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Genotyping *KRAS* and *EGFR* Mutations in Greek Patients With Non-small-cell Lung Cancer: Incidence, Significance and Implications for Treatment

HELENA LINARDOU[1], VASSILIKI KOTOULA[2,3], GEORGE KOUVATSEAS[4], GIANNIS MOUNTZIOS[5], VASILIOS KARAVASILIS[6], EPAMINONDAS SAMANTAS[7], ANNA KALOGERA-FOUNTZILA[8], DESPINA TELEVANTOU[3], KYRIAKI PAPADOPOULOU[3], XANTHIPI MAVROPOULOU[8], EMILY DASKALAKI[2], THOMAS ZARAMBOUKAS[2], IOANNIS EFSTRATIOU[9], SOFIA LAMPAKI[6], GRIGORIOS RALLIS[6], ELENI RES[7], KONSTANTINOS N. SYRIGOS[10], PARIS A. KOSMIDIS[11], DIMITRIOS PECTASIDES[12] and GEORGE FOUNTZILAS[3,13]

[1]*Oncology Unit, Metropolitan Hospital, Athens, Greece;*
[2]*Department of Pathology, Aristotle University of Thessaloniki, School of Health Sciences, Faculty of Medicine, Thessaloniki, Greece;*
[3]*Laboratory of Molecular Oncology, Hellenic Foundation for Cancer Research/ Aristotle University of Thessaloniki, Thessaloniki, Greece;*
[4]*Health Data Specialists Ltd, Athens, Greece;*
[5]*Second Oncology Department, Henry Dunant Hospital Center, Athens, Greece;*
[6]*Department of Medical Oncology, Papageorgiou Hospital, Faculty of Medicine, Aristotle University of Thessaloniki, School of Health Sciences, Thessaloniki, Greece;*
[7]*Third Department of Medical Oncology, Agii Anargiri Cancer Hospital, Athens, Greece;*
[8]*Department of Radiology, AHEPA Hospital, School of Health Sciences, Faculty of Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece;*
[9]*Department of Pathology, Papageorgiou Hospital, Thessaloniki, Greece;*
[10]*Oncology Unit GPP, Sotiria General Hospital, National and Kapodistrian University of Athens School of Medicine, Athens, Greece;*
[11]*Second Department of Medical Oncology, Hygeia Hospital, Athens, Greece;*
[12]*Oncology Section, Second Department of Internal Medicine, Hippokration Hospital, Athens, Greece;*
[13]*Aristotle University of Thessaloniki, Thessaloniki, Greece*

Abstract. *Background/Aim: KRAS mutations are reported in 20-25% of non-small cell lung cancer (NSCLC) and their prognostic role is unclear. We studied KRAS and EGFR genotyping in Greek NSCLC patients. Patients and Methods: KRAS and EGFR genotypes were centrally evaluated in 421 NSCLC patients (diagnosed September 1998 -June 2013) and associated with clinicopathological parameters. Outcome comparisons were performed in 288 patients receiving first line treatment. Results: Most patients were male (78.6%), >60 years old (63.9%), current smokers (51.1%), with adenocarcinoma histology (63.9%). EGFR and KRAS mutations were found in 10.7% and 16.6% of all histologies, respectively, and in 14.9% and 21.9% of adenocarcinomas. At 4.5 years median follow-up, KRAS status was an independent negative prognostic factor for overall survival (OS, p=0.016). KRAS mutations conferred 80% increased risk of death in patients receiving first-line treatment (p=0.002). Conclusion: The presence of KRAS mutations is an independent negative prognosticator among Greek NSCLC patients and an independent response predictor to first line treatment.*

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Correspondence to: Helena Linardou, Oncology Unit, Metropolitan Hospital, Ethn. Makariou 9, Athens, 18547, Greece. Tel: +30 2104809852, e-mail: elinardou@otenet.gr

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Non-small-cell lung cancer (NSCLC) is the primary cause of cancer-related mortality worldwide, with more than one million deaths per year (1). With our increasing understanding of lung cancer biology, we are now able to recognise many molecular subtypes of NSCLC, based on the

identification of diverse molecular events, which play a central role in lung cancer growth and metastasis. These genetic alterations include driver mutations in several genes, such as *EGFR, KRAS, BRAF, HER2*, or translocations, such as in ALK or ROS-1. Pivotal studies and comprehensive reviews have summarised data on the importance of *EGFR* mutations and other molecular alterations and their respective inhibitors that have changed the natural history of oncogene-driven NSCLC (2-4). Although the predictive role of activating *EGFR* mutations on treatment with EGFR tyrosine kinase inhibitors (EGFR-TKIs) is well established, evidence is still inconclusive on their prognostic value (5-7).

