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Outcome of EGFR inhibitors treatment in advanced NSCLC patients, not enrolled in clinical trials

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Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) have become a treatment after first-line chemotherapy in patients with advanced NSCLC. We assessed the predictive and prognostic role of EGFR and Kras mutations in NSCLC patients treated with TKIs after progression, not included in clinical trials. Gefitinib 250 mg or Erlotinib 150 mg per os were administered to 70 patients. Radiological assessment was performed every six weeks. EGFR and Kras mutations were found in 21.4% and 24.3% of patients, respectively. At multivariate analysis, Kras mutation was positively associated with progression-free survival (PFS; HR=0.71, 95% CI: 0.53-0.96; p=0.027) and, less clearly, with response (OR=1.84, 95% CI: 0.98-3.45; p=0.057) and survival (HR=0.74, 95% CI:0.54-1.02; p=0.066). EGFR mutation influenced positively PFS (HR=0.69, 95% CI: 0.47-1.02; p=0.06), but not survival. In conclusion, in our unselected patients mutation of Kras correlated with a better outcome. The small number of patients may explain some discrepancies with data in literature.

Key words: EGFR, Kras, Erlotinib, Gefitinib, mutation, non-small cell lung cancer

Most patients with non-small-cell lung cancer (NSCLC) present with advanced disease. Current treatment paradigms are chemotherapies and targeted therapies. As patient responses to these therapies vary, predictive biomarkers may be an important facet of a patient's diagnostic workup in personalized medicine: there is accumulating evidence that they may entail prognostication and prediction of therapeutic response. Biomarkers for the selection of patients with NSCLC most likely to benefit from epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), such as Gefitinib and Erlotinib, include mutations and single-nucleotide polymorphisms of the EGFR gene and mutations on the Kras gene [1,2]. EGFR-TKI treatment in metastatic NSCLC patients significantly improves progression-free survival (PFS) with acceptable toxicities [3,4], being effective and tolerated also in elderly patients with EGFR mutations [5]. Especially, NSCLC with exon 19 deletions had longer median PFS and overall survival (OS) than NSCLC with other mutations such as exon 21 L858R substitution [3,6], while the wild-type EGFR was associated with poorer outcomes, irrespective of Kras status [3].

The significance of the Kras mutant status on treatment response in NSCLC patients remains controversial and so far there is insufficient evidence to determine the association between KRAS status and tumor progression or survival [7]. Reviews of several studies suggested that Kras mutations could be used as a potential negative predictor of clinical benefit from EGFR-TKIs in unselected advanced NSCLC patients, but they are of limited value when EGFR status is considered [8,9]. Similarly, other papers showed that in lung cancer patients, in contrast with colorectal cancer, patients with mutant tumors did not benefit from adjuvant chemotherapy, and their disease did not respond to EGFR inhibitors [10]. Despite some conflicting findings it is clear that Kras mutations are characterized by a complex biology involving the interaction between different Kras amino acid substitutions, various growth factor pathways, and several tumor suppressor genes [10-12].

Anyway, in recent years, the management of lung cancer has moved towards molecular-guided treatment.

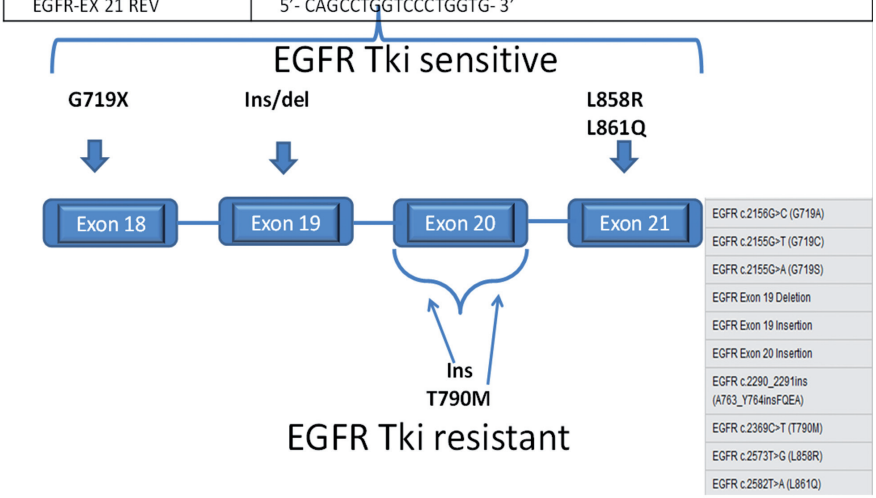
We studied a consecutive series of NSCLC patients not included in clinical trials, treated between 2007 and 2009 in 2

