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ORIGINAL ARTICLE

Optimization of patient selection for EGFR-TKIs in advanced non-small cell lung cancer by combined analysis of *KRAS*, *PIK3CA*, *MET*, and non-sensitizing *EGFR* mutations

V. Ludovini · F. Bianconi · L. Pistola · V. Minotti · R. Chiari · R. Colella · G. Bellezza · F. R. Tofanetti · A. Siggillino · E. Baldelli · A. Flacco · D. Giuffrida · A. Sidoni · L. Crinò

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Abstract
Background We present a comprehensive analysis of *KRAS*, *PIK3CA*, *MET,* and non-sensitizing *EGFR* mutations in advanced non-small cell lung cancer (NSCLC) patients treated with tyrosine kinase inhibitors (TKIs), with the aim of clarifying the relative contribution of these molecular alterations to resistance.
Patients and methods One hundred and sixty-six patients with advanced NSCLC treated with EGFR-TKIs with available archival tissue specimens were included. *EGFR* (exons 18–21), *KRAS* (exons 2, 3), *PIK3CA* (exons 9, 20), and *MET* (exons 14, 15) mutations were analyzed using PCR-based sequencing. Among all the mutations evaluated, only *KRAS*, *PIK3CA*, *MET,* and non-sensitizing *EGFR* mutations, defined as "TKI non-sensitizing mutations" were used for response, time to progression (TTP), and overall survival (OS) analysis.
Results TKI non-sensitizing mutations were associated with disease progression ($p = 0.001$), shorter TTP ($p < 0.0001$), and worse OS ($p = 0.03$). Cox's multivariate analysis including histology and performance status showed that TKI non-sensitizing mutations were independent factors for shorter TTP ($p < 0.0001$) and worse OS ($p = 0.01$).
Conclusions When *KRAS*, *PIK3CA*, *MET,* and non-sensitizing *EGFR* mutations are concomitant, up to 96.0% of NSCLC patients unlikely to respond to TKIs can be identified, and they represented independent negative prognostic factors. Comprehensive molecular dissection of EGFR signaling pathways should be considered to select advanced NSCLC patients for TKIs therapies.

Keywords *EGFR* · *KRAS* · *PI3KCA* · *MET* mutations · Response to TKIs · NSCLC

V. Ludovini (✉) · L. Pistola · V. Minotti · R. Chiari · F. R. Tofanetti · A. Siggillino · E. Baldelli · A. Flacco · L. Crinò
Medical Oncology Division, S. Maria della Misericordia Hospital, 1, Via G. Dottori, 06132 Perugia, Italy
e-mail: ludoviniv@hotmail.com

F. Bianconi
Department of Electronic and Information Engineering, University of Perugia, Perugia, Italy

R. Colella · G. Bellezza · A. Sidoni
Institute of Pathological Anatomy and Histology, University of Perugia, Perugia, Italy

D. Giuffrida
Medical Oncology, Istituto Oncologico del Mediterraneo, Catania, Italy

Introduction

Chemotherapy, the mainstay of treatment in advanced metastatic non-small cell lung disease, is only marginally effective [1, 2], but gefitinib and erlotinib, which target the epidermal growth factor receptor (EGFR) pathway, have shown promising activity for its treatment [3] with response rates of 10–20% when used as second- or third-line treatment for advanced disease [4–6].

Somatic activating mutations of *EGFR* gene and some clinical and pathological features such as women, never smokers, patients with adenocarcinoma, and Asians have been associated with tumor response and favorable clinical outcome with these agents [7].

The specific types of activating mutations that confer sensitivity to EGFR tyrosine kinase inhibitors (TKIs) are present in the TK domain of the *EGFR* gene. Exon 19 deletion mutations and the single-point substitution

mutation L858R in exon 21 are the most frequent (account for about 44 and 41%, respectively, of all *EGFR*-TK mutations) in non-small cell lung cancer (NSCLC) and are termed "classical" mutations. Other activating mutations result in a glycine-719 (G719) change to serine, alanine, or cysteine (4% of all *EGFR*-TK mutations), and missense mutations account for another 6% of *EGFR* mutations. Other mutations are in-frame duplications and/or insertions in exon 20 (5% of *EGFR*-TK mutations). A variety of other activating mutations have been detected with low frequency, including V765A and T783A (<1%) in exon 20 [8].

Although *EGFR* mutations are associated with an enhanced sensitivity to gefitinib and erlotinib, not all tumors that have activating mutations are associated with an enhanced response. Tumors that fail to respond to EGFR-TKIs despite the presence of an activating mutation might have an additional genetic lesion that relieves the tumor of its dependence on the EGFR signaling pathway. One mechanism that has been linked to insensitivity of NSCLC to EGFR-TKIs is the occurrence of insertion point mutations in exon 20 of the *EGFR* gene such as D770_N771 (ins NPG), D770_(ins SVQ), and D770_(ins G) N771T [8, 9]. In an in vitro model system, insertion mutations in exon 20 render transformed cells less responsive to EGFR-TKIs compared with the sensitizing mutations of exons 19 and 21 [9]. Different mechanisms of resistance to EGFR-TKIs have also been described. Mutations of *KRAS*, which occur in 20–30% of NSCLCs, mainly in adenocarcinomas (30%) and smokers, seem to confer resistance to EGFR-TKIs and have been reported to be mutually exclusive with *EGFR* or *HER2* gene mutations [10–13]. KRAS is a downstream mediator of EGFR-induced cell signaling, and ras mutations confer constitutive activation of the signaling pathways without EGFR activation. Several retrospective studies showed prolonged survival in gefitinib-treated patients with tyrosine kinase activating mutations [14–19].