In contrast to *EGFR* mutations that have been the paradigm shift in lung cancer management, the proto-oncogene *Kirsten Rat Sarcoma virus* (*KRAS*) mutations are the 'waterloo' of molecular targeting, a continuous story of fruitless attempts in identifying effective therapies, for what is the commonest known oncogene mutated in NSCLC. *KRAS* mutations are reported in approximately 20-25% of adenocarcinomas, and in a substantially lower number of squamous-cell carcinomas (5-8%) and are thought to be involved in many phases of cancer-cell transformation (8). The most common oncogenic mutations of the *KRAS* proto-oncogene are point mutations in codons 12 and 13, with the commonest types including G12C, G12V and G12D (9). Preclinical evidence has suggested a differential biological behaviour and chemosensitivity among different types of mutations, resulting in clinical attempts to identify a possible differential prognostic and predictive role (10, 11). *KRAS* mutations are generally mutually exclusive with EGFR and other oncogenic mutations in NSCLC; however, there are reports of co-existence of these diverse molecular events (12, 13).

Many clinicopathological features, such as gender, age, histology and smoking history have been correlated with *EGFR* and *KRAS* mutations. The latter are found more commonly in smokers; nevertheless, recently *KRAS* mutations have been reported with an incidence of up to 15% in never smokers with NSCLC (14), while there are reports of different mutation types associated with smoking status (15). Interestingly, although for EGFR it has been well established that mutation frequency is ethnicity dependent, very little is known about *KRAS* mutations frequency among different ethnic groups (16).

In view of the unclear picture of the role of *KRAS* in advanced NSCLC, and given that ethnicity may play a role on the mutational profiling of tumors, we report here on the first genotype mapping of NSCLC in Greek patients, aiming to investigate the incidence and prognostic significance of *KRAS* and *EGFR* mutational status.

Patients and Methods

Study population. In this retrospective analysis, performed by the Hellenic Co-operative Oncology Group (HeCOG), samples from patients with histologically confirmed NSCLC, who had been treated within HeCOG-affiliated centres from September 1998 through June 2013, were centrally evaluated for the presence of *KRAS* and *EGFR* mutations. All patients had available clinicopathological data at diagnosis. The following information was collected from the HeCOG clinical database: age at diagnosis, gender, smoking status, stage at diagnosis, histology, and details on treatments received (surgery, first line chemotherapy, platinum compounds, EGFR TKIs), best response achieved, as well as clinical outcomes of first line treatments (ORR, PFS, OS), and *EGFR* and *KRAS* mutation status at diagnosis. The study and all treatments were conducted in accordance with the Good Clinical Practice (GCP) guidelines, and the Helsinki Declaration and were approved by the Scientific Committee of HeCOG. The translational protocol was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Health Sciences, Faculty of Medicine (4.34/4-6-2010; A13064/16-7-2010). All patients had signed informed consent for the use of their biological material for translational research purposes.

Tissue processing. Formalin-fixed, paraffin-embedded tissue blocks were retrospectively retrieved from the HeCOG tumor repository. Cytologic material was prospectively submitted for genotyping in more recent years (2010-2013). All tumors were initially evaluated at local pathology laboratories. Subsequently, paraffin-embedded tumor blocks and cytologic material in CytoLyt containers (Hologic, Manchester, UK) were sent to the Laboratory of Molecular Oncology of HeCOG for central *KRAS* and *EGFR* testing, which was implemented following central histology review. Out of 441 submitted materials, 8 biopsy samples were excluded upfront due to absent or inadequate (<200 per sample) tumor cells on the provided paraffin block. Consequently, biological material was processed for 433 patients. Tumors were centrally reviewed for histology and tumor cell content (TCC%). Manual macrodissection was applied for enrichment in TCC wherever possible. DNA was extracted with a standard protocol using the QIAamp DNA mini kit (Qiagen, Hilden, Germany), measured in an Eppendorf Biophotometer, and normalized at 50 ng/μl.

KRAS and EGFR genotyping. All 433 samples were submitted for *KRAS* genotyping with a routinely used qPCR Taqman-MGB allelic discrimination assay targeting the 7 most common mutations in codons 12 and 13 (17). All samples were also analysed with dd-sequencing on nested PCR products with M13-coupled, intron-spanning primers for *KRAS* exon 2 (coordinates according to GRCh38 for KRAS on chr12: 25245453-25245233). Mutations in the ATP-binding pocket of the EGFR kinase domain were assessed with dd-sequencing as above for the following GRCh37 coordinates on chr7: exon 18 (55241512-55241795); exon 19 (55242380-55242570); exon 20 (55248954-55249194); and, exon 21 (55259354-55259591). Samples were sequenced in both directions with the BigDye Terminator v1.1 Cycle Sequencing Kit and analysed in an ABI3130XL system (Applied Biosystems/Life Technologies). Samples were considered as non-informative (a) with qPCR if the cycle threshold [CT; crossing point (CP)] was ≥36 for the control wild type allele contained in each assay, and (b) with dd-sequencing, for failed sense and antisense capillary electrophoresis for all targets in both genes. By using these criteria, informative sequencing data were obtained for 424 tumors (96% of all submitted tumors; 98% of analyzed samples). The 10 underperforming samples corresponded to 7 biopsies, 1 surgical specimen and 2 fine needle aspirates from the tumor.

