Assessment of *EGFR* and *K-ras* mutations in fixed and fresh specimens from transesophageal ultrasound-guided fine needle aspiration in non-small cell lung cancer patients

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Abstract. In non-small cell lung cancer (NSCLC) patients, somatic *EGFR* and *K-ras* mutations predict therapeutic effectiveness and resistance, respectively, to EGFR tyrosine kinase inhibitors (TKIs). Transesophageal ultrasound-guided fine needle aspiration (EUS-FNA) is a validated technique for diagnosis and staging of NSCLC. In the present study, we compared the feasibility and reliability of *EGFR* and *K-ras* gene mutation analysis in fixed and fresh mediastinal lymph nodes and extra-lymph nodal samples obtained by EUS-FNA in patients suspicious for NSCLC. Thirty-six patients were enrolled into the study. For each patient, DNA was extracted from both fresh samples and fixed cytological smears. Exons 18-21 of *EGFR* and exon 2 of *K-ras* were amplified by PCR and mutation status was determined by direct sequencing and pyrosequencing. All cases were eligible for analysis. NSCLC was diagnosed in 32 patients (25 adenocarcinomas and 7 squamous cell carcinomas) and 4 patients were free of malignancy. Of the 25 patients with adenocarcinoma, *EGFR* mutations were detected in 2 (8%) fresh tumor samples and in 3 (12%) fixed cytological smears. *K-ras* mutations were detected in 8 (32%) fresh samples, and in 9 (36%) fixed cytological smears. Fixed and stained cytological samples seem to be more reliable than fresh material for molecular analysis.

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Introduction

The presence of somatic mutations in the epidermal growth factor receptor (EGFR) gene in non-small cell lung cancer (NSCLC) patients correlates with a good response to tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib (1-4). Most *EGFR* mutations consist of small in-frame deletions or substitutions clustered around the ATP-binding site in exons 18, 19 and 21. Patients with these mutations show an 80% response rate to TKIs, compared to only 10% of patients with wild-type *EGFR* (1,4,5). On the other hand, *EGFR* exon 20 mutations and *K-ras* mutations, have been shown to be related to acquired or intrinsic resistance to TKIs (6-11). In view of these results, it is mandatory to perform *EGFR* mutation analysis in all adenocarcinomas, providing patients with a more effective and tailored therapy.

The first step in deciding the best treatment option is the correct staging of the disease, followed by molecular characterization. Despite the fact that NSCLC is primarily treated by surgery, only 30% of patients present with a resectable stage at the time of the diagnosis (12). Clinical staging of NSCLC 'classically' include imaging (CT and PET scans) and invasive procedures, e.g., mediastinoscopy, but recently it has been recognized that less invasive procedures, such as transbronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) or transesophageal ultrasound-guided fine needle aspiration (EUS-FNA) can be used. These less invasive cytological procedures are reported to have a similar high specificity and sensitivity to mediastinoscopy (13-16), and have recently been included in lung cancer staging guidelines, as an alternative to surgical staging of the mediastinum (17,18).

In recent years, many efforts have been made to verify if cytological specimens are suitable for molecular testing. It has been demonstrated that somatic *EGFR* mutations can be detected in fixed cytological samples obtained through different approaches such as bronchial washing, EBUS-TBNA and trans-thoracic needle aspiration (TTNA) (19-24). Moreover, recent studies have reported that *K-ras* and *EGFR* mutations, and also other gene mutations, e.g., *BRAF* and *PIK3CA*, can

be detected in archivial, fixed cytological smears or cell blocks obtained from both EBUS-TBNA or EUS-FNA specimens of lung adenocarcinoma (25,26).

The aim of the present study was to compare the feasibility and reliability of *EGFR* and *K-ras* mutation analysis in fixed and fresh mediastinal lymph node and extra-lymph nodal samples obtained by EUS-FNA in patients suspicious for NSCLC.

Patients and methods

Patients. A prospective study was conducted in the Pulmonology Unit, Department of Thoracic Diseases, Forlì, Italy, between October 2009 and March 2010, including patients referred for diagnosis and/or staging of known or suspected NSCLC using EUS-FNA. Thirty-six patients were selected for the EUS-FNA procedure based on: i) a computed tomography (CT) of the chest and mediastinum, showing enlarged lymph nodes (short-axis diameter >l0 mm); ii) no evidence of extra-thoracic metastases; and iii) CT scan evidence of mediastinal lymphadenopathy that is detectable from the esophagus (e.g., 2L, 4L, 5, 7). This study was approved by the local institutional review boards. All patients provided written informed consent to the procedure and to genetic analysis of the tissue samples. For 13 patients, paraffin-embedded samples derived from a histological sample or from a cytological cell block were also available.