Other mechanisms of resistance to EGFR inhibitors could involve activating mutations of the other main EGFR effector pathway, i.e., the PI3K/PTEN/AKT pathway. Phosphatidylinositol 3-kinase (PI3K) is an activator of Akt, which regulates cell tumoral survival. Akt has been shown to frequently be activated in NSCLC, suggesting that the PI3K/Akt pathway plays an important role in the oncogenesis and progression of human lung cancers.

It is well established that PI3K is activated by the stimulation of receptor tyrosine kinases. Recently, in addition to this mechanism, somatic mutations of *PIK3CA* clustering in exons 9 and 20, a gene which encodes the catalytic subunit, have been shown to play an important role in the activation of PI3K and the Akt signaling pathway [20]. In our previous study, *PIK3CA* mutations appeared to be the indicators of resistance and poor survival in NSCLC patients treated with EGFR-TKIs [21].

The NSCLC tumors insensitive to EGFR-TKIs also include those driven by the mesenchymal-epithelial transition (*MET*) factor oncogene. The *MET* receptor tyrosine kinase and its cognate ligand hepatocyte growth factor (HGF) promote cell proliferation, invasion motility, and angiogenesis in in vitro studies. In cell line studies, mutations of MET have been identified, which lead to its increased expression. Although *MET* mutations did not occur frequently, the study of Lim et al. [22] shows that *MET* mutations were significantly correlated with decreased survival. Taken together, these findings suggest that several different molecular alterations regulate the sensitivity of NSCLC cells to EGFR-TKIs, and that a comprehensive approach to this phenomenon is necessary for an appropriate selection of patients that should be treated with these drugs. Therefore, we present a comprehensive analysis of *KRAS*, *PIK3CA*, *MET,* and non-sensitizing *EGFR* mutations in advanced NSCLC patients treated with TKIs, with the aim of clarifying the relative contribution of these molecular alterations to resistance.

Materials and methods

Patient selection

This retrospective study was conducted in a cohort of 240 patients with NSCLC followed up at the Medical Oncology Department, S. Maria della Misericordia Hospital in Perugia (Italy) who had been treated with gefitinib or erlotinib after failure of platinum-based chemotherapy.

Patients were included into the analysis if they had histologically confirmed the diagnosis of NSCLC with locally advanced (stage III) or metastatic disease, progressing or relapsing after chemotherapy, or with medical contraindications for chemotherapy; availability of tumor tissue and full clinical data; and the presence of at least one measurable lesion according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria [23]. Patients were classified as nonsmokers (never smoker), light smokers (<10 pack year and quit >10 years ago), and smoker.

Patients had performance status ranging from grade 0 to 2. Performance status was defined according to the Eastern Cooperative Oncology Group [24]. Treatment consisted of 250 mg of gefitinib or erlotinib 150 mg administered orally once daily and continued until disease progression, intolerable toxicity, or patient refusal. Tumor response was assessed by computed tomography scan after 2 months, with a confirmatory evaluation to be repeated in responders

and in patients with stable disease at least 4 weeks after the initial determination of response. Time to disease progression was calculated from the date of initiation of TKIs treatment to the date of detection of progressive disease or to the date of last contact.

Survival was calculated from the date of therapy initiation to the date of death or to the date of last contact. Survival status was collected until the end of December 2009. Median duration of follow-up was 24.1 months (range: 4.9–118 months). The study was approved by the local Ethics Committee and was conducted in accordance with ethical principles stated in the most recent version of the Declaration of Helsinki. Written informed consent was obtained from each patient before enrollment.

DNA sequencing

Formalin-fixed paraffin-embedded tissues obtained before any cancer therapy were retrieved from the archives of the Department of Histology and Pathology of Perugia University (Italy). Serial sections (4 μm) containing representative malignant cells were stained with hematoxylin and eosin and classified based on the World Health Organization criteria [25]. The tumor samples for molecular analysis were obtained from broncoschopic biopsy or from computed tomography/ultrasound-guided needle biopsy, or from percutaneous aspiration (lymph nodes and skin metastasis), or surgery. Formalin-fixed paraffin-embedded (FFPE) tumor blocks were reviewed for quality and tumor

Table 1 Primers used in *EGFR*, *PIK3CA*, *KRAS,* and *MET* mutation analysis

Gene	Sense (5′–3′)	Antisense (5′–3′)
EGFR exon 18		
External	ATGGTGAGGGCTGAGGTGAC	TCCCCACCAGACCATGAGAG
Internal	GACCCTTGTCTCTGTGTTCTTGT	CCAGACCATGAGAGGCCCTG
EGFR exon 19		
External	GCAATATCAGCCTTAGGTGCGGCTC	CATAGAAAGTGAACATTTAGGATGTG
Internal	CCTTAGGTGCGGCTCCACAGC	CATTTAGGATGTGGAGATGAGC
EGFR exon 20		
External	CCATGAGTACGTATTTTGAAACTC	CATATCCCCATGGCAAACTCTTGC
Internal	GAAACTCAAGATCGCATTCATGC	GCAAACTCTTGCTATCCCAGGAG
EGFR exon 21		
External	CTAACGTTCGCCAGCCATAAGTCC	GCTGCGAGCTCACCCAGAATGTCTGG
Internal	CAGCCATAAGTCCTCGACGTGG	CATCCTCCCCTGCATGTGTTAAAC
PIK3CA exon 9		
External	TCTGTAAATCATCTGTGAAT	AATTCTGCTTTATTTATTCC
Internal	ATCATCTGTGAATCCAGAGG	TTTTAGCACTTACCTGTGAC
PIK3CA exon 20		
External	AACATCATTTGCTCCAAACT	TGTGGAATCCAGAGTGAGCT
Internal	GCTCCAAACTGACCAAACTG	ATCCAGAGTGAGCTTTCATT
KRAS exon 2		
External	GTTCTAATATAGTCACATTT	ACTCATGAAAATGGTCAGAGAAACCTTTAT
Internal	AGTCACATTTTCATTATTTT	AGAAACCTTTATCTGTATCAAAGAATG
KRAS exon 3		
External	GAAGTAAAAGGTGCACTGTA	AACTATAATTACTCCTTAAT
Internal	GTGCACTGTAATAATCCAGA	ACTCCTTAATGTCAGCTTAT
MET exon 14		
External	CTTTAACAAGCTCTTTCTTTCT	TGTATAGGTATTTCTCAGAA
Internal	CTTTAACAAGCTCTTTCTTTCT	TGTATAGGTATTTCTCAGAA
MET exon 15		
External	TTGTTCTTTAATAATTTTC	GAGTCGAAAAACAATTTATGCT
Internal	CCCATTAAATGAGGTTTTACTGT	AATCGTATTTAACAAAAAGCTGAG
M13	TGTAAAACGACGGCCAGT	CAGGAAACAGCTATGACC