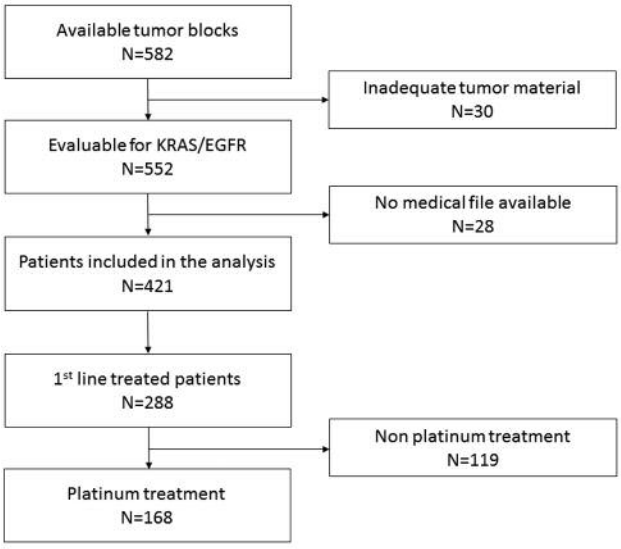

Figure 1. *REMARK diagram*.

Statistical methods. Associations between *KRAS*, *EGFR* mutations (mutated *vs*. wild-type) and clinicopathological parameters were performed in the entire cohort as well as in the first-line treated subgroup of patients and were evaluated with the chi-square or Fisher's exact test (where appropriate). Progression-free survival (PFS) was measured from the date of initiation of first line treatment until the first verified disease progression, death from any cause or date of last contact, whichever occurred first, while overall survival (OS) from the date of initial diagnosis until death from any cause or last contact. PFS was assessed in the subgroup of patients with available treatment and follow-up data, following first line treatment, while OS was examined in the entire cohort. Surviving patients (for OS and PFS) and patients without relapse (for PFS) were censored at the date of last contact. The Kaplan–Meier method was used for estimating time-to-event distributions, while log-rank tests were used for comparison of survival curves among groups. The associations between the examined factors and relapse/mortality rates were evaluated with hazard ratios estimated with Cox proportional hazards models.

Statistical significance was set at 5% (two-sided). The statistical analysis complied with the reporting recommendations for tumor marker prognostic studies (18) and was performed using the SAS software (SAS for Windows, version 9.3, SAS Institute Inc., Cary, NC, USA).

Results

Patients and genotype characteristics. *KRAS* and *EGFR* genotypes were evaluated in a total of 421 NSCLC patients. Among these, 288 patients had received first line treatment for advanced, inoperable disease (Figure 1); 18 patients had not received chemotherapy, while treatment status was not available for 17.6% of the patients, probably due to the retrospective nature of the analysis. Clinicopathological characteristics are detailed in Table I. More patients were males (78.6%), older than 60 years (63.9%), current smokers (51.1%) and had tumors with adenocarcinoma histology (63.9%). With respect to histologic type, because all tumors and genotyping methods were applied before the end of 2013, bronchioloalveolar carcinoma (BAC) was distinguished from adenocarcinomas; the term has been discontinued in the currently valid 2015 WHO Classification of Lung Tumors (19) and this tumor type is herein discussed as adenocarcinoma.

EGFR mutations were found in 45 tumors and *KRAS* mutations in 70 tumors, corresponding to 10.7% and 16.6% of all histological types, respectively, and to 14.9% and 21.9% of adenocarcinomas. Mutation details can be found in the following link (https://www.hecog.gr/images/stories/pdf/PAPERS_ONLINE/EGFR_and_KRAS_mutation_details_for_all_421_NSCLC_patients.pdf). Most *EGFR* mutations were of the classical type (72.6%), with an almost equal representation of the exon 19 p.E746_A750delELREA and the exon 21 p.L858R point mutation (Figure 2A); 41 of them were registered in the COSMIC database. The T790M resistance mutation was found in one tumor. The most common *KRAS* mutations were p.G12C, p.G12D and p.G12V (Figure 2B). *EGFR* and *KRAS* mutations were mutually exclusive except for one tumor that had both *EGFR* and *KRAS* mutations. Finally, it should be noted that 23 out of the 45 patients with an *EGFR* activating mutation received first line treatment with an EGFR TKI, probably since these drugs were not the standard of care for *EGFR* mutant NSCLC until 2011.

Associations. *EGFR* mutations were significantly associated with female gender, adenocarcinoma histology and non-smoking status, as previously described (Tables II and III). *KRAS* mutations were associated with adenocarcinoma histology in the population with available first line data. In our study, the presence of KRAS mutations was not associated with smoking status either in the whole study population or among patients with advanced disease receiving first-line treatment (Tables IV and V).

Effect of KRAS/EGFR mutational status on overall survival in the whole study population. Regarding overall survival (OS) in the whole patient population with available follow-up data (N=377), independent favourable prognostic factors were the best response to treatment, (CR or PR *vs*. other, Wald's p=0.017), platinum *vs*. non-platinum treatment p=0.001, earlier disease stage (I-II) *vs*. advanced disease stage (III-IV) (p<0.001), and surgical removal of the primary tumour *vs*. no surgery (p<0.001). At a median follow-up of 4.5 years, in the univariate analysis for the entire population, *KRAS* mutant status was an adverse prognostic factor for overall survival (p=0.016), while *EGFR* status did not show

Table I. *Clinicopathological patient characteristics*.

