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EGFR and *K-ras* mutations in cytologic samples from fine-needle aspirates in NSCLC patients

To the Editors:

Mutations in the epidermal growth factor receptor (EGFR) gene in patients with nonsmall cell lung cancer (NSCLC) have been correlated with tumour response to treatment with targeted tyrosine kinase inhibitors (TKIs), especially gefitinib and erlotinib [1, 2]. A large number of studies have reported a significantly higher overall response rate, overall survival and time-to-progression in patients with EGFR-activating mutations compared with those with wild-type tumours [3–5].

It has thus become mandatory to perform *EGFR* mutation analysis in all adenocarcinoma patients to improve treatment opportunities.

In addition to *EGFR* alterations, *K-ras* mutations are found in ~30% of adenocarcinomas [6] and each is usually mutually exclusive. About 70% of NSCLC patients present at first diagnosis with advanced disease, and in such cases, morphologic and molecular diagnoses are necessarily based on cytologic samples obtained by different methodological procedures [7]. It has been demonstrated that *EGFR* mutations can be detected in cytologic specimens containing >50% of neoplastic cells, and that analysis can be performed in samples with as little as 25% tumour cellularity [8]. Moreover, although the most reliable results have been obtained in samples from which ⩾100 cells were analysed, it has also been noted that mutation analysis can be performed in as few as 30 isolated cells [9].

In the present study, 66 consecutive NSCLC patients diagnosed between December 2009 and December 2010 were analysed. Patients underwent bronchoscopy with Wang needle aspiration (19-, 21- and 22-gauge needles; ConMed Endoscopic Technologies, New York, NY, USA) when lung cancer was suspected. Specimens were fixed in Citofix (Bio-Optica, Milan, Italy) and then stained with Papanicolau (Bio-Optica) or Fast Quick Rapid (Diapath, Bergamo, Italy) for rapid, on-site cytologic evaluation. Quantitative and qualitative cell analyses were performed on stained slides. Cell blocks were prepared in 20 cases that had enough available material. Histologic samples were also available for 19 patients (12 obtained by biopsy and seven by lobectomy).

Both cytologic and histologic specimens were accurately selected before DNA extraction. For cytologic smears, non-tumour cells were eliminated using a scalpel under an optical microscope (2.5× magnification) or a magnifying glass, while the remaining material, comprising almost 90% tumour cells, was scraped off the slides and placed in a test tube. We used 10× and 2.5× magnification for the selection and quantification of small groups of cells, respectively. Cells were lysed in 50 mM KCl, 10 mM Tris-HCl pH 8.0, 2.5 mM MgCl2 and Tween-20 0.45% supplemented with proteinase K. DNA quality and quantity were assessed using Nanodrop (Celbio, Milan, Italy).

For paraffin-embedded samples, areas containing ⩾70% of tumour cells were identified on haematoxylin and eosin-stained tissue sections, and 5-μM sections of corresponding areas were macrodissected and collected in specific tubes. DNA extraction was performed as described for cytologic smears.

DNA extracted was subjected to PCR amplification for exon 2 of the *K-ras* gene and for exons 18, 19 and 21 of the *EGFR* gene. Primers for *K-ras* amplification were forward 5′- GGT GAG TTT GTA TTA AAA GGT ACT GG -3′ and reverse 5′- GGT CCT GCA CCA GTA ATA TGC-3′, while primers for *EGFR* analysis were as previously reported [2]. The PCR reaction product was purified using MiniElute PCR purification kit (Qiagen, Hilden, Germany) and then submitted to sequencing using BigDye Terminator 3.1 Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequence reaction was purified using DyeEx 2.0 Spin kit (Qiagen) and separated by capillary electrophoresis with laser-induced fluorescence detection (3100 Genetic Analyser; Applied Biosystems).

For pyrosequencing analysis, exon 2 of the *K-ras* gene and exons 18, 19 and 21 of the *EGFR* gene were analysed on PyroMark Q96 ID (Qiagen), using anti-EGFR MoAb response (*K-ras* status), and EGFR TKI response (sensitivity), respectively (Diatech, Jesi, Italy), according to the manufacturers' instructions.