Italian Hospitals. These patients received EGFR-TKI inhibitors as second or third line therapy, as the approval of these drugs in a first line setting was not available at the time of the study. We correlated PFS, OS and objective response rate (ORR) to mutational status of EGFR and Kras and to some clinical features of patients.

Patients and methods

Seventy NSCLC metastatic patients were included in the study. They received Gefitinib 250 mg or Erlotinib 150 mg per os after first or second line or third line chemotherapy when progression occurred. Tumor response was assessed according

EGFR-EX 19 FOR	5′-GTGCATCGCTGGTAACAT 3′
EGFR-EX 19 REV	5′ - GGAGATGAGCAGGGTCT-3′
EGFR-EX 20 FOR	5′- CGCCATTCATGCGTCTTCA - 3′
EGFR-EX 20 REV	5′-CTATCCCAGGAGCGCAG- 3′
EGFR-EX 21 FOR	5′ - TGGCATGAACATGACCCT - 3′
EGFR-EX 21 REV	5′- CAGCCTGGTCCCTGGTG- 3′



Kras EX 2 FOR	5′-AAGGCCTGCTGAAAATGACTG-3′
Kras EX 2 REV	5′-CAAAGAATGGTCCTGCACCAG-3′

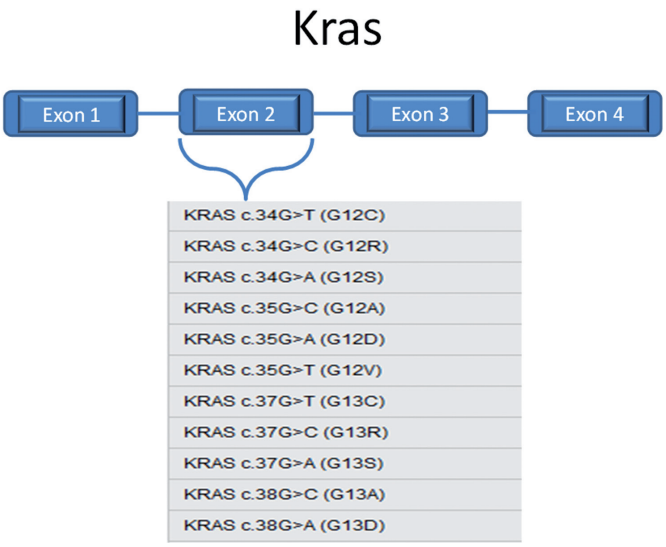


Figure 1. EGFR and Kras mutation analysis

to the Response Evaluation Criteria in Solid Tumors (RECIST): complete response (CR) was complete disappearance of all objective evidence of disease; partial response (PR) was ≥30% reduction in size (products of the two longest perpendicular diameters) of measurable lesions without any new lesion; progressive disease (PD) was ≥20% increase in size of known lesions or appearance of new lesions; stable disease (SD) was all other situations [13]. Radiological assessment was performed with CT scan every six weeks. Patients with a radiological objective response with either RECIST CR or PR or SD were considered as responders. All patients received TKI and then were tested for EGFR and Kras.