Patients	421
Age (N=420)	
Median	63.3
Min-Max	26.0-88.0
<60 years	151 (35.9%)
≥60 years	269 (63.9%)
Not reported	1 (0.2%)
Gender	
Male	331 (78.6%)
Female	90 (21.4%)
Smoking status	
Current smoker	215 (51.1%)
Former smoker	34 (8.1%)
Never smoker	118 (28.0%)
Not reported	54 (12.8%)
Histology	
Adenocarcinoma	269 (63.9%)
Squamous cell carcinoma	97 (23.0%)
Large cell	15 (3.6%)
Bronchioloalveolar carcinoma	39 (9.3%)
Not reported	1 (0.2%)
Stage	
I	31 (7.4%)
II	43 (10.2%)
IIIA	76 (18.1%)
IIIB	31 (7.4%)
IV	210 (49.9%)
Not reported	30 (7.1%)
Surgery	
Yes	141 (33.5%)
No	249 (59.1%)
Not reported	31 (7.4%)
Treatment type	
1st line	288 (68.4%)
3rd line	1 (0.2%)
Adjuvant	40 (9.5%)
No chemotherapy	18 (4.3%)
Not reported	74 (17.6%)
Platinum-based therapy*	
Yes	207 (62.9%)
No	121 (36.8%)
Not reported	1 (0.3%)
TKIs*	
Yes	60 (18.2%)
No	268 (81.5%)
Not reported	1 (0.3%)
Objective response rate*	
Complete or partial response	114 (34.7%)
No response	161 (48.9%)
Not reported	54 (16.4%)

*For patients that received chemotherapy of any line (N=329).

any significant associations with OS (p=0.45), most likely due to the small sample size. The effect of *KRAS* mutation was strong, with a median difference in OS of approximately 10 months between *KRAS* wt and mut patients (Figure 3).

Effect of KRAS/EGFR mutational status on outcomes in patients with advanced NSCLC receiving first-line treatment. In the subpopulation of patients receiving first line treatment for advanced disease (N=288), the presence of *KRAS* mutations, was also an independent adverse prognostic factor for survival with an 80% increase in the risk of death (HR=1.80, 95%CI=1.25-2.60, p=0.002, Figure 4), while *EGFR* status did not show any significant associations (p=0.53). In the same population, it seemed that patients receiving platinum-based chemotherapy had improved OS *vs*. those receiving non-platinum-based chemotherapy, irrespective of *EGFR* status (HR=0.45, 95%CI=0.14-1.54, p=0.21 and HR=0.86, 95%CI=0.64-1.15, p=0.31 for the *EGFR* mut and wt subgroups respectively, test for interaction p=0.31), even though significance was not reached. When we combined the effect of the presence of any mutation (either *EGFR* or *KRAS*) on OS in patients treated with first-line platinum-based chemotherapy, a marginally non-significant difference was observed, with patients harbouring either EGFRmut or KRASmut tumors bearing worse prognosis as compared to any wt (HR=1.52, 95%CI=0.97-2.38, p=0.066). Furthermore, *KRAS* mutational status showed shorter OS, as compared to either *EGFR* mut or wt status on both genes (Log-rank p=0.015) for all patients receiving first line treatment regardless of platinum or non-platinum regimen (Figure 5). Detailed analyses according to type of response (complete or partial response *vs*. other) did not show any significant differences based on *EGFR* or *KRAS* status, most likely due to smaller sample sizes.

The effect of different mutation types on outcome. No significant associations were observed in analyses per *EGFR* mutation type (*EGFR* classic *vs*. non-classic), most likely due to the small patient numbers. With respect to *KRAS* mutation type, analysis has shown that patients with the most common mutations (p.G12C/D/V) *vs*. other less common, had similar outcomes [median OS 12.6 months (95%CI=8.6-20.6) *vs*. 11.8 months (95%CI=0.2-16.4)] and again these fared significantly worse than *KRAS* wt patients (median OS 22.9 months (95%CI=19.2-28.4), log-rank p=0.009).

