Epidermal growth factor receptor and K-RAS mutations in 411 lung adenocarcinoma: A population-based prospective study

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Abstract. Targeting the epidermal growth factor receptor has played a central role in advanced non-small cell lung cancer research, treatment, and patient outcomes over the last several years; however, a number of questions about this approach remain to be addressed. Through the Istituto Toscano Tumori and the Italian Association of Women Against Lung Cancer Project, we collected 411 lung adenocarcinomas from several clinical centers in Tuscany. Mutations were assessed by sequencing exons 18-21 of the epidermal growth factor receptor gene, and by restriction fragment length polymorphism analysis of codons 12 and 13 of the K-RAS gene. Epidermal growth factor receptor mutations (12.6%) were more frequently observed in females (p<0.0001), in non-smokers (p=0.005), and in the presence of bronchioloalveolar features (p=0.0004). K-RAS mutations (17.9%) were more frequent in males (p=0.0007) and were associated with smoking habits (p=0.005). Epidermal growth factor receptor and K-RAS mutations were mutually exclusive (p=0.001). We focused on 21 female patients with advanced/metastatic lung adenocarcinoma who received gefitinib 250 mg/day (expanded access) or erlotinib 150 mg/die as second/third-line therapy; partial response was associated with classic epidermal growth factor receptor mutations (p=0.006) and with a non-smoking history (p=0.02). None of the female patients with partial response and/or stable disease showed K-RAS alterations. Although the data obtained in our study have yet to be analyzed and confirmed with a larger number of patients treated with tyrosine kinase inhibitors, they should provide useful information for targeted therapy, in particular for non-smoking female lung cancer patients.

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Introduction

Targeting the epidermal growth factor receptor (EGFR) has played a central role in advanced non-small cell lung cancer (NSCLC) research, treatment, and patient outcomes over the last several years. Recent findings have shown that mutations in the tyrosine kinase domain of the EGFR gene are significantly associated with a high sensitivity to tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib (1-3). Several clinicopathologic factors have been identified as being related to the frequency of EGFR mutations, including adenocarcinoma histology, female gender, non-smoking status, and East Asian ethnicity (4).

In spite of the strong association between EGFR genotype and TKI sensitivity, EGFR somatic mutations are neither sufficient nor necessary for a drug response (5). Studies have demonstrated that EGFR somatic mutations are found in only 81% of lung cancer patients who are both treated with gefitinib (or erlotinib) and experience partial responses or significant clinical improvement; furthermore, not all patients sensitive to gefitinib have EGFR mutations. These findings suggest that gender, smoking status, or interactions between these factors influence the EGFR mutational spectrum in an exon-specific manner in NSCLC cases.

Although a great deal has been learned since the initial discovery of EGFR mutations in lung cancer, a number of fundamental questions remain to be addressed. Different mutations in EGFR may confer different tumor activation profiles that lead to variations in both the natural history and clinical course of NSCLC after treatment with erlotinib or gefitinib. The clinical impact of EGFR-TKIs on patients with different mutations seems to depend on the specific EGFR mutation present (6-8). Moreover, mutations at other points of the EGFR pathway have been identified; in particular, K-RAS mutations are frequently found in lung adenocarcinomas (15-50%) (9). Most of these mutations are observed at codon 12 (GGT) or 13 (GGC) in exon 2, and the most frequent type of mutation involves a G to A transition or G to T transversion at the second position of codon 12. Several studies report that lung tumors with K-RAS mutations are resistant to EGFR-TKIs (10,11) but so far, these findings

have provided insufficient reason to prevent patients with K-RAS mutations from receiving EGFR-TKI therapy, as they may still derive clinical benefit from the treatment.

The lung cancer incidence recorded by the Tuscan Cancer Registry (RTT) was reported to be 2190 cases (1688 men, 77%, and 502 women, 23%) during a 1-year period (September 2006-September 2007); in women, the incidence of primary lung cancer is still increasing. The adenocarcinoma histotype accounts for 50% of the cases, with a high percentage in women. In this study, we carried out mutational profiling of 411 lung adenocarcinomas, assessing mutations in exons 18 to 21 of the EGFR gene and in codons 12 and 13 of the K-RAS gene. Moreover, we focused on 46 female patients in our series with advanced/metastatic lung adenocarcinoma, and analyzed their smoking history and clinical responsiveness to TKIs in relation to their EGFR and K-RAS mutational status.