EGFR epidermal growth factor receptor, *PIK3CA* phosphoinositide-3-kinase catalytic alpha, *KRAS* Kirsten rat sarcoma viral oncogene homolog, *MET* mesenchymal-epithelial transition factor

content. Tumor cells (at least 70%) were macro-dissected, and genomic DNA was isolated using the QIAmp DNA extraction kit and automatically purified by BioRobot EZ1 instrument (Qiagen S.p.A., Milan, Italy) according to the manufacturer's instructions. Nested PCRs were carried out using primers (Table 1) to amplify exons 18–21 of *EGFR*, exons 9 and 20 of *PIK3CA*, exons 2–3 of *KRAS,* and exons 14–15 of *MET*. PCR products were purified with Exonuclease 1 and Shrimp Alkaline Phosphatase (ExoSAP-IT) at 37°C for 15 min followed by heating at 80°C for 15 min to stop the enzymatic reaction. After purification, the PCR products were sequenced with forward and reverse M13 primers and Big Dye Terminator v1.1 Cycle Sequencing Kit. Sequencing fragments were detected by capillary electrophoresis using 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA). Electropherograms were analyzed for the presence of mutations using SeqScape v2.7 Software. In all cases, samples harboring mutations were re-amplified and re-sequenced using the same experimental conditions.

Statistical analysis

The statistical analyses of categorical variables were done using Pearson's χ^2 test or Fisher's exact test where appropriate. The median durations of overall survival and time to progression were calculated using the Kaplan–Meier method. Comparisons between different groups were made using the log-rank tests. We defined "TKI non-sensitizing mutations," all the mutations of *KRAS*, *PIK3CA*, *MET*, and non-sensitizing *EGFR* mutations (in exon 20), and they were used for response to TKIs, time to disease progression (TTP), and overall survival (OS) analysis. The response to TKIs, as a dependent variable, with clinical and molecular factors was also investigated by logistic regression analysis to account for the effect of the different variables. Multivariate analyses were done using a logistic regression model for response and stepwise Cox regression models for TTP and OS to identify independent biomarkers and to adjust for baseline characteristics. All statistical tests were two-sided, and statistical significance defined as $p < 0.05$ was considered significant. All analyses were done using Matlab software (The MathWorks ver. 7.2.0.232).

Results

Patient characteristics

Complete clinical information were available from 166 of 240 patients with advanced NSCLC. All the patients were whites, and clinical and demographic characteristics are presented in Table 2. Median age was 60.2 years (range: 25.6–84 years), the majority of patients were men (54.8%) and with good performance status (0–1: 95.8%). Adenocarcinoma was the most frequent histology (61.5%), most of the patients were never and former smokers (65.1%). Seventy-five (45.2%) patients were treated with gefitinib and 91 (54.8%) patients with erlotinib. Twenty-four patients received EGFR-TKIs therapy as first-line treatment, 99 as second-line, and 43 as third-line. Standard platinum-based doublets, as treatment for first-line, were offered to 125 of 142 (75.3%) patients. Seventeen (10.2%)

Table 2 Clinical and demographic characteristics of the patient population

Characteristics	No. of cases	%
All patient	166	100
Median age, years (range)	60.2 (25.6–84)	
Sex		
Male	91	54.8
Female	75	45.2
Stage		
III	25	15.1
IV	141	84.9
Histology		
ADC	102	61.5
BAC	17	10.2
SCC	28	16.9
LCC	6	3.6
Mixed[a]	7	4.2
Not otherwise specified NSCLC	6	3.6
Smoking history		
Never smokers	61	36.8
Former smokers	47	28.3
Current smokers	46	27.7
Unknown	12	7.2
Therapy		
Total patients treated with EGFR-TKI therapy	166	100
Patients treated with gefitinib	75	45.2
Patients treated with erlotinib	91	54.8
Total patients treated with previous chemotherapy	142/166	85.5
Patients treated with platinum-based chemotherapy	125	75.3
Patients treated with non-platinum compounds	17	10.2

ADC adenocarcinoma, *BAC* bronchioloalveolar carcinoma, *SCC* squamous cell carcinoma, *LCC* large cell carcinoma, *EGFR-TKI* epidermal growth factor receptor-tyrosine kinase inhibitor

[a] 6 patients had adenosquamous carcinoma and 1 patient had large cell neuroendocrine carcinoma (50%), adenocarcinoma 30%, and squamous cell carcinoma 20%

patients received single agent chemotherapy with gemcitabine or vinorelbine because of age (13 patients) or due to the presence of comorbidity contraindicating platinum-based chemotherapy (4 cases).