EUS-FNA procedure. Patients underwent EUS-FNA under deep sedation, with anesthesiologic assistance. The procedure was performed through the mouth using a linear esophageal endo-echoscope (Olympus GF UCT 160, EUS Exera, EU-C60, Hamburg, Germany). The left adrenal gland and the left liver lobe were identified, and when an enlarged lymph node was located under continuous real-time ultrasound imaging, a needle (22 G, Olympus, NA-200H-8022, Tokyo, Japan) was introduced through the biopsy channel of the endoscope. Before sampling, a power Doppler examination was performed to avoid the unintentional puncture of the vessel. After the needle was placed into the lesion, suction was applied with a syringe, under real-time ultrasonic guidance. An average of 6-7 specimens was obtained. Specimens were expelled onto glass slides, smeared and air-dried. A rapid on-site cytological examination (ROSE) was performed to assess the suitability of the sample. At ROSE examination, the sample was defined as: i) negative, when it contained predominantly lymphocytes and no neoplastic cells; ii) metastatic, when groups of neoplastic cells were identified; iii) adequate, when it was negative or metastatic; and iv) inadequate, when it contained bronchial or blood cells alone, in these cases the procedure was repeated up to three times.

A mean of 4-5 aspirates were performed. When a sample was identified as metastatic at ROSE examination, fresh material was collected in hypertonic solution (9%) and sent for molecular analysis. When possible, a sample obtained from metastatic nodes was processed as a histology core (cell block). All remaining material was sent to the Department of Pathology for final diagnosis. Fixed and stained cytological smears, which had undergone selection of the tumor cells, were also sent for molecular analysis.

DNA extraction from EUS/FNA and paraffin-embedded samples. Fresh EUS-FNA samples were centrifuged at 800 g and cell pellets were washed in saline solution. DNA was extracted using QIAamp DNA Micro kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. DNA quantity and quality were assessed by Nanodrop (Celbio, Milan, Italy).

For cytological smears, tumor cell areas were macroscopically removed from slides and placed in a test-tube. Cells were lysed in 50 mM KCl, 10 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$ and Tween-20 (0.45%) in the presence of 1.25 mg/ml proteinase K, overnight at 56˚C. Proteinase K was inactivated at 95˚C for 10 min, and then samples were centrifuged twice at 2,500 g to eliminate debris. The supernatant was purified using QIAamp DNA Micro kit (Qiagen) using the 'Cleanup of genomic DNA' protocol. DNA quality and quantity was assessed by Nanodrop (Celbio).

For paraffin-embedded samples, an area containing at least 50% of tumor cells was selected in hematoxylin-eosin-stained sections, and contiguous areas in 5 μM sections were macrodissected and collected in specific tubes. DNA extraction was performed as described for cytological smears.

Fifty nanograms of DNA were used for PCR amplification.

EGFR and K-ras mutation analysis. Exon 2 of *K-ras* and exons 18-21 of *EGFR* were amplified by PCR using the primers indicated in Table I. PCR products were purified using Minielute PCR purification kit (Qiagen) and then submitted to sequencing using BigDye Terminator 3.1 Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequence reactions were then purified using DyeEx 2.0 Spin kit (Qiagen) and separated by capillary electrophoresis with laser-induced fluorescence detection (3100 Genetic Analyzer, Applied Biosystems).

Exon 2 of *K-ras* and exons 18, 19 and 21 of *EGFR* were also analyzed by pyrosequencing using anti-EGFR MoAb response (*K-ras* status) and *EGFR* TKI response (sensitivity), respectively (Diatech, Jesi, Ancona, Italy), according to the manufacturer's instructions. Reactions were run on a PyroMark Q96 ID (Qiagen).

Results

Patient characteristics. Of the 36 patients enrolled in the study, 11 were females and 25 were males. Median age was 64 years (range 44-83). EUS-FNA samples were adequate for final diagnosis and molecular analysis in all cases. NSCLC was diagnosed in 32 patients (25 adenocarcinomas and 7 squamous cell carcinomas) and 4 patients were free of malignancy. Tumor stages were IIB in 1 patient, IIIA in 1 patient, IIIB in 15 patients, and stage IV in 15 patients. Metastatic EUS-FNA samples were obtained from station 4L (9 samples), station 5 (2 samples), station 7 (25 samples), and extra-lymph nodal metastases in 5 cases (2 mediastinal masses, 1 lung parenchyma tumor, 1 left adrenal metastasis and 1 liver metastasis). All samples, evaluated by ROSE, had at least five clusters of neoplastic cells in two slides.

