EGFR and *KRAS* mutational analysis in a large series of Italian non-small cell lung cancer patients: 2,387 cases from a single center

RICCARDO GIANNINI[1*], CRISTIANA LUPI[2*], ELISA SENSI[2*], GRETA ALÌ[2], AGNESE PROIETTI[2], LAURA BOLDRINI[1,2], ADELE SERVADIO[1], MIRELLA GIORDANO[1], ELISABETTA MACEROLA[1], ROSSELLA BRUNO[1], NICLA BORRELLI[1], ANTONIO CHELLA[3], FRANCA MELFI[4], MARCO LUCCHI[1,4], ALESSANDRO RIBECHINI[5], ENRICO VASILE[6], FEDERICO CAPPUZZO[7], ALFREDO MUSSI[1,4] and GABRIELLA FONTANINI[1,2]

[1]Department of Surgical, Medical, Molecular Pathology and Critical Area, University of Pisa, Pisa; Units of [2]Anatomic Pathology 3, [3]Pneumology, [4]Thoracic Surgery, [5]Thoracic Endoscopy and [6]Medical Oncology 2, Azienda Ospedaliero-Universitaria Pisana, Pisa; [7]Medical Oncology Department, Istituto Toscano Tumori, Ospedale Civile, Livorno, Italy

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Abstract. Activating *EGFR* mutations are important genetic alterations that have strong therapeutic implications for non-small cell lung cancer (NSCLC) patients. However, the role of *KRAS* mutations in this process is still under evaluation. Here, we report on the feasibility of a large-scale *EGFR* and *KRAS* mutation analysis in the daily routine of a single center. NSCLCs from 2,387 patients were screened for *EGFR* and *KRAS* mutations from January 2010 to September 2015. Mutational analyses were performed in a single laboratory using single strand conformation polymorphism (SSCP)-Sanger sequencing and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) on Sequenom platform for *EGFR* and pyrosequencing for *KRAS*. Activating *EGFR* mutations were found in 14.1% of all tumors, whereas *KRAS* mutations were found in 30.5% of all tumors. Direct sequencing showed analyzable cytological, small biopsy and surgical specimen percentages of 90.3, 90.9 and 98.1%, respectively, whereas the MALDI-TOF platform showed analyzable cytological samples, small biopsies and surgical specimens percentages of 94.6, 95.7 and 96.9%, respectively. The mean analytical turnaround times (TAT) were 4 and 3 days for direct sequencing and the MALDI-TOF platform, respectively. Our results confirm that small biopsy or cytological samples can be used for reliable *EGFR* and *KRAS* mutation testing and indicate that adopting the MALDI-TOF platform reduces the rate of missed samples among the samples. Moreover, the 3-day analytical TAT of the MALDI-TOF multi-target technique is appropriate for clinical management and reduces the overall treatment decision time.

Correspondence to: Professor Gabriella Fontanini, Department of Surgical, Medical, Molecular Pathology and Critical Area, University of Pisa, Via Roma 57, I-56126 Pisa, Italy
E-mail: gabriella.fontanini@med.unipi.it

[*]Contributed equally

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Introduction

EGFR molecular profiling predicts non-small cell lung cancer (NSCLC) patients' responsiveness to tyrosine kinase inhibitors (TKIs), such as erlotinib and gefitinib (reversible TKI *EGFR*) and afatinib (irreversible TKI *EGFR*) (1,2). *EGFR* mutational analyses are performed exclusively in patients with adenocarcinoma (ADC) or adenosquamous carcinoma (ADCSCC) because of their higher rates of *EGFR* gene mutations relative to other NSCLC types (3).

Caucasian NSCLC patients experience *EGFR* mutational rates ranging from 10 to 15.7%. The most representative *EGFR* mutations are the exon 19 deletions and the exon 21 L858R point mutations, which account for ~90% of cases (1,4). Differences in the reported incidence of *EGFR* mutations could be related to patient selection and the use of different methodologies to test for *EGFR*. Direct Sanger sequencing requires a proportion of >40-50% of tumor cells, whereas pyrosequencing and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) (5) using Myriapod Lung Status CE-IVD kits (Diatech Pharmacogenetics, Jesi, Italy) require as few as 20% tumor cells. Moreover, the DNA quantity and quality affect *EGFR* mutational analyses and may cause a proportion of cases to be missed (6,7).