Materials and methods

Sample collection and processing. Four hundred and eleven cytological/histological specimens of lung adenocarcinoma were consecutively collected at the Pathological Anatomy section of the Surgical Department of Pisa University from September 1, 2006 to September 1, 2007. The samples were provided by the following Pathological Anatomy Centers of Tuscany, Italy: U.O. Anatomia Patologica III AOU Pisa; U.O. Anatomia Patologica AOU Firenze; U.O. Citopatologia CSPO Firenze; Anatomia Patologica Spedali Riuniti Pistoia; U.O. Anatomia Patologica USL12 Viareggio; U.O. Anatomia Patologica USL6 Livorno; U.O. Anatomia Patologica USL1 Carrara.

Informed consent was obtained from individual patients for tissue collection and gene analyses for research purposes, in accordance with the protocol reviewed and approved by each center's Institutional Review Board.

Slides of all specimens were incubated in xylene overnight to remove the coverslip and paraffin, then washed in 100% and 70% ethanol. The cells were microdissected using a 25-gauge needle. While viewing the tissue through the microscope, the cell population of interest was gently scraped with the microdissecting needle. The tip of the needle with the procured tissue fragments was then placed into a small PCR tube containing the appropriate buffer. The tube was shaken gently to ensure detachment of the tissue from the tip of the needle.

Patients and tumor characteristics. The pathologic features (histologic subtype, grade, and post-surgical staging) of each tumor were evaluated according to the WHO 2004 histologic criteria (12) and the International Union Against Cancer tumor-node-metastasis classification (13) in 235 out of 411 tumors. In these cases, surgical resection was performed, and the tumor samples were suitable for post-surgical evaluation. Of the 235 patients, 184 patients (78.3%) had a lobectomy, 46 (19.6%) patients had a wedge resection, and the remaining 5 patients (2.1%) had a pneumonectomy. Classifying the 235 patients by tumor status, there were 69 T1 (29.3%), 104 T2 (44.3%), 26 T3 (11.1%), and 36 T4 (15.3%). Thirty-two patients were classified as N1 (13.6%), 71 as N2 (30.2%),

Table I. Clinicopathological features and EGFR and K-RAS mutational status of patients with lung adenocarcinoma.

Clinicopathological characteristics	No. of cases (%)
Age (years) (n=411)	
Range 37-88	
Average 65.7	
Gender (n=411)	
Male	235 (57.2)
Female	176 (42.8)
Smoking status (n=217)	
Current	110 (50.7)
Former	55 (25.3)
Never	52 (24.0)
EGFR status (n=411)	
Wild-type	359 (87.4)
Mutated	52 (12.6)
K-RAS status (n=374)	
Wild-type	307 (82.1)
Mutated	67 (17.9)

and 10 as Nx (4.3%), while 122 (51.9%) did not show metastatic nodal involvement at the time of diagnosis. The different histologic subtypes/patterns of adenocarcinoma were documented as follows: mixed (118/235, 50.2%), acinar (44/235, 18.7%), bronchioloalveolar mucinous/non-mucinous (14.5%, 3/235 and 31/235, respectively), papillar (16/235, 6.8%), solid (12/235, 5.1%), and mucinous (11/235, 4.7%).

Particular attention was paid to distinguishing tumors with bronchioloalveolar features (51/235, 21.7%, 34 single pattern and 17 mixed pattern) from those without. In 176 of the 411 tumor samples (82 histological and 94 cytological samples) the material was insufficient (or inadequate, in the cases of biopsy and cytological samples) for performing a complete characterization according to the different subtypes of adenocarcinoma.

Regarding gender, there were 235 males (57.2%) and 176 females (42.8%), with age at diagnosis ranging from 37 to 88 years (mean age 65.7 years, median 66 years). Smoking habits were known for 217 of the 411 patients: there were 52 non-smokers (24%, 14 men and 38 women), 55 former smokers (25.3%, 40 men and 15 women), and 110 current smokers (50.7%, 73 men and 37 women) (Table I).