Patient age ranged 36–88 yrs, with 40 patients <65 yrs and 26 patients ⩾65 yrs. 30 patients were females and 36 were males. 56 patients had adenocarcinoma, six had squamous cell carcinoma and four had poorly differentiated carcinoma. The new International Association for the Study of Lung Cancer (IASLC)/European Respiratory Society (ERS)/American Thoracic Society (ATS) classification was used for patients who underwent surgical treatment: five cases were classed as predominant with mucin production, one was classed as papillary predominant and one was classed as acinar predominant [10]. Four patients had stage I, 15 had stage II, and 18 had stage IIIa disease. 29 patients had inoperable lung cancer (stage IIIb or IV), according to TNM (tumour, node, metastasis) classification. 58 tumour samples were taken from primary tumours, four were taken from metastatic lymph nodes and four were from distant metastases (one hepatic lesion, two subcutaneous lesions and one submaxillary lymph node). Finally, 10 patients were never-smokers, 17 were former smokers and 39 were current smokers.

All cases were successfully screened for *EGFR* and *K-ras* mutations. Seven (12.5%) *EGFR* mutations were detected in adenocarcinoma, comprising four exon 19 deletions (delE746-A750), two exon 21 (L858R and L861Q) point mutations and one exon 18 (G719S) point mutation. The group comprised five females and two males of whom one was a current smoker, three were former smokers, and three had never smoked. Two patients had stage II, three had stage III and two had stage IV tumours. All mutations were also confirmed by pyrosequencing

TABLE 1 Characteristics of patients and *EGFR*/*K-ras* mutation status in cytologic smears, cell blocks and histologic material

Patient no.	Sex	Age yrs	Histotype	Stage	Smoker	*EGFR*			*K-ras*		
						CS	CB	HM	CS	CB	HM
1	F	75	ADC	I	Never	wt	wt		wt	wt	
2	F	84	ADC	IIIb	Never	wt	wt		G12C	G12C	
3	F	58	SCC	IIIb	Current	wt		wt#	wt		wt#
4	M	70	ADC	II	Current	wt	wt		wt	wt	
5	F	73	ADC	IIIa	Former	E746-A750	E746-A750		wt	wt	
6	M	68	ADC	II	Current	wt		wt¶	wt		wt¶
7	F	68	ADC	IIIa	Current	wt		wt#	G12C		G12C#
8	M	52	ADC	IIIa	Former	wt	wt		wt	wt	
9	M	78	ADC	II	Former	wt	wt	wt¶	wt	wt	wt¶
10	M	79	ADC	I	Current	wt		wt¶	wt		wt¶
11	F	80	ADC	II	Never	wt		wt#	wt		wt#
12	F	64	ADC	IV	Current	wt	wt		wt	wt	
13	F	65	ADC	II	Former	L858R		L858R¶	wt		wt¶
14	F	62	ADC	I	Current	wt		wt¶	wt		wt¶
15	M	71	ADC+	IIIb	Former	wt		wt#	G12D		G12D#
16	M	55	ADC	IV	Current	wt	wt	wt#	G12C	G12C	G12C#
17	M	82	ADC	I	Current	wt	wt		wt	wt	
18	F	81	ADC	II	Former	wt	wt		G12V	G12V	
19	F	55	ADC+	IV	Current	E746-A750	E746-A750		wt	wt	
20	M	56	ADC	II	Current	wt	wt	wt#	wt	wt	wt#
21	F	80	ADC+	IIIa	Never	E746-A750	E746-A750		wt	wt	
22	F	82	ADC	IIIa	Never	wt	wt	wt#	wt	wt	wt#
23	M	68	ADC	IIIb	Current	wt	wt	wt#	wt	wt	wt#
24	M	74	ADC	II	Current	wt	wt	wt#	G12C	G12C	G12C#
25	M	73	ADC	IV	Current	wt	wt		wt	wt	
26	F	77	ADC	II	Current	wt		wt#	wt		wt#
27	F	77	ADC	II	Current	wt		wt#	wt		wt#
28	F	77	ADC	IIIb	Former	wt	wt	wt#	wt	wt	wt#
29	F	75	ADC	II	Former	wt	wt	wt¶	wt	wt	wt¶
30	M	85	ADC+	IIIb	Former	wt	wt	wt#	wt	wt	wt#
31	F	87	ADC	IIIb	Never	wt	wt		wt	wt	
32	F	57	ADC	IIIb	Current	wt	wt		G12V	G12V	