The majority of recent lung cancer diagnoses have been based on small biopsies or cytological smears of patients who cannot undergo surgery because of advanced disease. Thus, small biopsy and cytosmear samples often represent the only source of NSCLC tumor cell DNA for molecular characterization. However, the validity of *EGFR* testing on small biopsies and cytological smears remains debatable (4,8,9).

KRAS mutations that are located mainly in codons 12 and 13 of exon 2 have been reported in up to 30.0% of NSCLC patients (10,11). Although the role of *KRAS* mutations as predictive markers of treatment response in NSCLCs is still under debate, TKI administration has recently been shown to have potentially detrimental effects on patients with *KRAS* mutations (12). Moreover, the clinical value of *KRAS* mutation testing may increase if the development of a MEK inhibitor in NSCLCs with *KRAS* mutations leads to drug approval (5).

The first aim of this study was to analyze a large consecutive and homogeneous series of Italian NSCLCs to determine the incidence of *EGFR* and *KRAS* mutations over the last 5 years. This study also aimed to compare two different routinely used testing methods and investigate the viability of a multi-target methodology for use in clinical practice by considering its feasibility for different specimen types (e.g., cytological, small biopsy and surgical samples). We also evaluated the effect of analytical turnaround time (TAT) on the success rate and clinical utility of a multi-target analysis in routine clinical care.

Materials and methods

Patients. Samples from 2,387 NSCLC patients between January 2010 and September 2015 were analyzed at the Molecular Pathology Laboratory of the Unit of Anatomic Pathology 3 of the Azienda Ospedaliero-Universitaria Pisana to determine their *EGFR* mutational status. The majority of patients were diagnosed and treated in northern Tuscany's Oncology Departments (Italy). The tumor samples were obtained from the respective Pathological Anatomy Departments. The oncologists required *EGFR* mutational testing based on the individual clinical situation of each patient, and the majority of patients were also recommended to undergo *KRAS* mutational testing.

Each sample was accompanied by a histological diagnosis performed by hematoxylin and eosin (HE) staining for FFPE sections and Papanicolaou staining for cytological smears. Clinical pathological information concerning gender and age were available for all patients. Informed consent was collected by the oncologist upon each patient's first visit.

DNA purification and mutation detection. DNA was extracted from the FFPE and cytological smears using a commercial kit (Qiagen, Milan, Italy) following the manufacturer's instructions.

The status of *EGFR* exons 18-21 from January 2010 to February 2013 was analyzed by the SSCP-Sanger sequencing method. The MALDI-TOF method was used on a Sequenom (Agena Bioscience, San Diego, CA, USA) platform. The SSCP-Sanger analysis [performed as previously described by Rotella *et al* (12)] revealed that all of the mutations were detected with a sensitivity of ~10% of the mutated allele. The MALDI-TOF dedicated Myriapod Lung Status CE-IVD kit (Diatech Pharmacogenetics) detects more representative *EGFR* mutations and has a sensitivity ranging from 2.5 to 10%. This kit can also simultaneously analyze *EGFR*, *KRAS*, BRAF, NRAS, PIK3CA, ALK, ERBB2, DDR2, RET and MAP2K1 mutations.

When required, the mutational status of KRAS codons 12 and 13 from 2010 to February 2013 was analyzed with a pyrosequencing Anti-EGFR MoAb response® (*KRAS* status) CE-IVD marked kit (Diatech Pharmacogenetics) following the manufacturer's instructions. This step showed a sensitivity of 5-10% of mutated alleles. The pyrosequencing method was replaced by the method using MALDI-TOF dedicated Myriapod Lung Status CE-IVD kits (Diatech Pharmacogenetics) in March 2013. Fig. 1 shows the operative flow chart for the tests described above. Cell/tissue sample enrichment was performed to ensure the highest tumor content. The SSCP-Sanger method required a tumor cell representativeness of >20%, whereas pyrosequencing and the Myriapod Lung Status CE-IVD kits each required tumor cell representativeness of >10%.