EGFR and K-ras mutations. Mutation analysis was successfully carried out in all fresh and fixed samples (non-tumor cells were excluded from fixed samples). Benign lesions did not show any *K-ras* or *EGFR* mutations. As shown in Table II, *EGFR* mutations were detected in 3 adenocarcinoma patients (12%):

Table I. Primer sequences.

	Forward primer	Reverse primer	Anneal temp. (˚C)	Product size (bp)
K-ras exon 2	GGT GAG TTT GTA TTA AAA GGT ACT GG	GGT CCT GCA CCA GTA ATA TGC	58	265
EGFR exon 18	CAA ATG AGC TGG CAA GTG CCG TGT C	GAG TTT CCC AAA CAC TCA GTG AAA C	58	399
EGFR exon 19	GCA ATA TCA GCC TTA GGT GCG GCT C	CAT AGA AAG TGA ACA TTT AGG ATG TG	58	371
EGFR exon 20	CCATGAGTACGTATTTTGAAACTC	CATATCCCCATGGCAAACTCTTGC	58	407
EGFR exon 21	CTA ACG TTC GCC AGC CAT AAG TCC	GCT GCG AGC TCA CCC AGA ATG TCT GG	58	415
EGFR exon 18 N	CAA GTG CCG TGT CCT GGC ACC CAA GC	CCA AAC ACT CAG TGA AAC AAA GAG	58	381
EGFR exon 19 N	CCT TAG GTG CGG CTC CAC AGC	CAT TTA GGA TGT GGA GAT GAG C	58	348
EGFR exon 21 N	CAG CCA TAA GTC CTC GAC GTG G	CAT CCT CCC CTG CAT GTG TTA AAC	58	373

Table II. *EGFR* and *K-ras* mutations in 32 NSCLC patients.

No.	Gender	Age	Smoking	Packs/year	Stage	Diagnosis	*EGFR* status	*K-ras* status
1	M	81	Yes	40	IV	SCC	wt[b]	wt[b]
2	M	64	Ex	40	IV	ADC	wt	G12C
3	F	82	No	-	IV	ADC	wt	wt
4	F	57	Ex	30	IIIB	SCC	wt	wt
5	M	50	No	-	IV	ADC	Del L747-T751[b]	wt[b]
6	F	54	Yes	30	IV	ADC	wt	G12C
7	F	63	Yes	30	IV	ADC	wt[b]	wt[b]
8	M	55	Yes	30	IV	ADC	wt	G12V
9	M	74	Ex	40	IIIB	ADC	wt[b]	wt[b]
10	M	53	Ex	80	IV	ADC	Del E746-A750[a]	wt
11	F	44	No	-	IV	ADC	wt[b]	wt[b]
12	M	59	Yes	30	IIIB	ADC	wt	G12A
13	M	68	Ex	30	IV	ADC	wt	wt
14	M	73	Ex	50	IIIB	SCC	wt[b]	wt[b]
15	M	63	Ex	40	IIIB	ADC	wt	G12C[a]
16	M	62	Yes	50	IIIB	ADC	wt	wt
17	M	58	Ex	40	IIIB	SCC	wt	wt
18	M	65	Ex	50	IIB	SCC	wt[b]	wt[b]
19	M	59	Yes	40	IV	SCC	wt	wt
20	M	73	Ex	30	IIIB	ADC	wt[b]	G12C[b]
21	M	76	Ex	35	IIIB	ADC	wt[b]	wt[b]
22	F	64	Ex	20	IIIB	ADC	wt	wt
23	M	71	Ex	40	IV	ADC	wt	wt
24	F	78	Ex	20	IIIB	ADC	wt[b]	G12C[b]
25	F	81	No	-	IV	ADC	Del E746-A750[b]	wt[b]
26	M	66	Yes	60	IIIA	ADC	wt[b]	wt[b]
27	M	73	Ex	40	IV	ADC	wt	wt
28	M	52	Yes	30	IIIB	ADC	wt[b]	wt[b]
29	M	48	Ex	20	IIIB	ADC	wt	wt
30	F	48	Yes	30	IV	ADC	wt	G12C
31	M	83	Ex	70	IIIB	ADC	wt	G12C
32	M	71	Ex	40	IIIB	SCC	wt	wt

[a]Mutations were only found in fixed cytological smears. [b]Analyses were confirmed in histological samples.