Discussion

The genomic diversity of NSCLC across different geographical areas and ethnic groups necessitates analyses in ethnically homogenous populations in order to elucidate epidemiological differences and to capture genomic alterations with clinical relevance. To our knowledge, this is the first publication of data on central *EGFR* and *KRAS* genotyping in the Greek population with NSCLC. We found that the incidence of *EGFR* mutations in Greek patients was 10.7% across all histological types and 14.9% in adenocarcinomas. A recent large meta-analysis of 456

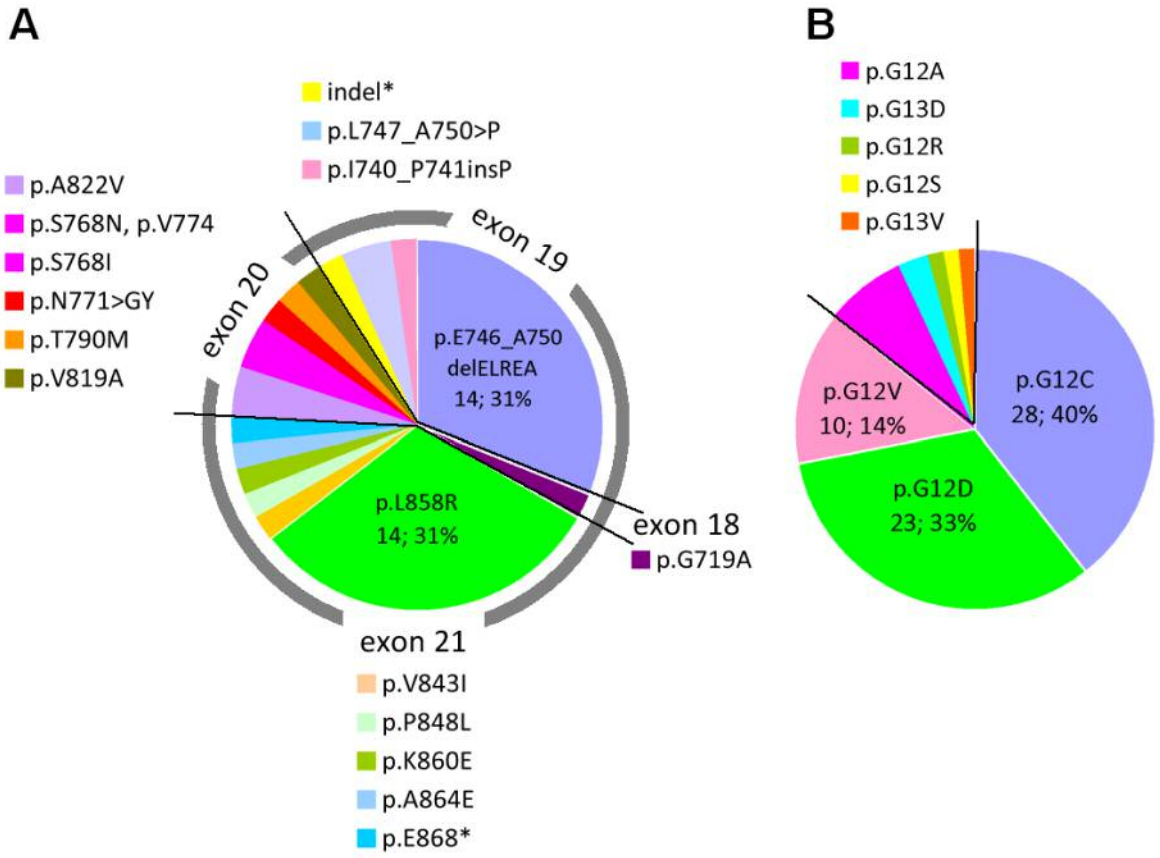

Figure 2. *Types of mutations detected. A: EGFR mutations, B: KRAS mutations.*

published studies on the prevalence of *EGFR* mutations (30,466 patients with an *EGFR* mutation reported among 115,815 NSCLC patients), showed an overall pooled *EGFR* mutation prevalence of 32.3%, which varied by geographical area, with Asia exhibiting the higher prevalence (38.4%), followed by North and South America (24.4%) and Europe showing the lowest prevalence (14.1%) (20). Results varied similarly in ethnic groups, with a prevalence of 17.4% in Caucasians rising to 19.2% in patients with adenocarcinoma (20). Furthermore, very few studies have reported on intra-ethnic differences across Europe. From the published data, the *EGFR* mutation incidence in Europe ranges between 6% in Switzerland, 11% in France to 37.5% in Germany, and is dependent on ethnicity and on clinicopathological characteristics (21, 22). Different incidences have been previously reported for Greek patient populations, from as low as 8.2% to as high as 15.83% (23). In these studies, however, analyses were performed in local laboratories and included patients with diverse characteristics, *i.e.* in the study with the higher prevalence, 82.5% of the patients had adenocarcinomas (23). In our population, all tumor samples were centrally genotyped and 63.9% of the patients had adenocarcinoma, which is representative for the Greek population; the reported incidence is within the expected range for a Caucasian population in Europe, and is consistent with the incidence reported in 2016 in a large cohort of approximately 1,500 Greek patients included in a National program for early access to EGFR-TKis (10.04%) (24). Furthermore, in agreement with previous knowledge, the presence of *EGFR* mutations in our study was significantly associated with female gender, adenocarcinoma histology and non-smoking status (25, 26).