DNA extraction, EGFR and Kras mutation analysis. DNA was extracted from FFPE tissue samples using QIAamp DNA FFPE Tessue Kit (Qiagen, Hamburg, Germany). Briefly, 3 sections (8μm) were cut from FFPE tissue sample. When it was necessary we performed a macro-dissection following the instructions anatomopathologist to obtain almost 50% of tumoral cells. Tissue sections were incubated in Buffer ATL (Qiagen) and proteinase K at 56°C until the tissue was completely lysed (1-3 hours), and then at 90°C (1 hour) to reverse formalin crosslinking. The DNA was extracted using manufacturer's protocol.

Sanger sequencing was used to analyse EGFR exons 18, 19, 20, and 21 and Kras exon 2 (Figure 1). DNA was amplified by PCR (Mastercycler, Eppendorf) in 25μl reactions containing 40 ng of template DNA, 12.5μl AMPLITAQ GOLD 360 (Life Technologies) and 0.5μM of forward and reverse primers. PCR conditions for EGFR amplification were 95°C 10min, 45X (94°C 45s, 65°C 45s, 72°C 45s) and 72°C for 7 min. PCR conditions for Kras amplification were 95°C 10min, 45X (95°C 30s, 58°C 30s, 72°C 40s) and 72°C for 7 min. Amplification of a single PCR product of the expected size was electroforetically confirmed on a 2% agarose gel by ethidium bromide staining and UV-light. The PCR product was purified by removal of residual primers and nucleotides in reactions containing 10μl PCR product and 2μl illustra ExoStar 1-Step. The PCR product was then used as a template for sequencing reactions in both forward and reverse directions in 20μl reactions containing; 0.5μM forward and reverse primer, 1μl BigDye Terminator (Applied Biosystems), 2μl BigDye Terminator Buffer (Applied Biosystems) and 2-4μl purified PCR product. The sequencing conditions were 25X (96°C 10s, 50°C 5s, 60°C 4 min). The amplicon was then precipitated by using Performa® DTR Ultra 96-Well Plate Kit (EdgeBio). DNA was analysed on an ABI 3130 Genetic Analyzer (Applied Biosystems).

Statistical analysis. Continuous variables distribution was reported as median (range). The study population was categorized according to the median of the age distribution (i.e., <=61 and >61 years), histology (adenocarcinoma and others), PS ECOG (0-1 and 2), smoking status as never smoker and smoker (former- and current smoker). EGFR and Kras genotypes were classified as wild type or mutated. EGFR was considered mutated when at least one mutation in 18-21 exons was present. The relationship between categorical variables was examined by means of the chi-square test. Odds Ratios (OR) and the corresponding 95% confidence intervals (95%CI) for Kras and EGFR status (mutation vs wild type), gender (female vs male) and smoking habits (smoker vs never smoker) were computed to predict therapy response using multiple logistic regression analysis. The Kaplan-Meier method was applied in univariate analysis to estimate survival and PFS probabilities and the log-rank test was carried out to assess heterogeneity within each prognostic factor. Multivariate analyses were conducted using Cox regression model including terms for EGFR and Kras status (mutation vs wild type) and for the other variables that reached a p<=0.2 in univariate analysis. PFS was defined as the time from TKIs initiation until documented disease progression or death. OS was defined as the time from TKI initiation until death from any cause or last follow-up. Statistical calculations were performed using the SPSS statistical package version 20. For all comparisons, a two sided p value of <0.05 was considered as statistically significant.

Results

Patients characteristics at enrollment were as follows: median age 62.6 years (range 37-80 years), 48 males, and 53 adenocarcinoma histological subtype. Fifty-nine patients (84.3%) were smokers (Table 1). All patients were tested for

Table 1. NSCLC patients characteristics.