Frequency of *EGFR*, *KRAS*, *PIK3CA,* and *MET* mutations and their correlation with patients' clinical and pathologic features

We performed mutation analysis of EGFR in 166 samples (84 from broncoschopic biopsy, 25 samples from surgery, 30 from computed tomography/ultrasound-guided needle biopsy, and 27 from percutaneous aspiration). We detected 41 *EGFR* mutations (24.6%) in the 166 patients evaluated: 36 sensitizing *EGFR* mutations (28 deletions in exon 19 and 8 point mutations in exon 21 (L858R)), 5 non-sensitizing *EGFR* mutations [4 insertion/duplication in exon 20 (768insNPH, 766insMAS)], and 1 point mutation in exon 20 (S784F). *KRAS* mutations at codons 12, 13, and 61 were evaluated in 162 samples (84 from broncoschopic biopsy, 25 samples from surgery, 30 from computed tomography/ultrasound-guided needle biopsy, and 23 from percutaneous aspiration) and were found in 11 (6.8%) patients (two G12C, G12V, G12D, G12R, and Q61H and one G12A). *EGFR* mutations and *KRAS* mutations were mutually exclusive, as reported by other authors [15], while an overlapping pattern was observed among other alterations.

PIK3CA mutations were evaluated in 145 samples (84 from broncoschopic biopsy, 25 samples from surgery, 20 from computed tomography/ultrasound-guided needle biopsy, and 16 from percutaneous aspiration) and were mutated in 6 patients (4.1%): two point mutations in exon 9 (E542K, E545K) and four point mutations in exon 20 (two M1043I and one H1047Y, G1049S, and each). Two patients with *PIK3CA* mutation in exon 20 (M1043I) had adenocarcinomas harboring an *EGFR* mutation S784F in exon 20, and an *EGFR* mutation (deletion in exon 19), respectively.

Owing to yielded insufficient DNA for mutation testing, *MET* mutations were evaluated in 106 samples (76 from broncoschopic biopsy, 25 samples from surgery, and 5 from computed tomography/ultrasound-guided needle biopsy) and were mutated in 4 (3.8%) patients: four point mutations in exon 14 (two T1010I, one R988C and G893S). One patient with *MET* mutation in exon 14 (T1010I) had adenocarcinomas harboring an *EGFR* mutation (768insNPH in exon 20).

Some sequence chromatograms of sensitizing *EGFR* mutations are shown in Fig. 1, whereas those of *KRAS*, *PIK3CA*, *MET,* and non-sensitizing *EGFR* mutations are shown in Fig. 2.

EGFR mutations were significantly more frequent in ADC/BAC/Mixed histology (30.2 vs. 10.0%, $p = 0.01$). The associations with sex and smoking status were not statistically significant ($p = 0.10$, $p = 0.10$, respectively), although *EGFR* mutations were more frequent in women and in nonsmokers. No significant associations of *PIK3CA*, *KRAS,* and *MET* mutations were detected with the clinical and pathological features. No difference was found according to inhibitor administered (gefitinib or erlotinib) and the presence or absence of *EGFR* mutations (data not shown).

Response to EGFR-TKIs according to molecular and clinical parameters

For the entire group, the objective response rate to EGFR-TKIs was 33.1% (13 complete and 42 partial responses),

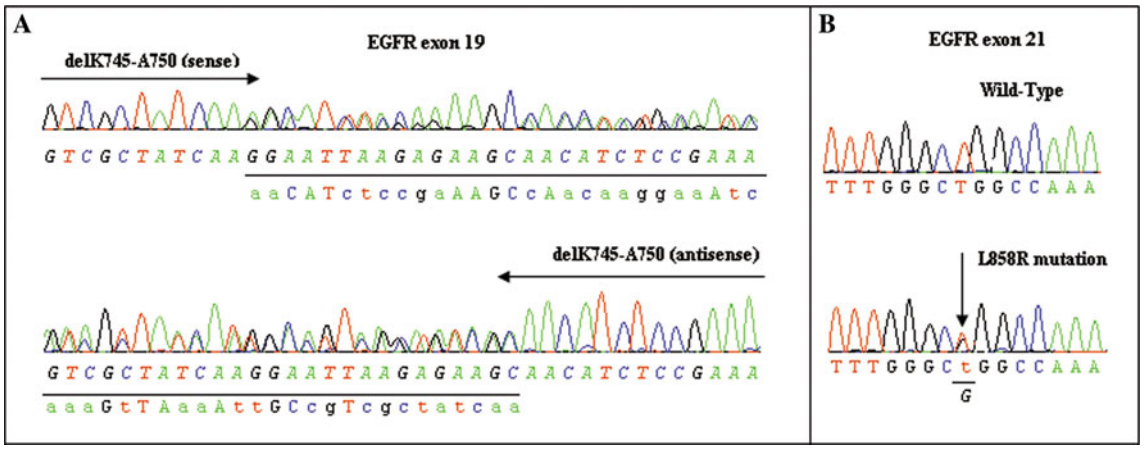

Fig. 1 Sensitizing *EGFR* mutations. **a** Shows the nucleotide sequence of the *EGFR* gene in tumor specimens with heterozygous in-frame deletion (delK745-A750) in exon 19 (double peaks). Tracing in both sense and antisense directions are shown to demonstrate the two breakpoints of the deletion; the wild-type nucleotide sequence is shown in *capital letters* and the mutant sequence is in *lowercase letters*. **b** Shows the heterozygous mutation of *EGFR* in exon 21: a T to G base-pair change (*arrow*), resulting in a substitution of Leucine for Arginine at position 858