Female patients with advanced disease. To ascertain the degree to which, in women, mutations in the EGFR tyrosine kinase domain and in the K-RAS codons 12-13 are predictive of an effective response to TKIs, we focused our attention on 46 women in our series with histologically and cytologically proven stage IV adenocarcinoma of the lung. Twenty-one patients were treated with gefitinib 250 mg/day (expanded access) or erlotinib 150 mg/die as second/third-line therapy. Single agent gefitinib 250 mg/day (expanded access) or erlotinib 150 mg/die (according to indication) were admi-

Table II. Epidermal growth factor receptor (EGFR) mutation in 411 samples of lung adenocarcinoma.

EGFR Exon	Type of mutation	Nucleotide number and sequence (5'-3')	Amino acid	No. of mutation (%)
				52 (12.6)
18	Single-nucleotide substitution	2156 G→C	G719A	1 (0.24)
19	In-frame deletion	2235-2249 del GAATTAAGAGAAGC	E746-A750 del	21 (5.1)
		2237-2254 del AATTAAGAGAAGCAACAT	E746-S752 del	2 (0.48)
		2254-2277 del TCTCCGAAAGCCAACAAGGAAATC	S752-I759 del	1 (0.24)
		2252-2276 del CATCTCCGAAAGCCAACAAGGAAAT	T751-I759 del	2 (0.48)
	Deletion	2239-2256 del TTAAGAGAAGCAACATCT	I747-S752 del	1 (0.24)
20	Single-nucleotide substitution	2339 T→G	I780S	1 (0.24)
		2353 A→C	T785P	1 (0.24)
		2387 G→A	G796D	3 (0.72)
		2387 G→C	G796A	1 (0.24)
	Insertion	2403 ins G	Y801 stop	1 (0.24)
21	Single-nucleotide substitution	2507 G→A	R836H	1 (0.24)
		2563 G→A	D855N	1 (0.24)
		2573 T→G	L858R	14 (3.4)
	Nucleotide substitution	2573-4 TG→GA	L858R	1 (0.24)

nistered orally until the onset of progression, unacceptable toxicity, or refusal. Restaging occurred every two months. Radiographic tumor responses to treatment were evaluated according to the Response Evaluation Criteria for Solid Tumors (14). Age at diagnosis ranged from 40 to 86 years (mean age 60.8 years, median 58 years). Histology demonstrated bronchioloalveolar features in 7 out of 46 samples (15.2%). Smoking habits were known for all of them: there were 29 non-smokers (63%), 3 former smokers (6.5%), and 14 current smokers (30.5%). Twenty-six women (26/46, 56.5%) demonstrated 2 different metastatic sites, and the other 20 more than two sites; the dominant metastatic sites were in the lungs (18/46, 39.1%), bone (13/46, 28.3%), central nervous system (8/46, 17.4%), and liver (7/46, 15.2%).

DNA extraction. Genomic DNA was isolated from cytological specimens and histological tissues by a standard method. Paraffin was removed by xylene extraction, and the sample was subsequently lysed by proteinase K. DNA extraction was then performed using a spin column procedure (QIAamp Tissue kit, Qiagen).

Mutational profiling of EGFR and K-RAS. Mutational profiling of EGFR (exons 18-21) and K-RAS (codons 12 and 13) was performed as previously reported (15). Briefly, the eluted DNA was used as template in a standard 20 µl PCR reaction mixture. The PCR product sizes for EGFR exons 18, 19, 20, and 21 were 207, 194, 247, and 235 bp, respectively.

Because both primers had similar melting temperatures, the same PCR conditions were used to simultaneously amplify the four exons (in separate reaction tubes). The PCR conditions to amplify EGFR exons were initial denaturation at 94˚C (7 min) followed by 35 cycles of denaturation at 94˚C for 60 sec, annealing at 58˚C for 60 sec, and synthesis at 72˚C for 60 sec, followed by a final extension for 7 min. In negative controls, the DNA template was omitted from the reaction. The amplification products were separated on 2% agarose gels and visualized by ethidium bromide staining. For the detection of mutations, PCR products were purified with the QIAquick PCR Purification Kit (Qiagen), and sequenced using a cyclic sequencing kit (ALFexpress II, Amersham Biosciences) following the manufacturer's recommendations.