	No. of patients (70)	%
Gender		
Male	48	68.6
Female	22	31.4
Age, median (range)	62.6 (37-80)	
Histology		
Adenocarcinoma	53	75.7
Squamous	13	18.6
NSCLC unspecified	4	5.7
PS ECOG		
0-1	56	80.0
2	14	20.0
Smoking status		
Never smoker	11	15.7
Smoker	59	84.3
Best response		
Progression	40	57.1
Stable disease	26	37.2
Response	4	5.7
EGFR genotype		
Wild type	55	78.6
Mutated	15	21.4
Kras genotype		
Wild type	53	75.7
Mutated	17	24.3

EGFR exons 19 and 20, while exon 18 and exon 21 were tested in 22 and 52 of them, respectively. Fourteen patients (20%) were positive for at least one EGFR mutation (1 in exon 18, 4 in exon 19, 8 in exon 20 and 1 in exon 21). A mutation in both exon 20 and 21 was detected in one case. A mutation was found in 27.3% of females and in 18.8% of males (p=0.5) and in 15.3% of smokers versus 54.5 of never smokers (p=0.01). No differences were observed with regard to histology. A Kras mutation was found in 17 (24.3%) patients (31.8% of females and 20.8% of males, p=0.4). Never smokers (n=11) were all mutation negative, while mutation was found in 28.8% of smokers (p=0.055). According to histology, mutation was found in 32.1% of adenocarcinomas, but not in the other or not specified histotypes (p=0.007). EGFR and Kras mutations were mutually exclusive.

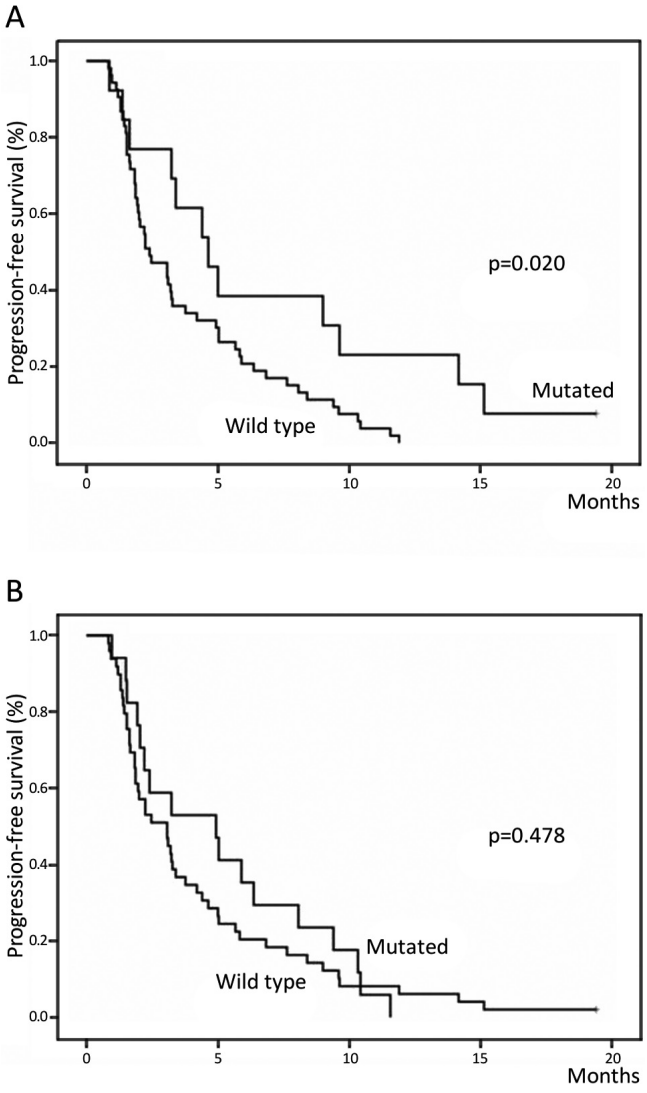


Figure 2. Progression-free survival of NSCLC patients according to EGFR (A) and Kras (B) status

Patients underwent TKI therapy after a median period of 14.0 months from diagnosis (range 0.7-70.7 months). On average, patients had received 1 prior line of chemotherapy (range 1-4). ECOG PS was 0-1 in 56 (80.0%) patients.