Evaluation of the analytical TAT. The analytical TAT is the mean time from sample receipt to results interpretation. This value was recorded for each sample according to the different methods of analysis and then compared.

Statistical analyses. Patients were classified as mutated or wild-type based on the presence of *EGFR* and *KRAS* mutations. The quality and quantity of the material were measured by the total number or percentage of neoplastic cells to determine whether the samples could be analyzed.

Differences in the variables between the two groups and their association with the clinical data were tested using Fisher's exact test or a two-sided Chi-square test. A p-value of 0.05 or less was considered significant. All of the statistical analyses were performed with STATISTICA software (Dell Software, Tulsa, OK, USA).

Results

Clinicopathological characteristics. Our analysis revealed that 1993 (83.5%) patients presented lung adenocarcinoma, 190 (8.0%) presented squamous cell carcinoma (SCC) and 204 (8.5%) presented not otherwise specified NSCLC (NSCLC-NOS). In addition, 1,539 patients were males (64.5%) and 848 (35.5%) were females, and the mean age of the entire series was 68.1 years (range, 25-91).

Molecular testing adequacy. Table I shows the overall efficiency of the *EGFR* molecular tests according to the method of analysis and type of material. The SSCP-Sanger method showed analyzable cytological, bioptic and surgical specimen percentages of 90.3, 90.9 and 98.1%, respectively, whereas the MALDI-TOF platform showed analyzable cytological, bioptic and surgical specimen percentages of 94.6, 95.7 and 96.9%, respectively. The MALDI-TOF platform showed a reduction in the percentage of non-analyzable cases because of the low amount of input DNA needed. A significant increase (p=0.03) of analyzable samples was observed.