For the K-RAS mutational analysis, previously extracted DNA was amplified by a mutagenic PCR assay. We used a mismatched upstream primer for codon 12 amplification and a mismatched downstream primer for codon 13 amplification, which introduced a *Bst*NI or a *Hae*III restriction site into the wild-type allele, respectively.

Using RFLP analysis (restriction fragment length polymorphism analysis), *Bst*NI digestion of the wild-type codon 12 allele yields two bands of 133 and 29 bp, while the mutant remains intact (162 bp). *Hae*III digestion of the wild-type codon 13 allele yields fragments of 85, 48, and 26 bp, while the mutant produces only two fragments of 85 and 74 bp (a common *Hae*III site at nucleotide 85 yields an 85 bp fragment in all samples).
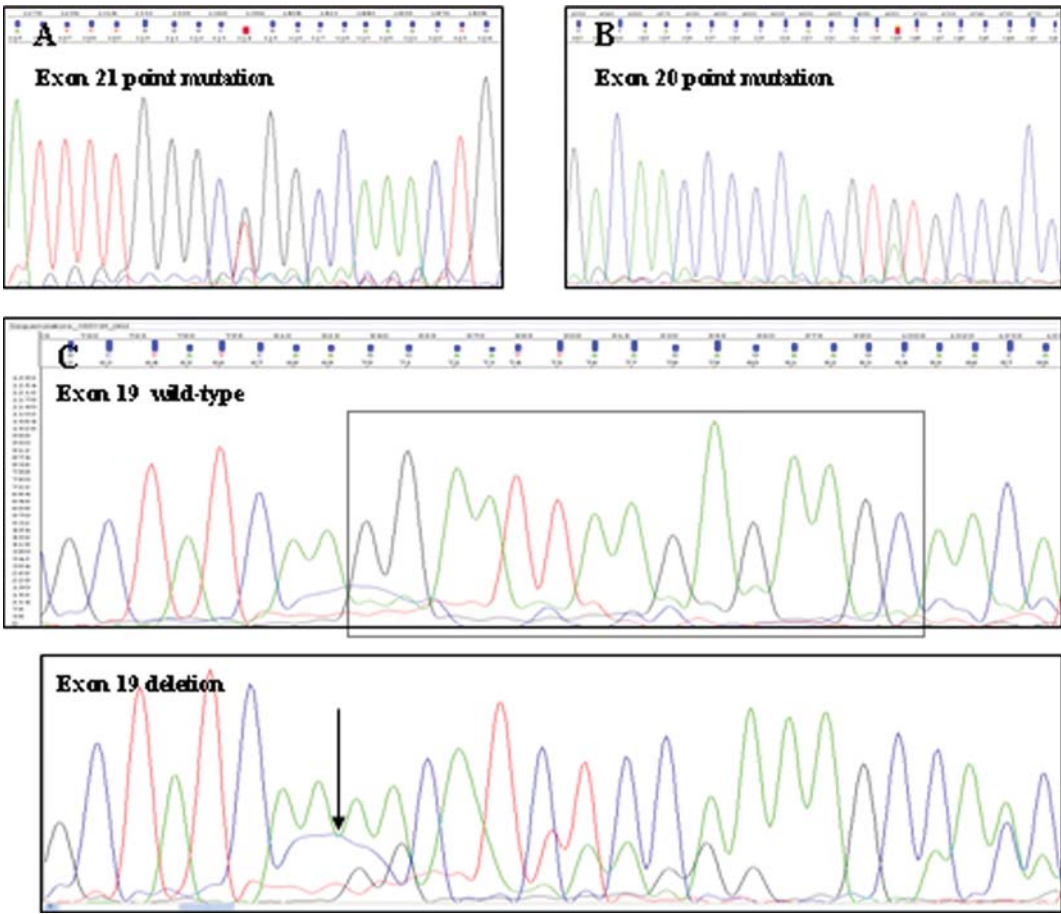
Figure 1. Representative electropherograms of mutations in the EGFR gene in lung adenocarcinomas. (A) L858R (T→G) mutation in EGFR exon 21 (B) G796D (G→A) mutation in EGFR exon 20 (C) in-frame deletion E746-A750 in EGFR exon 19 in comparison to the wild-type sequence.