Response to therapy. Forty-four patients (57.1%) had PD as their best response. Only 4 patients (all adenocarcinoma, median age 56.6 years) achieved a PR. Gender, age, histology and PS were not associated with the probability of response, while an objective response was achieved in 72.7% of never smokers versus 37.3% of smokers (p=0.045). Patients with at least an EGFR mutation had a response rate of 40%, similar to that of patients with wild type EGFR (43.6%). ORR was higher in patients with Kras mutation respect to wild type patients (58.8% vs 37.7%), but the difference was not significant (p=0.2) (Table 2). The response rate was 36.8% in patients with both Kras and EGFR wild type tumors. According to the EGFR genotype, response was obtained in the only one patient with mutated exon 18 (vs 23.8% of response in wild type), in 75% of mutated exon 19 (40.9% in wild type) and in 25% of the mutated exon 20 cases (45.9% in wild type). No response was obtained in the case with mutation in exon 21 (50% in wild type) and in the case of mutation of both exon 20 and 21.

Logistic regression analysis confirmed the inverse association between objective radiological response and smoke, showing an OR= 0.33 (95%CI: 0.13-0.85, p= 0.02). In addition, a non statistically significant association was found with

Table 2. Individual characteristics and radiological objective response in NSCLC patients

	NO response N (%)*	Response N (%)*	p
Gender			0.203
Male	30 (62.5)	18 (37.5)	
Female	10 (45.5)	12 (54.5)	
Age (years)			0.338
≤61	14 (50.0)	14 (50.0)	
>61	26 (61.9)	16 (38.1)	
Histology			1.000
Adenocarcinoma	29 (54.7)	24 (45.3)	
Squamous cell ca	7 (53.8)	6 (46.2)	
PS			0.562
0-1	33 (58.9)	23 (41.1)	
2	7 (50.0)	7 (50.0)	
Smoking status			0.045
Never smoker	3 (27.3)	8 (72.7)	
Smoker	37 (62.7)	22 (37.3)	
EGFR genotype			1.000
Wild type	31 (56.4)	24 (43.6)	
Mutated	9 (60.0)	6 (40.0)	
Kras genotype			0.163
Wild type	33 (62.3)	20 (37.7)	
Mutated	7 (41.2)	10 (58.8)	
Overall	40 (57.1)	30 (42.9)	

Kras mutation (OR=1.84, 95%CI:0.98-3.45; p = 0.057) (data not shown).

Progression-free survival. Overall, median PFS was 3.1 months (95% CI: 2.1-4.1 months) (Table 3). A statistically significant prolonged PFS was observed in female (median 4.6 vs 2.2 months for males, p=0.04), never smoker (5.8 months vs 2.2 months for smokers, p=0.011) and EGFR mutated status (median 4.6 months vs 2.4 months for wild type genotype, p=0.02). Kras mutation was not associated with PFS (median 4.9 months for mutated vs 3.1 months for wild type genotype, p=0.5). Figures 2A-B show PFS according to EGFR and Kras status, respectively. Median PFS was 2.0 months (95% CI: 1.5-2.5 months) in patients with both Kras and EGFR wild type tumors. According to the EGFR genotypes, the patient with mutation in exon 18 had a PFS of 15.1 months. Median PFS was 4.6 months (95% CI: 0.5-10.3 months) in patients with mutation in exon 19 and 4.4 months (95%CI:1.8-7.0 months) in mutated exon 20 cases. The patient with mutation in exon 21 and the case of mutation of both exon 20 and 21 had a PFS of 26 days and 3.5 months, respectively.

Multivariate Cox regression analysis for PFS included EGFR and Kras status (mutation vs wild type), gender (female vs male), PS (2 vs 0-1) and smoking habits (smoker vs never smoker). Only Kras mutation was statistically associated with PFS, with a favorable outcome (HR=0.71, 95% CI: 0.53-0.96; p=0.027). A non-significant positive association was found for EGFR mutation (HR=0.69, 95% CI: 0.47-1.02; p=0.06) (data not shown).