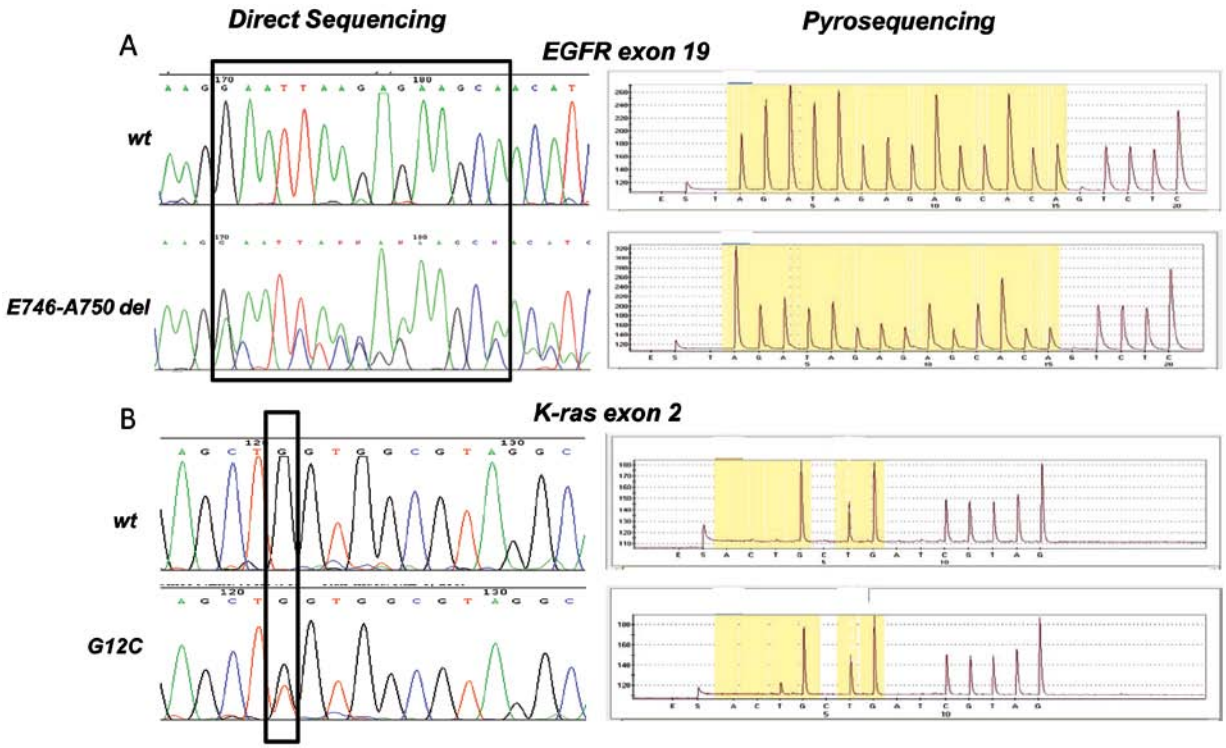

Figure 1. Example of *EGFR* (A) and *K-ras* (B) mutation analysis by direct sequencing and pyrosequencing. Black panels indicate the sites of mutation for direct sequencing results. With regard to pyrosequencing, allelic alteration frequencies were 59.5% for *EGFR* exon 19 mutation and 29.2% for *K-ras* exon 2 mutation.

1 never-smoker female (E746-A750del) and 2 males, 1 never smoker and 1 former smoker (L747-T751del; E746-A750del). *K-ras* mutations were detected in 9 adenocarcinoma patients (36%). Six patients were male (2 current smokers and 4 former smokers), and 3 females (2 current smokers and 1 former smoker). Seven cases had the G12C mutation, 1 case G12V, and 1 case G12A. Mutation status in fixed cytological smears was confirmed in all but 2 fresh fine needle aspirate samples, in which 1 *EGFR* (E746-A750del) and 1 *K-ras* (G12C) mutation were missed (Table II). This translates to 1 of 3 cases (33.3%) being missed for *EGFR*, and 1 of 9 cases (11%) for *K-ras*. All these mutations were confirmed by pyrosequencing analysis (Fig. 1).

For 13 patients, histological samples were also available. Histology was obtained from lymph nodal cell blocks in 6 cases, endobronchial biopsies in 3 cases, resected lung parenchyma in 2 cases, transbronchial lung biopsy in 1 case, and CT-guided lung biopsy in 1 case. The results of the mutation analysis were the same for paraffin-embedded sections and EUS/FNA samples (Table II).