Statistical analysis. All statistical analyses were carried out using Statistica software (Stat-soft). The χ^2 test was used to analyze the associations between the different variables. The *a priori* level of significance was set at a p-value of <0.05.

Results

EGFR mutations. EGFR mutations were present in 52 patients (12.6%) (Table I). The mutations were as follows: 1 in exon 18, 27 in exon 19, 7 in exon 20, and 17 in exon 21. Mutations of exon 19 consisted of in-frame deletions involving five amino acids from codons 746 through 750 (ELREA), and six to nine amino acids around codons 747 to 759. Most of the mutations in exon 21 were missense substitutions resulting in a change from leucine to arginine at codon 858 (L858R). Other mutations in exons 18, 20, and 21 were single nucleotide substitutions (Table II).

One hundred and seventy-one (171/411, 41.6%) silent mutations occurred in exon 20: at 2316, a G→A transition, which did not alter the encoded amino acid of glutamine at codon 787 (Q787Q). The allele frequencies of polymorphisms at codon 787 CAG/CAA were G/G (60.6%), G/A (22.7%), and A/A (16.7%). Twelve silent mutations of 2316 G→A were found to coexist (χ^2 test; p=0.01) with other mutations in exons 18, 19, and 21, and in a single case, with a mutation in

Table III. Association between EGFR mutations and clinical variables.

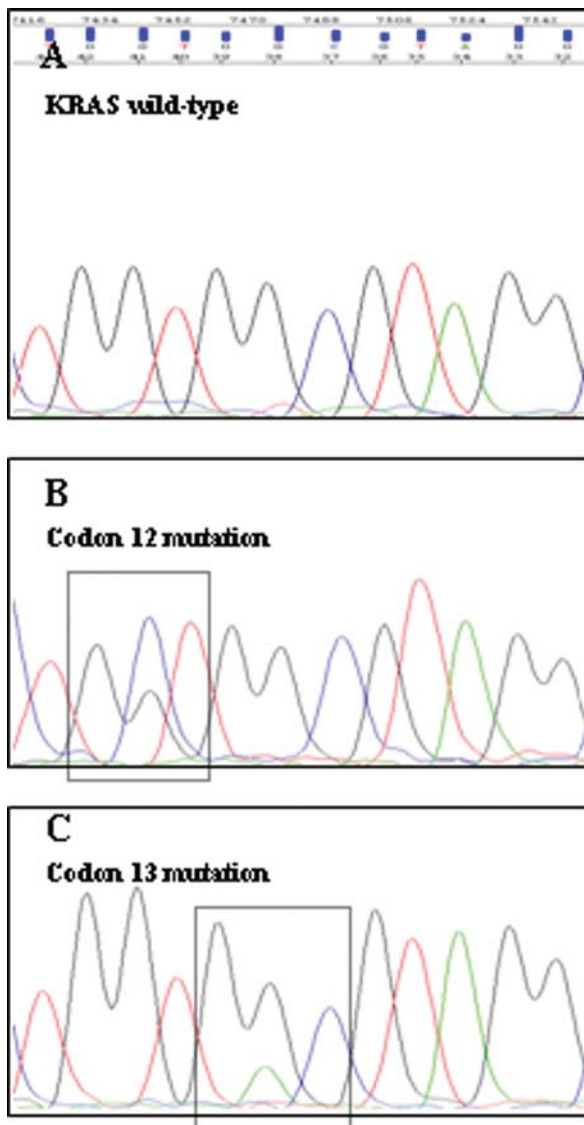| Variable	EGFR status		p-value
	Mutated n (%)	Wild-type n (%)	
Gender			**<0.0001**
Male	15 (28.8)	220 (61.3)	
Female	37 (71.2)	139 (38.7)	
Age (years)			0.33
≤66	24 (46.1)	191 (53.2)	
>66	28 (53.9)	168 (46.8)	
Smoking			**0.005**
Current	9 (30.0)	101 (54.0)	
Former	7 (23.3)	48 (25.7)	
Never	14 (46.7)	38 (20.3)	
Stage			0.21
<IIIA	16 (53.3)	134 (65.4)	
≥IIIA	14 (46.7)	71 (34.6)	
BAC features			**0.0004**
Present	14 (46.7)	37 (18.0)	
Absent	16 (53.3)	168 (82.0)	

Figure 2. Representative electropherograms of mutations in the K-RAS gene in lung adenocarcinomas. (A) Wild-type sequence (B) heterozygous sample for codon 12 mutation (C) heterozygous sample for codon 13 mutation.