Overall survival. During the study period, 67 patients (95.7%) died. For the whole group, median survival time was 6.6 months (95% CI: 4.7-8.4 months). OS at 6 and 12 months was 21% and 11%, respectively. Survival of patients according to the various prognostic factors is shown in Table 4. In univariate analysis, a statistically significant association with survival was found for gender and smoke. Females survived longer than males (11.1 versus 5.0 months, p=0.002) and never smokers survived longer compared to smokers (11.1 months vs 6.5 months, p=0.02). Patients with stable disease or response survived 9.6 months compared to 4.0 months of those undergoing progression (p<0.001). Median OS was 5.1 months in mutated EGFR vs 6.8 months in wild type patients (Figure 3A, p=0.5) and 8.3 months in mutated Kras vs 5.1 months in wild

Table 3. Progression-free survival (PFS) of NSCLC patients according to individual characteristics

	N	Median PFS (months)	95% CI	p
Overall	70	3.1	2.1-4.1	
Age (years)				0.982
≤61	28	3.1	2.0-4.2	
>61	42	2.5	1.1-3.9	
Gender				0.043
Male	48	2.2	1.0-3.4	
Female	22	4.6	1.4-7.8	
Histology				0.562
Adenocarcinoma	53	3.1	1.9-4.2	
Squamous cell ca	13	3.2	0.6-7.2	
NSCLC unspecified	4	1.6	0.1-1.8	
PS ECOG				
0-1	56	3.2	2.4-4.1	0.137
2	14	2.2	1.4-3.0	
Smoking status				0.011
Never smoker	11	5.8	1.1-10.5	
Smoker	59	2.2	1.1-3.3	
EGFR genotype				0.020
Wild type	55	2.4	1.3-3.4	
Mutated	15	4.6	2.7-6.5	
Kras genotype				0.478
Wild type	53	3.1	1.8-4.3	
Mutated	17	4.9	1.4-8.5	

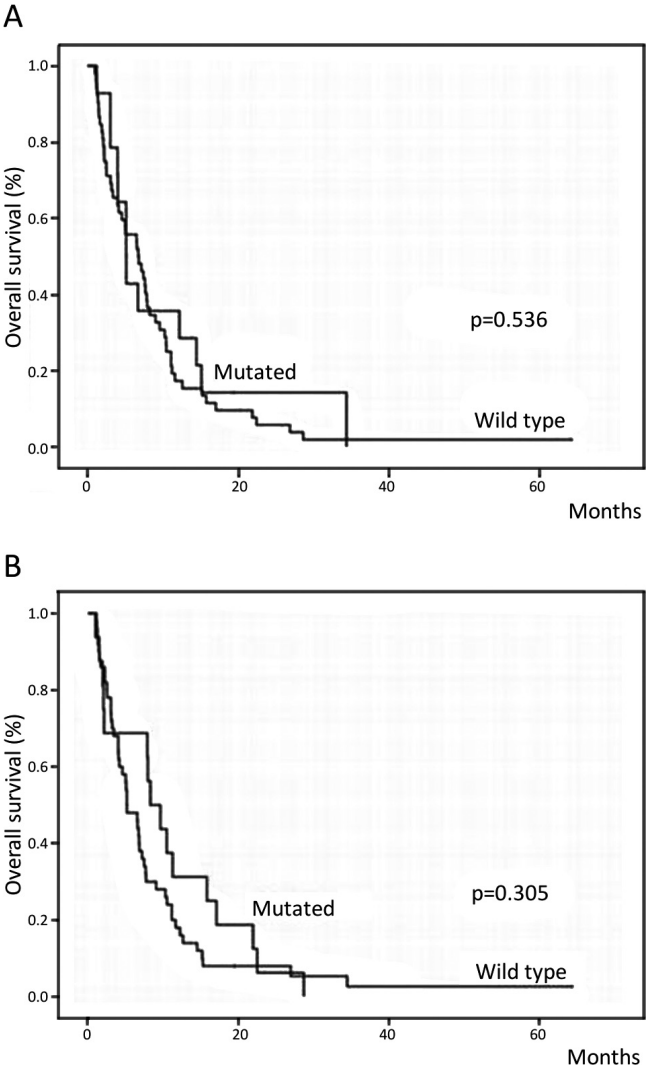


Figure 3. Overall survival of NSCLC patients according to EGFR (A) and Kras (B)