Discussion

In this study, we compared the feasibility of *EGFR* and *K-ras* mutation analysis on EUS-FNA cytology samples processed in two different ways, fixed and stained versus fresh material. For fixed samples, an accurate cancer cell selection was performed before testing, whereas, for fresh material, although the presence of tumor cells was verified, an estimation of cell numbers was not possible. Based on a case series of 36 patients with suspected NSCLC, we were able to perform molecular analysis on all the lymph nodal and extra-lymph nodal EUS/FNA aspirate samples. Fixed and stained samples were more reliable than fresh specimens, and this was probably due to the presence of contaminating non-tumor cells, leading to false-negative results in 2 cases of fresh samples.

Cytologic specimens have not been widely used for mutation analysis, due primarily to intra-sample heterogeneity, which precludes manual microdissection (23), and the possibility of low sample cellularity. One of the most common methods for *EGFR* mutation analysis is direct sequencing. However, this technique is thought to lack the sensitivity of other methods, and is limited by the presence of non-neoplastic cells in heterogeneous tissue samples. Many other techniques are used including high resolution melting (20,22), real-time PCR methods (27), and pyrosequencing (28), all of which are characterized by a higher sensitivity for mutation detection.

In recent years, a number of studies have evaluated the suitability of cytological specimens for molecular analysis. A recent report on a series of 35 adenocarcinomas demonstrated that *EGFR* and *K-ras* mutation analysis were successfully performed in approximately 80 cytological samples, Giemsa- or Papanicolau-stained smears, obtained by EUS and/or EBUS (26). Similarly, Garcia-Olivè *et al* (19) showed that *EGFR* mutations could be detected in 72% of EBUS-TBNA cell block samples. Other reports have demonstrated the feasibility of molecular analysis on cytological samples using different methodologies, all reporting different sensitivities (20,22,23). However, in these reports, results were not compared with those obtained from histological materials. In the study by Savic *et al* (21), *EGFR* mutation analysis was performed on 84 cytological specimens and, in 43 of these, a matched biopsy was available for comparative analysis. More recently, van Eijk *et al* (25) performed *K-ras*, *EGFR*, *BRAF* and *PIK3CA* mutation analysis

in both histological and cytological samples using allele-specific qPCR. In this study, a high concordance was observed when cytologic specimens were obtained from the primary tumor, and discordant results when cytologic specimens were derived from the mediastinal lymph node.

In our study, we confirmed the reliability of EUS/FNA samples for molecular analysis in NSCLC patients. Specifically, all cytological samples, both fresh needle aspirates and fixed cytological smears, provided sufficient DNA quantity and quality for *EGFR* and *K-ras* mutation analysis. The frequencies of *K-ras* (36%) and *EGFR* (12%) mutations obtained by both direct sequencing and pyrosequencing techniques were similar to those reported previously (29). These percentages were obtained with fixed and stained cytological smears, in which tumor cell areas were accurately selected for DNA extraction. Slightly lower *K-ras* and *EGFR* mutation frequencies of 32 and 8%, respectively, were found in fresh fine needle aspirate samples. These results demonstrate that accurate selection and enrichment of tumor cells in samples is mandatory for accurate analysis. Moreover, although a comparative analysis between cytological and histological samples was performed in a small patient subgroup, results were concordant in all cases, confirming the reliability of molecular analysis on cytological specimens.

The main advantage of fresh material is that DNA is in optimal conditions, free from possible artifacts of the fixation and paraffin-embedded processes. This improves the efficiency of amplification, without the need for DNA purification or nested PCR reactions. The main limit is that in fresh material it is impossible to accurately estimate the tumor cell density, and this can lead to false negative results due to the lack of tumor cells compared to the other 'contaminating' cells. The ROSE analysis performed simultaneously on another sample from the same lymph node could give a guarantee on the presence of, but not on the fraction of tumor cells. In our study, the 2 false-negative results on fresh samples show a somewhat lower sensitivity of this approach compared to the fixed cytological smears, in which an accurate selection of tumor cells can be made.

In conclusion, we have demonstrated that EUS/FNA samples are suitable to define tumor *EGFR* and *K-ras* mutation profiles aimed at a personalized treatment, and that the accurate selection of tumoral cells is mandatory for an accurate analysis. To this purpose, fixed and stained samples are more reliable than fresh materials.

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