Table IV. Association between K-RAS mutations and clinical variables.

| Variable | K-RAS status | | p-value |
	Mutated n (%)	Wild-type n (%)	
Gender			**0.0007**
Male	51 (76.1)	164 (53.4)	
Female	16 (23.9)	143 (46.6)	
Age (years)			0.94
≤66	35 (52.2)	159 (51.8)	
>66	32 (47.8)	148 (48.2)	
Smoking			**0.005**
Current	19 (46.3)	81 (51.6)	
Former	18 (43.9)	34 (21.7)	
Never	4 (9.8)	42 (26.7)	
Stage			0.67
<IIIA	26 (60.5)	117 (63.9)	
≥IIIA	17 (39.5)	66 (36.1)	
BAC features			0.53
Present	8 (18.6)	42 (23.0)	
Absent	35 (81.4)	141 (77.0)	

were analyzed in association with EGFR mutational status (Table III). No association with age and clinical stage was found, while EGFR mutations were more frequently observed in females (p<0.0001), in non-smokers (p=0.005), and in the presence of bronchioloalveolar features (p=0.0004).

Association between K-RAS mutations and clinical variables. K-RAS mutational status was analyzed in association with clinical variables (Table IV); we observed that K-RAS mutations were more frequent in males (p=0.0007) and were associated with smoking habits (current and former) (p=0.005). No statistically significant association was found with the other clinical characteristics.

EGFR and K-RAS mutations in female patients with advanced disease. EGFR sequencing analysis revealed that mutations were present in 12 out of 46 (26.1%) female patients with advanced disease. There were 7 mutations in exon 19, 2 in exon 20, and 3 in exon 21 (Table V). Fourteen (14/46, 30.43%) silent mutations (2316 G→A transition) occurred in exon 20 without altering the encoded amino acid of glutamine at codon 787 (Q787Q). For the K-RAS gene, mutations were identified in 15.8% (6 out of 46) of patients: four cases involved codon 12 and two involved codon 13.

Twenty-one female patients (21/46, 45.6%) among those with advanced disease received TKIs, gefitinib 250 mg/day (expanded access) or erlotinib 150 mg/die, as second/third-line therapy; 9/21 (42.9%) showed progressive disease, 4/21 (19%) showed stable disease, and 8/21 (38.1%) showed a partial response. The median duration of response was 8 months (range 2-24 months). Seven out of 21 (33.3%) female

exon 20. Representative nucleotide sequences of the EGFR mutations are shown in Fig. 1.

K-RAS mutations. There was no available material for K-RAS mutational analysis in 37 cases. K-RAS mutations were detected in 67 samples (67/374, 17.9%) (Table I); about 80% of the mutations (54/67) involved codon 12. Examples of sequencing of representative RFLP products for K-RAS mutational analysis are shown in Fig. 2. The occurrence of EGFR and K-RAS mutations was strictly mutually exclusive (χ^2 test; p=0.001).

Association between EGFR mutations and clinical variables. We categorized the clinical variables for analysis as follows: age, younger (≤66 years old) vs. older (>66 years old); TNM stage, early (< stage IIIA) vs. advanced stage (≥ stage IIIA).

Clinical variables, such as gender, age, smoking habits, TNM stage, and presence of bronchioloalveolar features,

Table V. EGFR mutations in female patients with advanced disease.