CS: cytologic smear; CB: cell block; HM: histologic material; F: female; M: male; ADC: adenocarcinoma; SCC: squamous cell carcinoma; wt: wild-type. #: HM derived from biopsies; ¶: HM derived from lobectomies; +: derived from metastatic lymph node.

analysis. *K-ras* mutations were found in 14 (25%) cases of adenocarcinoma; 13 were detected by direct sequencing and the remaining mutation was only detected by pyrosequencing analysis. Detected mutations were G12C (eight cases), G12V (four cases), G12D (one case) and G12A (the case identified by pyrosequencing). This group comprised eight females and six males, of whom 11 were current smokers, two were former smokers and one had never smoked. Two had stage II, seven had stage III and five had stage IV disease. None of the patients showed both *EGFR* and *K-ras* mutations.

In five patients with gene mutations, two *EGFR* and three *K-ras*, we performed the analysis on a scalar number of cells isolated from cytologic samples by pyrosequencing (from 100 to 20 cells) to verify whether mutations could be detected in a small number of cells. *EGFR* and *K-ras* mutations were detectable in samples with as few as 20 cells. In 32 patients for whom cell blocks (20 samples) and/or histologic material (19 samples) were available, comparative analyses confirmed results obtained from cytologic smears (table 1).

In conclusion, we demonstrated that cytologic specimens from archival material are adequate for *EGFR* and *K-ras* molecular analyses and that results are concordant with those obtained from histologic material. We also showed that very few cells are required for mutation detection, thus enabling molecular analyses to be performed on patients for whom very little biological material is available, and leaving open the possibility of using different methodologies to analyse other potentially interesting molecular targets.

Paola Ulivi*, Wainer Zoli*, Elisa Chiadini*, Laura Capelli*, Piero Candoli#, Daniele Calistri*, Rosella Silvestrini* and Maurizio Puccetti¶

*Biosciences Laboratory, IRCCS Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST), Meldola, #Pneumology Unit, Lugo Hospital, Lugo, and ¶Pathology Unit, S. Maria delle Croci Hospital, Ravenna, Italy.

Correspondence: W. Zoli, IRCCS Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST), Via Maroncelli 40, 47014 Meldola, Italy. E-mail: w.zoli@irst.emr.it

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Ambulatory oxygen in idiopathic pulmonary fibrosis: of what benefit?

To the Editors:

We read with interest the Letter by VISCA *et al.* [1], recently published in the *European Respiratory Journal*, which described improvement in 6-min walk distance with ambulatory oxygen in patients with interstitial lung disease (ILD). We have looked specifically at the effects of ambulatory oxygen on walk distance in patients with idiopathic pulmonary fibrosis (IPF), and here describe a practical way of ensuring patients are prescribed an optimum flow rate of ambulatory oxygen.

Between 2004 and 2007, we conducted a retrospective review of anonymised data, studying the effect of ambulatory oxygen on the distance walked in patients with IPF in the ILD clinic of the University Hospital of South Manchester (Manchester, UK). 70 patients performed an adapted 6-min walk test (6MWT) on air or their usual flow rate of oxygen. If their oxygen saturation fell to <90%, the test was terminated and repeated with a 2 $L \cdot min^{-1}$ increase in oxygen flow rate. This continued until patients did not desaturate to <90% or reached a 6 $L \cdot min^{-1}$ flow rate. Diagnosis of IPF was based on the