Table 4. Overall survival (OS) of NSCLC patients according to individual characteristics

	N	Median survival (months)	95% CI	P
Overall	70	6.6	4.7-8.4	
Age (years)				0.925
≤61	28	6.8	0.5-13.1	
>61	42	6.6	4.6-8.6	
Gender				0.002
Male	48	5.0	2.4-7.6	
Female	22	11.1	9.2-13.0	
Histology				0.824
Adenocarcinoma	53	6.5	4.5-8.6	
Others	13	6.7	2.8-10.6	
NSCLC unspecified	4	3.4	0.2-4.3	
PS ECOG				0.204
0-1	56	6.8	5.7-7.9	
2	14	2.2	0.5-5.1	
Smoking status				0.019
Never smoker	11	11.1	3.5-18.7	
Smoker	59	6.5	4.7-8.4	
Best response				<0.001
Progression	40	4.0	2.9-5.1	
Stable dis/response	30	9.6	6.2-13.0	
EGFR genotype				0.536
Wild type	55	6.8	3.9-9.6	
Mutated	15	5.1	3.7-6.5	
Kras genotype				0.305
Wild type	53	5.1	3.3-6.9	
Mutated	17	8.3	5.0-11.5	

type patients (Figure 3B, p=0.3). Median OS was 5.0 months (95% CI: 2.1-8.0 months) in patients with both Kras and EGFR wild type tumors. According to the EGFR genotypes, the patient with mutated exon 18 survived 15.1 months. Median OS was 5.2 months (95% CI: 0.5-25.0 months) in the patients with mutated exon 19 and 5.1 months (95%CI: 2.4-7.8 months) in mutated exon 20 cases. The patient with mutation in exon 21 and the case of mutation of both exon 20 and 21 survived 1.3 months and 4 months, respectively. Multivariate analysis for OS included EGFR and Kras status (mutant vs wild type), gender (female vs male), PS (2 vs 0-1) and smoke (smoker vs never smoker). In this case, female gender and Kras mutation confirmed to be favorable prognostic factors, but without reaching the significance (HR=0.74, 95% CI: 0.53-1.03, p=0.07 and HR=0.74, 95% CI: 0.54-1.02; p=0.066, respectively). No influence on survival was observed with regard PS or EGFR mutation (HR=1.24, 95% CI: 0.90-1.71; p=0.2 and HR=1.03, 95% CI: 0.73-1.46; p=0.8, respectively) (data not shown).

Discussion

In the present study we evaluated the role of EGFR and Kras on response to TKIs therapy, progression-free survival and overall survival in NSCLC patients undergoing second or third line therapy with EGFR-tyrosine kinase inhibitors and not included in clinical trials. Multivariate analysis showed only a tendency towards a positive association between EGFR and PFS. There was a non significant trend towards benefit for response and survival from Kras mutations, although Kras mutation had a significant positive impact on PFS.