Patient no.	EGFR exon	Nucleotide number and sequence (5'-3')	Amino acid	Smoking	Response
1	19	2235-2249 del GAATTAAGAGAAGC	E746-A750 del	No	PR
2	19	2235-2249 del GAATTAAGAGAAGC	E746-A750 del	No	PR
3	19	2237-2254 del AATTAAGAGAAGCAACAT	E746-S752 del	No	NE
4	19	2254-2277 del TCTCCGAAAGCCAACAAGGAAATC	S752-I759 del	No	PR
5	19	2235-2249 del GAATTAAGAGAAGC	E746-A750 del	No	NE
6	19	2235-2249 del GAATTAAGAGAAGC	E746-A750 del	No	PR
7	19	2235-2249 del GAATTAAGAGAAGC	E746-A750 del	No	NE
8	20	2387 G→A	G796D	Yes	NE
9	20	2387 G→C	G796A	Yes	NE
10	21	2507 G→A	R836H	Yes	PD
11	21	2573 T→G	L858R	No	PR
12	21	2573 T→G	L858R	No	PR

PR, partial response; NE, not evaluated; PD, progressive disease.

Table VI. Associations between EGFR and K-RAS mutations, smoking status and tumor response in TKIs-treated patients.

	Partial response n=8	Stable disease n=4	Progressive disease n=9
EGFR			
Wild-type	2	4	8
Mutated	6	0	1
K-RAS			
Wild-type	8	4	7
Mutated	0	0	2
Smoking history			
No	8	1	5
Yes	0	3	4

patients treated with TKIs showed EGFR mutations. In a patient with progressive disease, there was a mutation at codon 836 in exon 21 which has never been previously reported in the literature; six classic EGFR mutations were observed in women with a partial response, while none of the 4 patients with stable disease after TKIs treatment showed EGFR alterations (p=0.006). For the K-RAS gene in TKIs-treated patients, mutations were identified in 2 out of 21 (9.5%) cases. None of the female patients with partial response and/or stable disease showed K-RAS mutations. Moreover, partial response was associated with a non-smoking history (8/8), while 4/9 patients with progressive disease, and 3/4 with stable disease were current smokers (p=0.02) (Table VI).

The group of twenty-five female patients with advanced disease who were not treated with TKIs (25/46, 54.4%) showed five EGFR (5/25, 20%) and four K-RAS (4/25, 16%) mutations.

Discussion

Although a great number of studies have been performed on EGFR and K-RAS status in several human cancer models, no prospective study has been conducted on a homogeneous and large series of patients. Within the Istituto Toscano Tumori and the Italian Association of Women Against Lung Cancer (AIDA[CP]), we saw the need to evaluate EGFR and K-RAS status in a wide population of patients diagnosed with lung carcinoma. Based on the high incidence of lung carcinoma in Tuscany (particularly in certain areas) and on the fact that EGFR and K-RAS mutations seem to have a major impact on the development and progression of certain subtypes of adenocarcinoma, we designed our study with the aim of determining the actual incidence of EGFR mutations in a wide group of lung adenocarcinomas, prospectively collected from different areas of Tuscany. In our series, EGFR mutations were more frequently found in cases with bronchioloalveolar features, in agreement with results recently published by Kim *et al* (16), suggesting a possible correlation between pathological characteristics and molecular changes to EGFR. We found that the frequency of EGFR mutations (12.6%) is in the range of 3-13%, as reported in the literature (1-3), is higher in women (37/176, 21%) than men (15/235, 6.4%), and is more prevalent in non-smokers (14/52, 26.9%) than in current (9/110, 8.2%) or former smokers (7/55, 12.7%). Data from other studies in Europe corroborate our results, showing a similar incidence of EGFR mutations in subgroups of patients with lung adenocarcinoma. In contrast, our observations differed significantly from those in several Asian studies, due to the greater prevalence of EGFR mutations in Asian populations (35-63% vs. 6-12.6%) (Table VII) (16-20). The implication of these findings is that ethnicity may determine different genetic backgrounds in common tumors, thereby influencing clinical outcome and response to therapy.