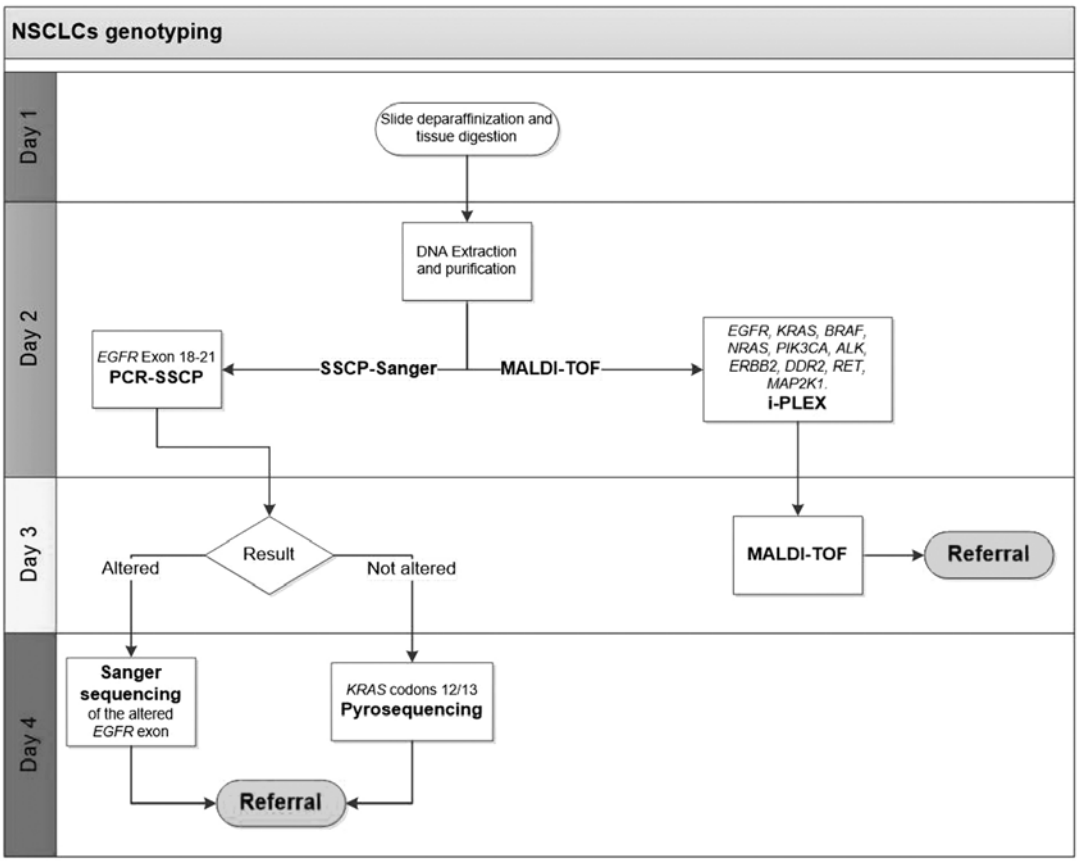

Figure 1. Flow chart of activity related to both analytical methods.

Table I. Overall efficiency of molecular testing.

			Not analyzable cases		Analyzable cases	
Type of material	Method	Total	NE n (%)	NA n (%)	n (%)	P-value
Cytology	S-S	425	37 (8.7)	4 (0.9)	384 (90.3)	0.012
	M-T	554	29 (5.2)	1 (-)	524 (94.6)	
Biopsy	S-S	265	18 (6.8)	6 (2.2)	241 (90.9)	0.026
	M-T	304	10 (3.3)	3 (0.9)	291 (95.7)	
Surgical specimen	S-S	421	1 (-)	7 (1.6)	413 (98.1)	0.6
	M-T	357	3 (0.8)	8 (2.2)	346 (96.9)	
Total	S-S	1111	56 (5.0)	17 (1.5)	1038 (93.5)	0.030
	M-T	1215	42 (3.4)	12 (1.0)	1161 (95.6)	

P-value, Chi-square (χ^2) test. S-S, SSCP-Sanger; M-T, MALDI-TOF; NE, not enough DNA; NA, non-amplifiable DNA.

EGFR mutational status. *EGFR* exon 18-21 mutations were found in 311 of 2,199 (14.1%) cases, and a histological analysis showed that 290 (15.8%) were ADCs, 15 (8.2%) were NSCLCs-NOS and 6 (3.3%) were SCCs.

Table II summarizes the *EGFR* mutational analysis results with regard to the method and type of analyzed material. The *EGFR* mutational ratios were 14.5 and 13.8% for the SSCP-Sanger and MALDI-TOF platforms, respectively. The MALDI-TOF platform and SSCP-Sanger method showed ratios of mutated samples for surgical, small biopsy and cytological samples of 12.0, 16.2 and 17.1%, respectively, and 15.3, 11.4 and20.8%, respectively.

Table III shows the different types of *EGFR* mutations according to the method of analysis. For the ADCs, the number of *EGFR* exon 18, 19, 20 and 21 mutations were 6, 71, 21 and 70 with the MALDI-TOF platform, respectively, and

Table II. Comparison of the *EGFR* mutational rate according to the analytical method and sampling type.

		ADCs		SCCs		NSCLCs-NOS		Total	
Type of material	Method	Total	MUT n (%)	Total	MUT n (%)	Total	MUT n (%)	Total	MUT n (%)
Cytology	S-S	298	62 (20.8)	32	1 (3.1)	54	8 (14.8)	384	71 (18.5)
	M-T	457	78 (17.1)	20	2 (10.0)	47	3 (6.4)	524	83 (15.8)
Biopsy	S-S	175	20 (11.4)	29	2 (6.9)	37	3 (8.1)	241	25 (10.4)
	M-T	229	37 (16.2)	28	0	34	1 (2.9)	291	38 (13.1)
Surgical specimen	S-S	352	54 (15.3)	54	1 (1.9)	7	0	413	55 (13.3)
	M-T	324	39 (12.0)	19	0	3	0	346	39 (11.3)
Total	S-S	825	136 (16.5)	115	4 (3.5)	98	11 (11.2)	1038	151 (14.5)
	M-T	1010	154 (15.2)	67	2 (3.0)	84	4 (4.8)	1161	160 (13.8)

S-S, SSCP-Sanger; M-T, MALDI-TOF; MUT, mutated.