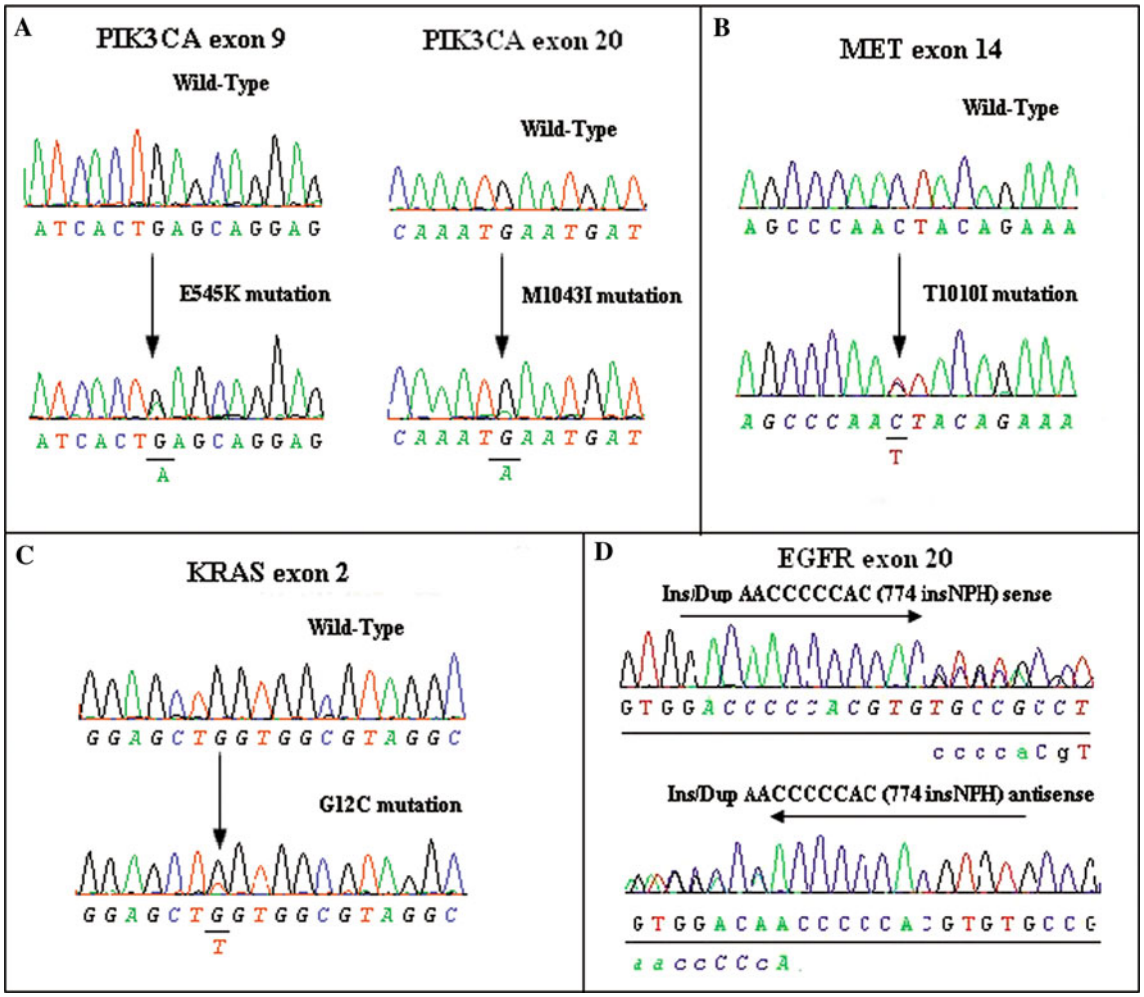

Fig. 2 Mutations in *PIK3CA*, *MET*, *KRAS,* and non-sensitizing EGFR genes. **a** Shows the heterozygous mutation of *PIK3CA* in exons 9 and 20: a G to A base-pair change (*arrow*), resulting in a substitution of Glutamic Acid for Lysine at position 545, a G to A base-pair change (*arrow*), resulting in a substitution of Methionine for Isoleucine at position 1043. **b** Shows the heterozygous mutation of *MET* in exon 14: a C to T base-pair change (*arrow*), resulting in a substitution of Treonine for Isoleucine at position 1010. **c** Shows the heterozygous mutation of *KRAS* in exon 2: a G to T base-pair change (*arrow*), resulting in a substitution of Glycine for Cysteine at position 12. **d** Shows the nucleotide sequence of the *EGFR* gene in tumor specimens with heterozygous in duplication (774ins NPH) in exon 20 (double peacks). Tracing in both sense and antisense directions is shown to demonstrate the duplication; the wild-type nucleotide sequence is shown in *capital letters* and the mutant sequence is in *lowercase letters*

disease control rate (complete response + partial response + stable disease) was 57.2%, median time to progression was 8.9 months, median survival was 31.9 months, and 1-year survival was 78.3%.

As summarized in Table 3, the presence of sensitizing *EGFR* mutation was significantly associated with objective response to TKIs treatment (75.0 vs. 23.1%, $p < 0.0001$). None of the patients with non-sensitizing *EGFR* mutations showed a response to TKIs treatment. The presence of *KRAS* mutation was significantly associated with the lack of response to TKIs treatment (0.0 vs. 35.0%, $p = 0.01$) while *PIK3CA* and *MET* mutations did not show a statistical association with response ($p = 0.34$ and $p = 0.30$, respectively). Patients with "TKI non-sensitizing mutations" showed a significant association with the lack of response (96.0%, $p = 0.001$) confirmed at multivariate analysis ($p = 0.008$, Table 4). The demographic and histology data for response to TKIs were similar to those previously reported (data not shown) [21].