EGFR mutations were the first molecular aberrations found in lung cancer, and were found to be more frequent

Table VII. Epidermal growth factor receptor (EGFR) and K-RAS gene mutations in patients with lung adenocarcinoma.

| | | | | EGFR mutations in subgroups of patients with adenocarcinoma | | | | |
| | | | | | Sex | | Smoking history | |
Author	Ethnicity	N	K-RAS mutation (%)	EGFR mutation (%)	Male (%)	Female (%)	Yes (%)	No(%)
Kim *et al* (16)	Asian	71	7	35	24	45	23	45
Haneda *et al* (17)	Asian	95	ND	39	33	45	30	45
Sakuma *et al* (18)	Asian	118	8.5	63	39	81	44	78
Marchetti *et al* (19)	Italian	375	28.8	10	6	30	7	25
Savic *et al* (20)	Swiss	49	ND	6	3.6	16	ND	ND
Present study	Italian	411	17.9	12.6	6	21	8	27

ND, non determined.

among non-smoking patients than among patients who smoked (21). However, this does not suggest that smoking has a preventive effect on EGFR mutations; rather, it is reasonable to assume that EGFR mutations are caused by carcinogens other than those contained in tobacco smoke. The apparent negative correlation with smoking likely results from diluting the number of tumors with EGFR mutations amidst a higher number of tumors with wild-type EGFR associated with higher levels of smoking, as suggested in a recent case-control study by Matsuo *et al* (22).

The influence of gender has also been an issue of interest, with the suggestion that female gender is associated with EGFR mutations (23-25). In contrast, Huang *et al* (26) reported no strong association between female gender or smoking status and the presence of a mutation in a group of adenocarcinomas. It is not clear whether female gender is directly associated with EGFR mutation *per se*, or whether this association results from erroneous analyses confounded by the higher prevalence of adenocarcinoma among women. The results in our study support the dominant finding that gender, smoking, and the interaction of these two factors differentially affect the site of somatically acquired EGFR mutations. However, the reasons for the impact of gender difference on the mutational spectrum are unclear, and thus warrant further investigation into the basis of this association, including the possible involvement of sex hormones.

The clinical effect of EGFR-TKIs on patients with various mutations seems to depend on the specific EGFR mutant present. Mitsudomi *et al* (27) first reported that the response rate of gefitinib was higher in patients with deletional EGFR mutations than in those with other types of mutations, which were predominantly L858R; recently, Pallis *et al* (28) demonstrated that classic (but not 'other') mutation variants of the EGFR gene are associated with response to TKIs.

Greulich *et al* (6) showed that one of the insertion mutations (D770insNPG) in exon 20 is associated with *in vitro* resistance to erlotinib. In that study, G719S of exon 18 showed intermediate sensitivity *in vitro*, lower than exon 19 or exon 21 mutant cells. In a transgenic mouse model, L858R mutants were associated with more aggressive adenocarcinomas compared to exon 19 deletion mutants (delL747-S752) (29). Mulloy *et al* (30) showed that Del747-753 kinase had a higher autophosphorylation rate and higher sensitivity to erlotinib than the L858R kinase. All these data reflect the differences in clinical response rates between exon 19 deletional mutations and L858R. Our study on 21 female patients receiving TKIs also supported the postulation that the type of mutation was correlated with tumor response, with 75% of the patients showing partial response having classic EGFR mutations, none of the patients with stable disease displaying EGFR mutations, and only one non-classic EGFR mutation among patients with progressive disease. Moreover, our observations correlate extremely well with the clinical response data, with EGFR mutation carriers being sensitive to tyrosine kinase inhibition and having a higher frequency of non-smokers (31).

Other factors also affect tumor response to TKIs; an activating mutation of the K-RAS gene was one of the earliest discoveries of genetic alterations in lung cancer. Huncharek *et al* (32) analyzed a series of studies and concluded that there is an approximately 25% incidence ratio of K-RAS mutations in Caucasian populations; our percentage (67 out of 374 patients, 17.9%) is within this range (Table VII) (16,18,19).

The incidence of simultaneous K-RAS mutations and EGFR mutations has been investigated by several groups, following the realization that there is a correlation between K-RAS mutations and smoking status (33) and that certain K-RAS mutations correlate with resistance to TK inhibition of EGFR signaling (10). It has been shown that EGFR and K-RAS mutations are mutually exclusive. We have seen a similar result in our patient population, and have observed that males and smokers have a higher incidence of K-RAS mutations, while no K-RAS mutations were found in female patients with stable and/or regressive disease.

Moreover, we and others have reported the existence of silent polymorphisms in the EGFR exons, as well as their co-occurrence with other mutations (31,34). Additional information, either retrospective and/or prospective, is required to determine the significance of not only the classic exonic EGFR mutations, but also the intronic and silent mutations.

Acknowledgments

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