Table III. Overall *EGFR* mutations revealed by SSCP-Sanger sequencing and MALDI-TOF.

	ADCs		NSCLCs NOS		SCCs		Total	
Exon/mutation	S-S	M-T	S-S	M-T	S-S	M-T	S-S	M-T
Exon 18								
E709A	1						1	
G719A	4	2					4	2
G719C	2						2	
G719S		1						1
DEL/INS	1						1	
E709A+G719A			1				1	
E709A+G719S	1						1	
E709K+G719S	1						1	
Exon 19								
L747P	1						1	
DEL/INS	81	71	7	2	3	2	91	75
Exon 20								
S768I		2						2
T790M[a]	7	11				1	7	12
Insertion	6	6					6	6
Exon 21								
V834L			1				1	
H835L					1		1	
P848L		1						1
L858R	35	64	2	2			37	66
L861Q	2	4					2	4
Other								
E709K+L858R		1						1
G719A+L858R	1						1	
G719C+S768I		1						1
G719S+S768I		1						1

[a]All the T790M mutations are associated to an exon 19 deletion or to an exon 21 L858R mutations. S-S, SSCP-Sanger; M-T, MALDI-TOF.

Table IV. *EGFR* exon 19 deletions/insertions revealed by SSCP-Sanger sequencing and MALDI-TOF.

	S-S	M-T
EGFR exon 19 DELETIONS/INSERTIONS	77	63
p.E746_E749>T (c.2236_2246>TAC)	1	1
p.E746_A750delELREA (c.2235-2249del15)	33	30
p.E746_A750delELREA (c.2236-2250del15)	21	17
p.E746_A750>NP (c.2235_2248>TC)	1	
p.E746_T751>A (c.2237_2251del15)	1	1
p.E746_T751>Q (c.2236_2253>CAA)	1	
p.E746_T751>S (c.2235_2252>ATT)	1	
p.E746_S752>I (c.2236_2256>ATC	1	
p.E746_S752>V (c.2237_2255>T)	9	9
p.E746_P753>VS (c.2237_2257>TCT)	1	
p.E746_A750>VP (c.2237_2248>TAC)	1	
p.L747_E749delLRE (c.2239_2247del9)	3	
p.L747_A750>P (c.2239_2248>C)	1	
p.L747_T751delLREAT (c.2240_2254del15)	5	
p.L747_T751>P (c.2239_2251>C)	1	
p.L747_S752delLREATS (c.2239_2256del18)	1	
p.L747_P753>S (c.2240_2257del18)	7	
p.L747_A755>AN (c.2239_2264>GCCAA)	1	
p.S752_I759delSPKANKEI (c.2254_2277del24)	1	
p.L747_P753>S/p.L747_T751delLREAT		8
p.L747_A750>P/p.L747_S752		4
p.E746_E749 delELRE/p.K745_E746insIPVAIK		1
p.E746_A750>QP/p.E746_S752		1
p.E746_T751>VA/E746_T751>V		1
p.K745_E746insIPVAIK		2

S-S, SSCP-Sanger; M-T, MALDI-TOF.

11, 82, 13 and 38 with the SSCP-Sanger method, respectively. Table IV shows the *EGFR* exon 19 deletions and insertions

Table V. Comparison of *KRAS* mutational rates according to the analytical methods and sampling type.

		ADCs		SCCs		NSCLC-NOS		Total	
Type of material	Method	Total	MUT n (%)	Total	MUT n (%)	Total	MUT n (%)	Total	MUT n (%)
Cytology	PYRO	193	56 (29.0)	26	3 (11.5)	42	11 (26.2)	261	70 (26.8)
	M-T	444	145 (32.7)	20	1 (5.0)	47	13 (27.7)	511	159 (31.1)
Biopsy	PYRO	139	40 (28.8)	22	4 (18.2)	30	11 (36.7)	191	55 (28.8)
	M-T	218	69 (31.7)	28	2 (7.1)	34	7 (20.6)	280	78 (27.9)
Surgical specimen	PYRO	281	96 (34.2)	40	2 (5.0)	7	1 (14.3)	328	99 (30.2)
	M-T	322	122 (37.9)	19	0	3	1 (33.3)	344	123 (35.8)
Total	PYRO	613	192 (31.3)	88	9 (10.2)	79	23 (29.1)	780	224 (28.7)
	M-T	984	336 (34.1)	67	3 (4.5)	84	21 (25.0)	1135	360 (31.7)