Time to progression (TTP) and overall survival (OS) according to molecular and clinical parameters

"TKI non-sensitizing mutations" were associated with shorter TTP (2.2 vs. 6.6 months, $p < 0.0001$, Fig. 3; Table 5) and worse OS (16.1 vs. 30.2 months, $p = 0.03$, Fig. 4; Table 5). Longer median TTP was observed in patients with sensitizing *EGFR* mutations ($p = 0.007$). Median TTP was

Table 3 Response to TKIs according to molecular alterations

	Whole study population TKIs			
	Total No. (%)	CR + PR No. (%)	SD + PD No. (%)	p^*
Sensitizing *EGFR* (Del19, L858R)				
Mut	36 (21.6)	27 (75.0)	9 (25.0)	<0.0001
Other group	130 (78.4)	30 (23.1)	100 (76.9)	
Non-sensitizing *EGFR* (other mutations)				
Mut	5 (3.1)	0 (0.0)	5 (100.0)	0.11
Other group	161 (96.9)	55 (34.2)	106 (65.8)	
KRAS				
Mut	11 (6.8)	0 (0.0)	11 (100.0)	0.01
W T	151 (93.2)	53 (35.1)	98 (64.9)	
PIK3CA				
Mut	6 (4.1)	1 (16.8)	5 (83.2)	0.34
W T	139 (95.9)	49 (35.2)	90 (64.8)	
MET				
Mut	4 (3.8)	0 (0.0)	4 (100.0)	0.30
W T	102 (96.2)	35 (34.3)	67 (65.7)	
All mutations "TKI non-sensitizing"				
Mut	25 (15.1)	1 (4.0)	24 (96.0)	0.001
Other group	141 (84.9)	54 (38.3)	87 (61.7)	

CR complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *EGFR* epidermal growth factor receptor, *PIK3CA* phosphoinositide-3-kinase catalytic alpha, *KRAS* Kirsten rat sarcoma viral oncogene homolog, *MET* mesenchymal-epithelial transition factor, *Mut* mutated, *WT* wild type

*$p < 0.05$ statistically significant

Table 4 Logistic regression and multivariate analyses of molecular and clinical parameters for response to TKIs, time to progression, and overall survival

Variables	Response to TKIs		
	OR	95% CI	p^*
Logistic regression			
All mutations "TKIs non-sensitizing" (mutated vs. other group)	6.08	2.09–12.3	0.008
ECOG-PS (1-2-3 vs. 0)	2.00	0.93–4.28	0.07
	HR	95% CI	p^*
Time to progression			
Cox proportional hazard model			
Sensitizing *EGFR* (del19, L858R) (mutated vs. wild type)	0.29	0.12–0.68	0.005
All Mutations "TKIs non-sensitizing" (mutated vs. other group)	2.76	1.61–4.74	<0.0001
ECOG-PS (1-2-3 vs. 0)	2.47	1.55–3.95	<0.0001
Overall survival			
Cox proportional hazard model			
All mutations "TKIs non-sensitizing" (mutated vs. other group)	1.79	1.12–2.86	0.01
Histology (ADC/BAC/mixed vs. other)	0.63	0.41–0.88	0.009
ECOG-PS (1-2-3 vs. 0)	1.98	1.37–2.87	<0.0001

ECOG eastern cooperative oncology group, *PS* performance status, *EGFR* epidermal growth factor receptor, *TKI* tyrosine kinase inhibitors, *ADC* adenocarcinoma, *BAC* bronchioloalveolar, *OR* odds ratio, *HR* hazard ratio, *CI* confidence interval

*$p < 0.05$ statistically significant

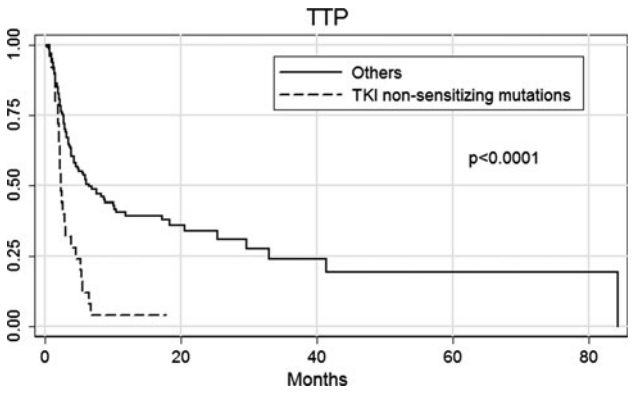

Fig. 3 Time to progression according to "TKI non-sensitizing mutations" from TKIs start

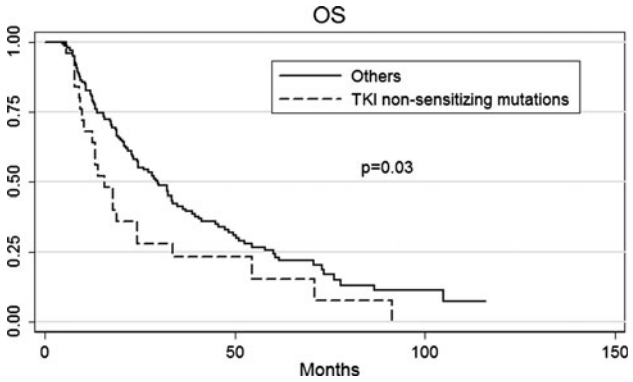

Fig. 4 Overall survival according to "TKI non-sensitizing mutations" from TKIs start

significantly shorter in the patients with mutated *PIK3CA* ($p = 0.01$), whereas TTP did not differ according to *MET* and *KRAS* mutation ($p = 0.06$ and $p = 0.12$, respectively).

A statistically significant shorter OS was observed for the patients with mutant *PIK3CA* ($p = 0.01$), whereas OS did not differ according to *KRAS*, *MET*, sensitizing *EGFR* mutation, and non-sensitizing *EGFR* mutation ($p = 0.47$, $p = 0.66$, $p = 0.12$, and $p = 0.77$, respectively).