With respect to *KRAS* mutations, the corresponding prevalence was 16.6%, rising to 21.9% among adenocarcinomas. In various studies in the European population, the reported incidence varies from 15-25%, depending on the geographical area, ethnicity and clinicopathological characteristics (9, 27). Given the high prevalence of *KRAS* mutations among smokers and the fact that Greece is one of the European countries with the highest percentage of smokers (51%) (28), one could expect a higher incidence than the one observed. Since, however, this is a centrally genotyped population, these results are valid and the low incidence might be attributed to epidemiological

Table II. *Associations of EGFR mutation status with clinicopathological parameters in all patients.*

	EGFR		
	wt (N=376)	mut (N=45)	*p*-Value
Gender			<0.001
Man	305 (81.1)	26 (57.8)	
Woman	71 (18.9)	19 (42.2)	
Histology			0.003
Adenocarcinoma	229 (61.1)	40 (88.9)	
BAC	37 (9.9)	2 (4.4)	
Large cell	14 (3.7)	1 (2.2)	
SCC	95 (25.3)	2 (4.4)	
Stage			0.012
I	25 (7.1)	6 (14.6)	
II	43 (12.3)	0 (0.0)	
IIIa	72 (20.6)	4 (9.8)	
IIIb	29 (8.3)	2 (4.9)	
IV	181 (51.7)	29 (70.7)	
Smoking status			<0.001
Current smoker	205 (62.1)	10 (27.0)	
Former smoker	28 (8.5)	6 (16.2)	
Never smoker	97 (29.4)	21 (56.8)	
TKIs*			<0.001
No	255 (87.6)	13 (35.1)	
Yes	36 (12.4)	24 (64.9)	
Platinum-based therapy*			<0.001
No	93 (32.0)	28 (75.7)	
Yes	198 (68.0)	9 (24.3)	

*For patients that received chemotherapy of any line. mut: Mutant; wt: wild-type.

Table III. *Associations of EGFR mutation status with clinicopathological parameters in patients treated with first line chemotherapy.*

	EGFR		
	wt (N=253)	mut (N=35)	*p*-Value
Gender			<0.001
Man	213 (84.2)	20 (57.1)	
Woman	40 (15.8)	15 (42.9)	
Histology			0.016
Adenocarcinoma	157 (62.3)	31 (88.6)	
BAC	23 (9.1)	2 (5.7)	
Large cell	10 (4.0)	1 (2.9)	
SCC	62 (24.6)	1 (2.9)	
Stage			0.031
I	9 (3.6)	4 (11.8)	
II	16 (6.5)	0 (0.0)	
IIIa	44 (17.8)	2 (5.9)	
IIIb	25 (10.1)	2 (5.9)	
IV	153 (61.9)	26 (76.5)	
Smoking status			<0.001
Current smoker	151 (65.4)	9 (30.0)	
Former smoker	20 (8.7)	4 (13.3)	
Never smoker	60 (26.0)	17 (56.7)	
TKIs*			<0.001
No	217 (86.1)	12 (34.3)	
Yes	35 (13.9)	23 (65.7)	
Platinum-based therapy*			<0.001
No	92 (36.5)	27 (77.1)	
Yes	160 (63.5)	8 (22.9)	
Objective response			0.12
CR or PR	96 (41.7)	15 (57.7)	
Else	134 (58.3)	11 (42.3)	

*For patients that received chemotherapy of any line. mut: Mutant; wt: wild-type.

differences and genomic diversity. As previously described, *KRAS* mutations were associated with adenocarcinoma histology and younger age (<60 years), while the frequencies of different *KRAS* mutation types were also within the previously reported ranges in European studies (23, 29). Smoking status is known to correlate with *KRAS* mutations and there are recent reports associating different smoking habits with different mutation types (14, 15).

One of the main aims of our study was to investigate the prognostic and predictive role of *KRAS* and *EGFR* mutations in NSCLC patients, both in the whole study population and in the subgroup receiving first line chemotherapy. We have found that at a median follow-up of 4.5 years, in the univariate analysis for the entire population, *KRAS* status was prognostic for worse overall survival (p=0.016). This effect was consistent, with a 10-month difference in OS between *KRAS* mutated and wild-type patients. When the effect of *KRAS* mutations on OS of patients treated with platinum-based therapy, was compared among different mutational groups (*EGFR* mut *vs*. *KRAS* mut *vs*. *EGFR+KRAS* wt), it was shown that patients with *KRAS* mutations retained the worst outcome regardless of the type of first line treatment

Table IV. *Associations of KRAS mutation status with clinicopathological parameters in all patients.*

	KRAS		
	wt (N=351)	mut (N=70)	*p*-Value
Age			0.048
<60	119 (33.9)	32 (46.4)	
≥60	232 (66.1)	37 (53.6)	
Stage			0.28
I	27 (8.4)	4 (5.9)	
II	38 (11.8)	5 (7.4)	
IIIa	67 (20.7)	9 (13.2)	
IIIb	24 (7.4)	7 (10.3)	
IV	167 (51.7)	43 (63.2)	
Smoking status			0.39
Current smoker	174 (57.4)	41 (64.1)	
Former smoker	27 (8.9)	7 (10.9)	
Never smoker	102 (33.7)	16 (25.0)	

mut: Mutant; wt: wild-type.