PYRO, pyrosequencing; M-T, MALDI-TOF; MUT, mutated.

according to the SSCP-Sanger and MALDI-TOF methods. Two simultaneous mutations of the *EGFR* gene were found in 23 patients, and the majority of double mutations were represented by T790M exon 20 mutations concomitant to exon 19 deletion (11 samples) and T790M mutation concomitant to L858R exon 21 mutation (7 cases).

Among the 6 SCCs with *EGFR* alterations, we found 4 exon 19 deletions, 1 H835L exon 21 mutation and 1 T790M mutation concomitant with exon 19 deletion.

Of the 15 NSCLCs-NOS *EGFR* mutated cases, we found 9 exon 19 deletions, 5 exon 21 mutations (4 L858R and 1 V834L) and 1 exon 18 mutation in two different codons (E709A and G719A).

EGFR mutations versus gender and age. EGFR mutations were found in 193 (24.5%) of 786 female patients and 118 (8.3%) of 1,412 male patients. A significant correlation ($p<0.0001$) was found between *EGFR* mutations and female patients. The mean age of the patients with mutation was 67.9 years, which was identical to the mean age of the patients without mutation. However, the *EGFR* mutational rate was significantly higher ($p=0.02$) in female patients 65 years and over (28.6%; 132 of 462) compared with younger women (18.9%; 60 of 316). Age-dependent differences were not observed for the male *EGFR* mutation rates.

KRAS status. Within the *KRAS* codons, 12/13 mutations were found in 584 (30.5%) lung carcinomas, including 528 of 1597 (33.0%) ADCs, 44 of 163 (26.9%) NSCLCs-NOS and 12 of 155 (7.7%) SCCs.

Table V summarizes the results of the *KRAS* mutational analysis in ADCs according to the method and type of analyzed material. The KRAS mutational ratios by pyrosequencing and the MALDI-TOF platform were 28.7 and 31.7%, respectively. The MALDI-TOF platform demonstrated that in the ADCs, mutated samples occurred in 122 of 322 (37.9%) surgical samples, 69 of 218 (31.7%) small biopsies and 145 of 444 (32.7%) cytological specimens, whereas pyrosequencing demonstrated that mutated samples occurred in 96 of 281 (34.2%) surgical samples, 40 of 139 (28.8%) small biopsies and 56 of 193 (29.0%) cytological specimens. MALDI-TOF indicated that there were 27 exon 3 codon 61 mutations, including 19 p.Q61H, 7 p.Q61L and 1 p.Q61R.

KRAS mutations versus gender and age. KRAS mutations were found in 173 (25.2%) of 686 female patients and 412 (33.5%) of 1,229 male patients. A significant correlation ($p=0.0002$) was observed between the *KRAS* mutations and male patients. Significant correlations were not observed between the mutated and not-mutated samples according to patient age ($p=0.21$).

Other gene mutations. The Myriapod Lung Status CE-IVD kit (Diatech Pharmacogenetics) analysis revealed 4 NRAS gene mutations (1 p.G12D, 2 p.Q61L and 1 p.Q61K), 26 BRAF gene mutations (2 p.G466A, 1 p.G466E, 2 p.G466V, 1 p.D594G and 20 p.V600E), 5 ERBB2 gene mutations (p.A775 G776insYVMA), 16 PIK3CA gene mutations (2 p.E542K, 6 p.E545K and 8 p.H1047R), 1 ALK gene mutation (p.C1156K) and 1 MAPK2K1 gene mutation (p.Q56P).

Analytical TAT. The mean TAT for the SSCP-Sanger/pyrosequencing analyses was four working days (Fig. 1). Overall, the throughput of this protocol was limited by the number of analyzable samples from the SSCP and Sanger sequencing, and only 5 patients could be simultaneously tested because of limitations of the SSCP precasting gel. The MALDI-TOF platform produced a mean TAT for the simultaneous *EGFR* and *KRAS* analyses of three working days (Fig. 1), and 10 patients could be simultaneously analyzed. At least one additional day was required for cytological smear demounting.