The EGFR signaling pathway may be pivotal in the progression of NSCLC. Molecular targeted therapy based on EGFR-TKIs have become a treatment option after first-line chemotherapy in subgroups of patients with advanced NSCLC and mutations in EGFR and in other genes such as Kras [1,14], nevertheless the topic is still debated. In a first line setting, it has been shown that the EGFR-TKIs treatment has improved response, PFS and quality of life in patients harboring specific EGFR mutations [15-19]. Data from a meta-analysis quantifying the magnitude of benefit with upfront EGFR-TKIs showed a pooled hazard ratio of 0.45 (95%CI: 0.36-0.58) for PFS and of 2.08 (95%CI: 1.75-2.46) for overall response in EGFR mutation positive patients over chemotherapy [20]. Nevertheless, smoking history should be considered, since smoking was associated with shorter PFS after EGFR-TKIs treatment in advanced NSCLC patients with EGFR mutations [21]. The impact of EGFR status on survival is debatable, especially in patients treated beyond first-line therapy [3,16,22-27]. The effect on progression, as well as on OS could be limited to adenocarcinoma histotype [17]. Recent meta-analyses showed that EGFR-TKIs treatment prolonged PFS in EGFR mutation positive patients in all settings, with an HR of 0.43 (95% CI: 0.38-0.49) for front-line, of 0.34 (95% CI: 0.20-0.60) for second-line and of 0.15 (95% CI: 0.08-0.27) for maintenance therapy. Nevertheless, EGFR-TKIs did not seem to have impact on OS [19], while the probability of obtaining a response increased in patients with EGFR mutation, in female or smokers patients who had never smoked (28). In contrast to the positive results, it is however possible that patients acquire resistance during a first- or second generation oral EGFR TKI, also after an initial response [29].

The role of Kras mutations remains to be clearly elucidated, mainly because of the small samples size of the studies and of the low prevalence of Kras mutations [2,11]. In contrast with our data, Kras mutations have been associated with EGFR-TKI resistance [30], while no influence or a low response rate was found in some studies in second- and third-line settings [25,31,32].

It remains unclear whether there is an association between Kras mutation and progression-free and overall survival [2,17]. According to some studies, Kras status impacts negatively survival [22,26,27,31]. In addition, Kras mutant patients experienced a significantly shorter PFS compared with those carrying a wild genotype [33,34]. A meta-analysis on l1470 NSCLC patients from 22 studies showed a higher frequency of Kras mutations among smokers than among never smokers (25% versus 6%) and among adenocarcinomas compared to

other histologies (26% versus 16%). Kras mutations were associated with a lack of response to TKIs therapy. The objective response rate was 3% and 26% in mutation positive patients and in patients with wild-type genotype, respectively. The pooled relative risk for the objective response rate was 0.29 (95% CI: 0.18-0.47). In spite of these results, it has been argued that the selection of patients on the basis of Kras status for EGFR-TKIs sensitivity in NSCLC patients has a limited value because of a mutually exclusive relationship between Kras and EGFR mutation and the lack of difference in survival between Kras mutant/EGFR wild-type and Kras wild-type/EGFR wild-type NSCLC [7].

In our series we found an EGFR and Kras mutation in 21% and 24% of patients, respectively. Published data show a large variation of mutations in tumor samples, ranging between 7% and 45% for EGFR [26,35,36] and between 7% and 23% for Kras [25,26]. In Western countries, a higher percentage of Kras mutations (30-50%) was found in adenocarcinomas with respect to the other histotypes, while the mutation rate of EGFR tended to be low (3% to 12%) [37,38]. We did not find differences among histotypes with regard EGFR status, while Kras mutations were found only among adenocarcinomas. Consistently with literature, EGFR mutations were more frequent in never smokers, while Kras mutations were present only in smokers [7,36,38].

Finally, our data of these second-third line setting patients not participating to any clinical trial showed a trend toward benefit for disease progression in patients with mutated EGFR Moreover, in our patients mutation of Kras oncogene was correlated to better PFS, in contrast with most of the other studies. The conflicting results regarding the role of Kras mutations on treatment response and patient outcomes have been recently attributed to a great molecular heterogeneity in tumors with mutated Kras, It is has been shown that disease stage at the time of diagnosis, specific Kras codon mutation and co-occurring genomic alterations may identify subgroups of mutant NSCLC with distinct biology and efficacy of targeted therapies [39,40].

In our opinion, these discrepancies between our findings and those reported in the scientific literature are mostly due to the small number of patients and do not reflect real differences between patients enrolled in clinical studies and our "daily practice" patients. Given the burden of lung cancer, more confirmative results on the actual role of EGFR and Kras in NSCLC therapy are needed.

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