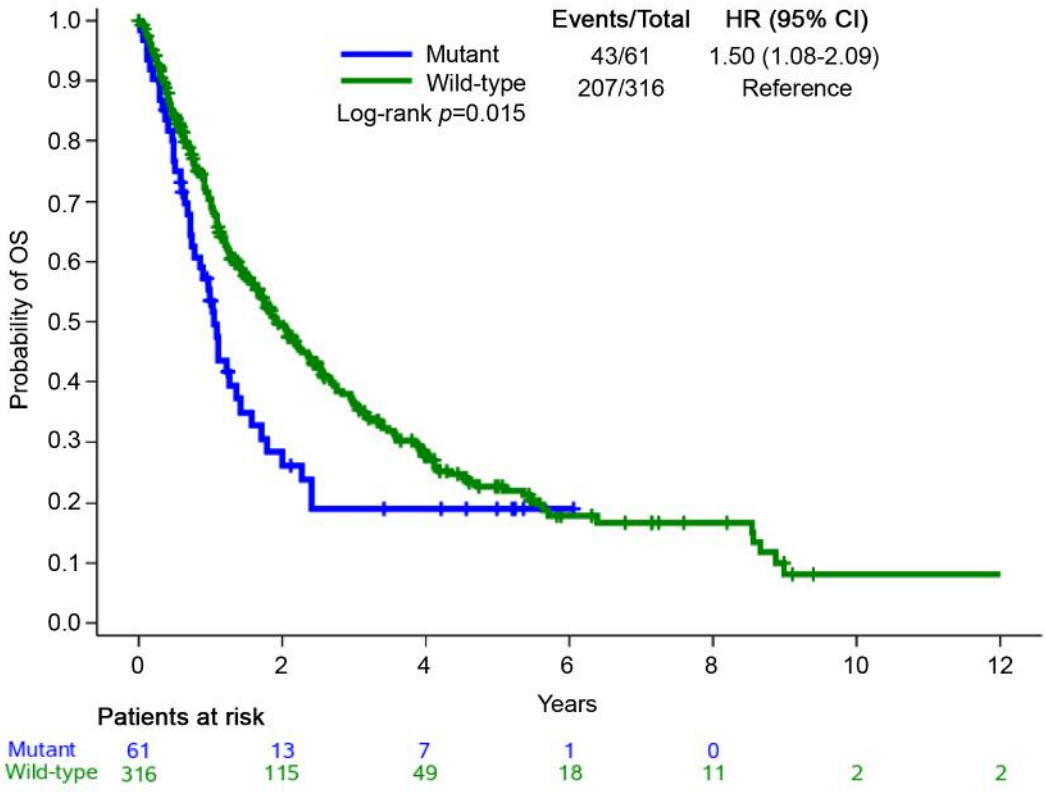

Figure 3. *Effect of KRAS mutational status on overall survival (OS) in the whole study population.*

(platinum or non-platinum). On the other hand, *EGFR* status did not show any significant associations with OS, most likely due to the small sample size in our study. These results come to add to the existing evidence on the prognostic role of *KRAS* mutations in advanced NSCLC, which remains largely controversial. In the early disease setting, a meta-analysis of four large adjuvant trials reported that *KRAS* mutational status was not prognostic for neither PFS nor OS (15). On the other hand, data in the metastatic setting are more persuasive on the negative prognostic impact of *KRAS* mutations, although contradictory studies have been published (9, 29-34). A recently published meta-analysis in the advanced disease setting suggested a detrimental effect of *KRAS* mutations on survival in patients with adenocarcinoma histology (9); more recent publications, focusing on the detection of *KRAS* mutations in cell-free DNA (cfDNA) in blood samples, attempted to provide stronger supportive evidence on the adverse prognostic role of *KRAS* in advanced NSCLC (31, 35, 36). Finally, recently published data have suggested that *KRAS*-mutation incidence and prognostic value are metastatic site-specific in lung adenocarcinoma, implicating *KRAS* mutations as independent predictors for the development of brain and bone metastases (37, 38).

Table V. *Associations of KRAS mutation status with clinicopathological parameters in patients with available first line clinical data.*

	KRAS		
	wt (N=241)	mut (N=47)	*p*-Value
Age			0.13
<60	87 (36.1)	22 (47.8)	
≥60	154 (63.9)	24 (52.2)	
Histology			0.011
Adenocarcinoma	149 (62.1)	39 (83.0)	
BAC	23 (9.6)	2 (4.3)	
Large cell	8 (3.3)	3 (6.4)	
SCC	60 (25.0)	3 (6.4)	
Smoking status			0.079
Current smoker	127 (58.8)	33 (73.3)	
Former smoker	19 (8.8)	5 (11.1)	
Never smoker	70 (32.4)	7 (15.6)	
TKIs			0.32
No	189 (78.8)	40 (85.1)	
Yes	51 (21.3)	7 (14.9)	
Platinum-based therapy			0.076
No	105 (43.8)	14 (29.8)	
Yes	135 (56.3)	33 (70.2)	

mut: Mutant; wt: wild-type.