Discussion

To the best of our knowledge, this is one of the larger studies to have performed an *EGFR* mutational analysis in a

homogeneous series of patients with metastatic NSCLCs from a single center. These results directly reflect the daily *EGFR* testing routine.

Our study presents an appropriate assessment of the epidemiologic and methodological information related to *EGFR* mutational testing of metastatic NSCLC patients in a clinical setting.

A total of 2,387 patients from Northern Tuscany (Italy) with metastatic lung cancer were analyzed, and they yielded an overall *EGFR* mutational rate of 14.1%. These data are consistent with that of previous reports for Caucasian patients (1,2,10,11,13-16). The predominant *EGFR* alteration was the exon 19 deletion, which was followed by the exon 21 L858R point mutation (13,17). The *EGFR* mutation rate was significantly higher in ADC patients (15.8%) than in NSCLC-NOS (8.2%) and SCC (3.3%) patients (3,18-20). The strong association between EGFR mutations and female patients was confirmed in this study. Moreover, a significant association between *EGFR* mutations and older age (≥65 years) in female patients was observed. This finding supports that of Gahr *et al* (13).

In our 5 years of experience with daily *EGFR* analyses, we have changed our method of analysis for *EGFR* and KRAS from the Sanger sequencing and pyrosequencing methods to the Sequenom multi-marker MALDI-TOF platform.

Recent advances in multiplex genotyping and high throughput genomic profiling by multi-marker sequencing offer the possibility of rapidly and comprehensively interrogating individual patient cancer genomes from small tumor biopsies and cytological samples. Particular emphasis can be placed on daily molecular diagnoses of lung cancers. Our experience with the two different methodologies in a large series of NSCLCs has helped emphasize certain important factors in genotyping and genomic profiling.

The overall rates of *EGFR* and *KRAS* mutations were not significantly changed after the adoption of a multi-target technique, although the MALDI-TOF platform nearly doubled the rate of detection of the L858R mutation.

The number of failed analyses because of low quantity or damaged DNA and reaction inhibition significantly decreased within cytological samples and small biopsies. Assessing the status of multiple genes requires a small amount (as low as 40 ng) of DNA template; however, this amount is crucial for performing reliable *EGFR* mutation analyses of cytological samples and small biopsies, and these samples are often the only material available to establish a diagnosis and perform a molecular analysis.

The adoption of the MALDI-TOF platform reduced the mean analytical TAT, which has important implications for the management and treatment of patients.

MALDI-TOF testing revealed an insignificant increase in the *KRAS* mutational rate. Furthermore, the strong association between *KRAS* mutations and male patients and the mutual exclusivity of *EGFR* and *KRAS* gene mutations were confirmed (21).

The Myriapod Lung Status CE-IVD kit revealed several mutations in *KRAS* at exon 3 as well as in *NRAS*, *BRAF*, *PIK3CA*, *ERBB2*, *ALK* and *MAPK2K1*. This finding suggests that a more comprehensive approach to predictive biomarker analysis is needed. The ability to simultaneously test several relevant genes may be beneficial to patients because of the potential to identify alternative treatment options.

In conclusion, although, this study brings nothing new to the field of NSCLC mutational screening, our results underline important concepts from a methodological point of view, first of all it confirms that small biopsy or cytological samples are adequate for multiple mutational testing of NSCLCs in a large series of cases. *EGFR* mutations are detectable with a similar frequency in the surgical, small biopsy and cytological samples by using the SSCP-Sanger or MALDI-TOF platforms, even if the MALDI-TOF method reduces the rate of missed samples when the DNA quality and quantity is low in the small biopsy and cytological samples. Moreover, this method is also able to detect a wider range of mutations using a small amount of DNA. Furthermore, the MALDI-TOF platform allows for the rapid implementation and application of newly identified biomarkers for target therapies and does not negatively affect the time and cost effectiveness of the analytical procedure.

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