For clinical variables, the patients with a PS of 0 had statistically significant longer TTP and better OS than patients with a PS > 1 ($p = 0.0003$ and $p = 0.0003$, respectively). On the other hand, patients with adenocarcinoma/bronchioloalveolar histologies had statistically significantly better OS and a trend toward longer TTP than patients with other histological types ($p = 0.009$ and $p = 0.06$, respectively), (data not shown).

Multivariate analyses of molecular and clinical factors for survival

A multivariate Cox regression model for OS and TTP was built using the variables that were found significant at the univariate analysis. "TKI non-sensitizing mutations" (hazard ratio (HR) 1.79, 95% CI 1.12–2.86; $p = 0.01$) and performance status (1–2–3 vs. 0) (HR 1.98, 95% CI 1.37–2.87; $p < 0.0001$) were statistically significant predictors of worse OS and histology (ADC/BAC/Mixed vs. Other) (HR 0.63, 95% CI 0.41–0.88; $p = 0.009$) of better OS (Table 4).

"TKI non-sensitizing mutations" (HR 2.76, 95% CI 1.61–4.74; $p < 0.0001$) and performance status (1-2-3 vs. 0) (HR 2.47, 95% CI 1.55–3.95; $p < 0.0001$) were statistically significant predictors of shorter TTP, whereas sensitizing *EGFR* mutation (HR 0.29, 95% CI 0.12–0.68; $p = 0.005$) of longer TTP (Table 4).

Discussion

Various clinical characteristics and molecular factors have been associated with sensitivity and resistance to EGFR-TKIs. The discovery and characterization of *EGFR* activating mutations and their relationship to sensitivity to gefitinib and erlotinib have provided a basis for transforming NSCLC from a disease treated with conventional combination chemotherapy to one in which subsets of patients with specific *EGFR* mutations can be effectively treated with targeted therapy. It is reasonable to suggest that personalized therapy for NSCLC patients should include a genetic assessment of the *EGFR* mutational status for individual patients. In this study, we evaluated the mutations of *EGFR*, *KRAS*, *PIK3CA,* and *MET* genes in advanced NSCLC patients treated with TKIs with the aim of clarifying the relative contribution of these molecular alterations to resistance.

However, the relative and overall contribution of each of these molecular alterations to clinical decision making remains unclear. Furthermore, whether and to what extent the occurrence of multiple molecular alterations affects clinical response and patient survival is presently unknown.

In this study, we exploited the comprehensive molecular analysis of EGFR downstream effectors to ascertain their role in predicting response/resistance to TKIs in advanced NSCLC. The overall response rate to TKIs of 33.1% observed in our study was higher than the previously reported rate in a Caucasian population [4–6]. This result can partially be attributed to the fact that the physicians tended to select patients with characteristics known to be predictive for TKIs sensitivity: women, never smokers, and patients with adenocarcinoma. Moreover, the *EGFR* mutation rate of 23% found in our study should be the consequence of the larger number of never smoker patients included.

Our findings that sensitizing *EGFR* mutations were a significant predictor of favorable response to EGFR-TKIs ($p = 0.007$) and that sensitizing *EGFR* mutations were associated with a trend toward longer OS ($p = 0.12$;

Table 5 Time to progression and overall survival according to molecular alterations

	Time to progression				Overall survival			
	Months	HR	95% CI	*p*	Months	HR	95% CI	*p**
Sensitizing *EGFR* (del19, L858R)								
Mut	6.7	0.31	0.13–0.72	0.007	36.9	0.67	0.41–1.10	0.12
Other group	2.7				24.8			
Non-sensitizing *EGFR* (other mutations)								
Mut	5.6	1.85	0.67–5.09	0.23	27.6	1.15	0.42–3.14	0.77
Other group	4.63				18.3			
KRAS								
Mut	2.7	1.83	0.84–4.02	0.12	19.3	1.26	0.66–2.42	0.47
W T	5.6				28.6			
PIK3CA								
Mut	2.3	3.18	1.26–7.98	0.01	9.9	3.35	1.34–8.36	0.01
W T	6.0				30.2			
MET								
Mut	5.1	3.11	0.95–10.19	0.06	18.5	1.24	0.45–3.43	0.66
W T	6.6				29			
All mutations "TKI non-sensitizing"								
Mut	2.2	2.81	1.65–4.68	<0.0001	16.1	1.67	1.05–2.65	0.03
Other group	6.6				30.2			

EGFR epidermal growth factor receptor, *PIK3CA* phosphoinositide-3-kinase catalytic alpha, *KRAS* Kirsten rat sarcoma viral oncogene homolog, *MET* mesenchymal-epithelial transition factor, *WT* wild type, *Mut* mutated, *HR* hazard ratio, *CI* confidence interval

*$p < 0.05$ statistically significant

Table 5) are in line with the existing literature [19, 26, 27]. Furthermore, four recent Asian randomized controlled trials compared gefitinib to doublet chemotherapy for the treatment for first-line metastatic or locally advanced NSCLC in clinically selected patients (adenocarcinoma, never or former light smokers) or in EGFR-TK-mutated patients [28–30]. In all four trials, gefitinib showed a significant reduction in HR for time to progression with a statistically significant benefit in progression-free survival and better outcomes in absolute terms in OS and response rate. We document that concomitant detection of *KRAS*, *PIK3CA*, *MET*, and non-sensitizing *EGFR* mutations in advanced NSCLC patients has remarkable clinical implications by increasing the ability to predict the outcome of EGFR-targeted therapies in patients harboring the EGFR-TKIs sensitizing mutations.

By the concomitant assessment of four molecular alterations, we were able to identify up to 90% of non-responder patients, a result that has never been achieved before. Our data indicate that multiple mutations of *KRAS*, *PIK3CA*, *MET,* and non-sensitizing *EGFR*, unfavorably affect the clinical outcome of gefitinib- or erlotinib-based therapies; however, the possibility that these molecular alterations could be negative prognostic biomarkers independent of targeted therapies should be taken into account.

