL747-E749, A750P	19	F	48	BC-FNA	AC	Yes	SS
15	delL747-E749, A750P	19	F	61	BC-FNA	AC	No	SS
16	delL747-T751insS	19	F	67	BC-FNA	AC	No	SS
17	delL747-E749, A750P	19	F	72	Direct FNA	AC	No	SS
18	delL747-E749, A750P	19	F	72	Direct FNA	AC	No	SS
19	L747P	19	F	60	BC-FNA	AC	Yes	SS
20	N771insH	20	F	61	BC-FNA	AC	Yes	SS
21	L858R	21	F	47	EUS-FNA	AC	Yes	SS
22	L858R	21	F	60	Direct FNA	AC	No	SS
23	L858R	21	F	60	BC-FNA	AC	No	SS
24	L858R	21	F	72	BC-FNA	AC	No	SS
25	L858R	21	F	82	BC-FNA	AC	No	SS
26	L858R	21	F	87	BC-FNA	AC	No	Cell block

^aStained slides received for consultation.

Abbreviations: AC, adenocarcinoma; BAC, bronchioloalveolar carcinoma; BC-FNA, bronchoscopy-guided FNA; CT-FNA, CT scan-guided FNA; EUS-FNA, endoscopic ultrasound-guided FNA; F, female; FNA, fine-needle aspiration; M, male; SS, stained smear.

The prior literature on *EGFR* mutation analysis relies primarily on larger tumor biopsies, but our results are comparable with regard to the frequency and distribution of *EGFR* mutations according to sex, histology, and prior smoking history, as well *EGFR* TKI activity [1–3, 6]. The 75% response rate and the median PFS interval of 12.3 months compare favorably with the 70.6% response rate and 14-month PFS interval reported in one of the most relevant series published to date [6]. Moreover, mutation status was determined in cytology as well as in biopsy samples in a subgroup of patients, and the agreement between the two methods was perfect. *EGFR* mutations were never observed in patients who had *K-ras* mutations, as has previously been reported [22].

Currently, there is ongoing debate in the oncologic com-

munity regarding the adequacy of samples obtained for molecular analysis from NSCLC patients. Although initial data suggested that cytological samples were not adequate for molecular analysis [16], recent findings suggest that minimally invasive techniques might suffice [10]. Some studies have evaluated the feasibility of assessing *EGFR* mutation status in paraffin-embedded cell blocks and histological cores from NSCLC patients obtained by TBNA and FNA [24–26]. Smouse et al. [27] recently showed that cytological cell blocks provide equivalent, if not higher, sensitivity when compared with surgical specimens, suggesting that the suitability of a sample should be based on tumor cell proportion and abundance, rather than on the technique used to obtain the sample. We agree with this concept, and we believe that the quality of the sample is a

Table 4. Summary of *K-ras* mutation types, histological diagnosis, and patient characteristics

Patient	Sex	Age	<i>K-ras</i> mutation	Sampling technique	Diagnosis	Smoking status	DNA source
1	F	54	G12D	BC-FNA	AC	Yes	SS
2	M	61	G12D	BC-FNA	AC	Yes	SS
3	M	43	G12D	BC-FNA	SqCC	Yes	SS
4	M	59	G12C	Imprints	AC	Yes	SS
5	M	66	G12V	BC-FNA	AC	Yes	ThinPrep®
6	F	49	G13D	EUS-FNA	AC	Yes	SS
7	F	41	G12C	BC-FNA	AC	Yes	SS
8	M	55	G12F	CT-FNA	AC	Yes	SS
9	M	45	G12C	BC-FNA	AC	Yes	SS
10	M	62	G13C	BC-FNA	NSCLC	Yes	SS
11	M	59	G12D	BC-FNA	NSCLC	Yes	SS
12	F	68	G12V	IO-FNA	AC	Yes	SS
13	F	70	G12V	BC-FNA	AC	Yes	SS
14	M	82	G12A	BC-FNA	LCC	Yes	ThinPrep®
15	F	67	G12C	BC-FNA	LCC	Yes	SS
16	M	68	G12V	Imprints	AC	Yes	SS
17	M	55	L19F	BC-FNA	AC	Yes	SS
18	M	67	G12C	BC-FNA	AC	Yes	SS

Abbreviations: AC, adenocarcinoma; BC-FNA, bronchoscopy-guided FNA; CT-FNA, CT scan-guided FNA; EUS-FNA, endoscopic ultrasound-guided FNA; F, female; FNA, fine-needle aspiration; IO-FNA, intraoperative FNA; LCC, large cell carcinoma; M, male; NSCLC, non-small cell lung cancer; SqCC, squamous cell carcinoma; SS, stained smear.

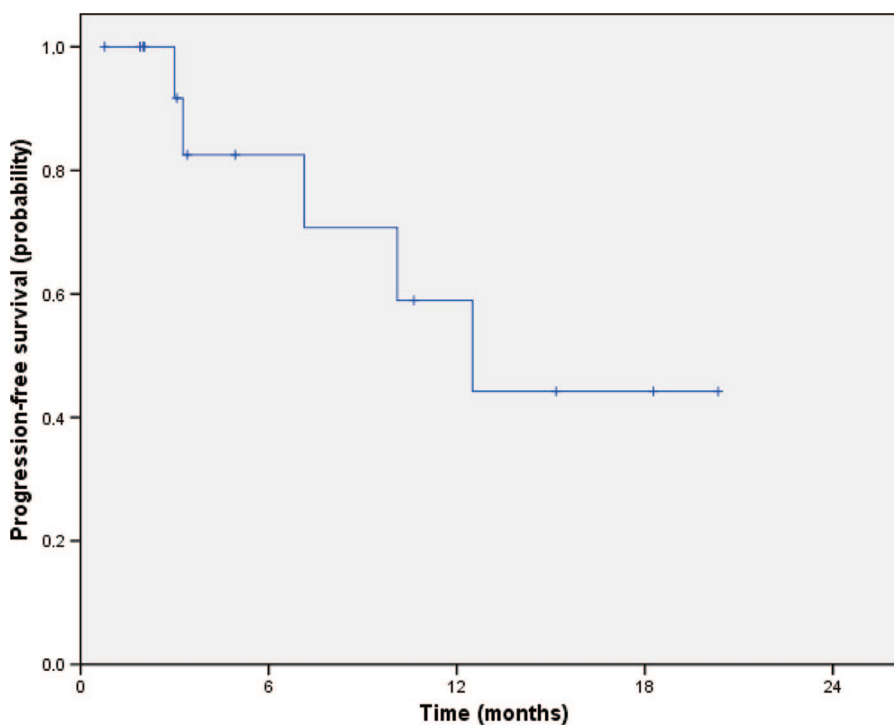


Figure 2. Progression-free survival of patients presenting with epidermal growth factor receptor gene mutations treated with erlotinib and gefitinib.

critical factor in order to obtain adequate results. In our study, we assessed mutations only from slides that contained $\geq 50\%$ malignant cells.

To our knowledge, this is the largest series analyzing *EGFR* mutations in exons 18–21 as well as *K-ras* mutations in cytological samples. Molecular analysis results were validated with clinical outcomes as well as simultaneous assessment using traditional biopsy samples. A few small series have assessed mutations in exons 19 and 21 of *EGFR* using cytological samples, some of them including Papanicolaou-stained smears. Boldrini et al. [28] reported their retrospective results from a small series of 23 cytological samples. Nomoto et al. [29] and Smith et al. [30] analyzed the feasibility of a high-resolution melting analysis to perform *EGFR* mutational analysis in small specimens, including cytological samples. Fukui et al. [31] reported their results with small diagnostic samples and biopsies from 92 patients. They reported better results from PCR analysis using cytological slides rather than biopsy specimens, regardless of the amount of tumor cells analyzed. They explained this fact by differences in the method of sample fixation between the two types of specimens, favoring methanol over formalin for mutational studies, thus highlighting the importance of preserving high-quality DNA. Savic et al. [32] studied the presence of *EGFR* mutations in NSCLC samples, including some Papanicolaou-stained slides from NSCLC patients, using sequencing methods. None of the previous studies provided clinical information on the outcome of the patients treated with TKIs to confirm their predictive value. Oshita et al. [33] evaluated a novel heteroduplex method to study some of the most frequently described mutations in exons 19 and 21 of *EGFR* in cytology specimens obtained by transbronchial abrasion from 52 NSCLC patients. They assessed the clinical activity of gefitinib in 26 patients, reporting a 91% response rate in the 11 patients that had mutations. Our study added a determination of the concordance between mutation assessment in biopsies and in cytological samples.

The main limitation of our study is the limited sample size. Although we showed that assessment of mutations in cytological samples is feasible, further studies are necessary to determine the specificity and sensitivity of this tech-

nique. In addition, this methodology, as any other, requires a learning curve during which simultaneous assessment of mutations in paraffin samples, if available, can provide quality control.

CONCLUSION

Our results show that assessment of *EGFR* mutations in exons 18–21 and *K-ras* mutation status in cytological samples, including Papanicolaou-stained slides, from NSCLC patients is feasible and results are comparable with those obtained from biopsies. Identification of *EGFR* mutations in cytological samples predicts clinical response to targeted therapies, thus extending the benefits of individualized treatment selection to NSCLC patients from whom tumor biopsies are not available. This finding may facilitate large-scale screening of patients with NSCLC for *EGFR* mutations with minimally invasive techniques, avoiding more invasive procedures. In addition, this strategy can be used for research purposes.

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