Our study has some limitations: it is a retrospective study, and the used method to detect molecular analysis by direct sequencing has a relatively sensitivity. Although various methods are used to detect EGFR mutations, there is no consensus on which method is most efficacious. Direct DNA sequencing is the current gold standard for the detection of EGFR mutations and is still widely used to uncover novel mutations. However, the type of EGFR mutation identified in our study is in accordance with previous studies, and the patients with sensitizing EGFR mutation showed a positive clinical response to EGFR-TKI. Moreover, the study of Kim et al. [31] compared two methods (direct DNA sequencing and peptide nucleic acid (PNA)-mediated real-time PCR clamp) to evaluate the correlation between EGFR mutation status and clinical response to EGFR-TKI in NSCLC patients showed that clinical outcomes were not significantly different between the groups harboring activating mutations detected by each method, even if PNA-mediated real-time PCR clamp exhibited high sensitivity than direct DNA sequencing.

In light of the nature of our patient series, the most reliable indicator of the predictive value of biomarker(s) is objective tumor response. Interpretation of survival analyses should indeed take into account a possible limitation due to patients treated with mixed previous line(s) of chemotherapy including the 15% (24/166) of patients

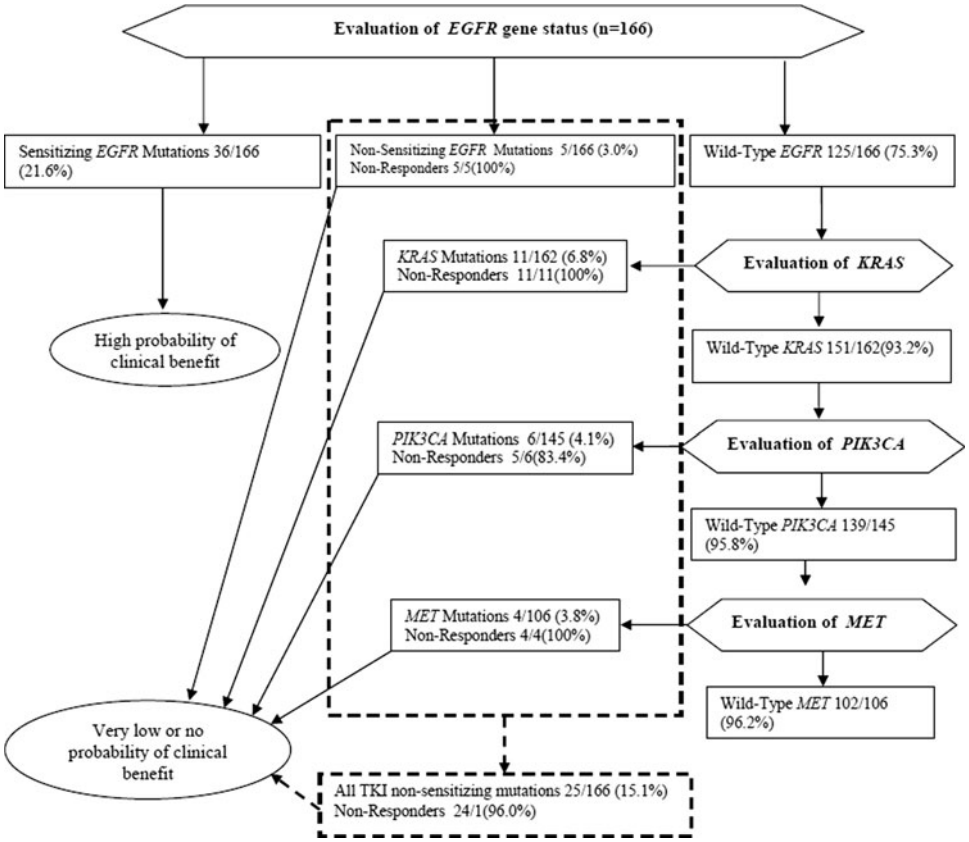

Fig. 5 Following evaluation of *EGFR* status in individual tumor enhancement of predictability of very low or no probability of clinical benefit may derive from the assessment of the status of *KRAS*, *PIK3CA,* and *MET*, as simulated here based on the analyses of subgroups from the present cohort ($n = 166$). We propose to define as "TKI non-sensitizing mutations" the NSCLCs alterations in *KRAS*, *PIK3CA*, *MET,* and non-sensitizing *EGFR* mutations

treated with first-line TKIs monotherapy. On the other hand, the study of such patients represents a unique opportunity to ascertain the predictive value of a given biomarker without the influence of chemotherapy, either concurrent or previous, as well as of selection exerted by other treatments. In view of these considerations, we propose here a new algorithm for deciding the clinical use of EGFR-targeted molecular therapies that is based on objective response rates (Fig. 5). This novel approach deserves validation in prospective studies with TKIs-based therapies in NSCLC before having an impact in changing clinical practice. Importantly, we found that approximately 96% of NSCLC non-responders harbored mutations of *KRAS*, *PIK3CA*, *MET,* and non-sensitizing *EGFR,* and we propose to define these tumors as "TKI non-sensitizing mutations". Further molecular dissections of the EGFR-initiated oncogenic signaling cascade are likely to be helpful in improving the tailoring of EGFR-targeted therapies. Identifying specific combinations of genetic alterations in tumors will be of importance in selecting synergistic combinations of inhibitors. As more drugs targeting transduction pathway proteins become available and as we increase our knowledge of the complexity of these signaling networks, the burden of selecting the correct drug combination for each individual cancer patient will ultimately depend on tumor alterations. Overall, our results underscore the relevance of using molecular-based algorithm to shift the treatment for solid tumors into the era of personalized cancer medicine.

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