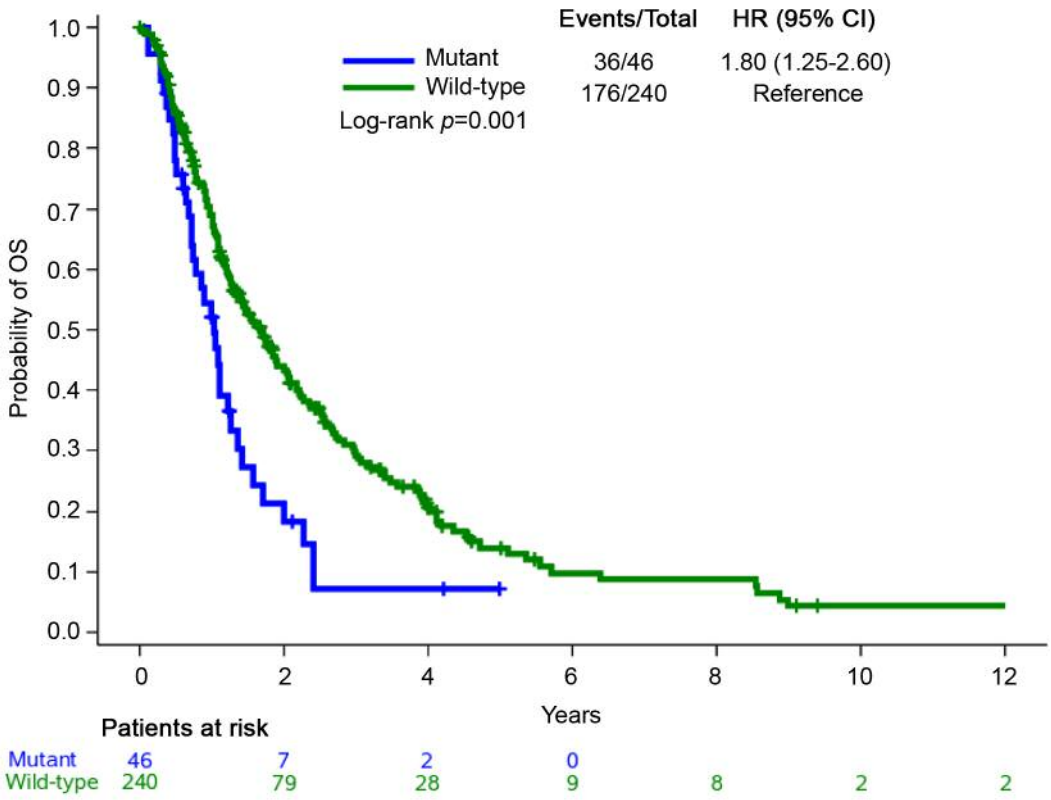

Figure 4. *Effect of KRAS mutational status on overall survival (OS) in the subgroup of first line treated patients.*

Another open issue with respect to *KRAS* status is its predictive value for response and outcome to first line therapies in NSCLC. In colorectal cancer it is well established that KRAS mutational status adversely affects response to EGFR-targeted agents (27); however, in NSCLC, a similar view is not generally accepted for response prediction to EGFR TKIs (39), although a few studies have proposed *KRAS* mutations as a predictive factor of poor response to first line chemotherapy (40-42). Of note, among patients with advanced NSCLC treated with immune check-point inhibitors, the presence of *KRAS* mutations was predictive for response to immunotherapy (43), probably due to the higher incidence of *KRAS* mutations in smokers, who usually carry a larger tumor mutation load (9). On the other hand, in patients with tumors harbouring activating EGFR mutations and treated with EGFR-TKIs, the presence of KRAS mutations was not associated with prognosis (44).

Previous reports have indicated that different point mutations might confer different biological behaviour, resulting in differential prognostic and possibly predictive effect for the various mutations. Although preclinical data are highly suggestive of this idea of a diverse molecular entity (10), the clinical evidence is not yet there (9, 25). In our study, when analyses were performed according to *KRAS* mutation type, it was shown that patients with the most common mutations (p.G12C/D/V) *vs*. other less common had similar outcomes (median OS 12.6 months *vs*. 11.8 months) and again these fared significantly worse than *KRAS* wt patients (median OS 22.9 months). Similarly, we were not able to observe significant associations or differences when analyses were performed per *EGFR* mutation type (EGFR classic *vs*. non-classic), most likely in both cases due to small patient numbers.

In the present study, *EGFR* and *KRAS* genotype incidences and prognostic significance are presented for Greek patients with metastatic NSCLC. Our results confirm the adverse prognostic significance of *KRAS* mutations for survival, both in the whole study population and importantly among patients receiving platinum-based first line treatment, while we were not able to detect prognostic differences among diverse types of mutations. These results add to the existing body of evidence for the role of *KRAS* mutations and support the negative prognostic value of this biomarker in advanced NSCLC. Furthermore, our results showed that the presence of *KRAS* mutations can strongly predict the response to first line platinum-based therapy, as patients with *KRAS* mutant tumours faired significantly worse than those with *KRAS* wt tumours,

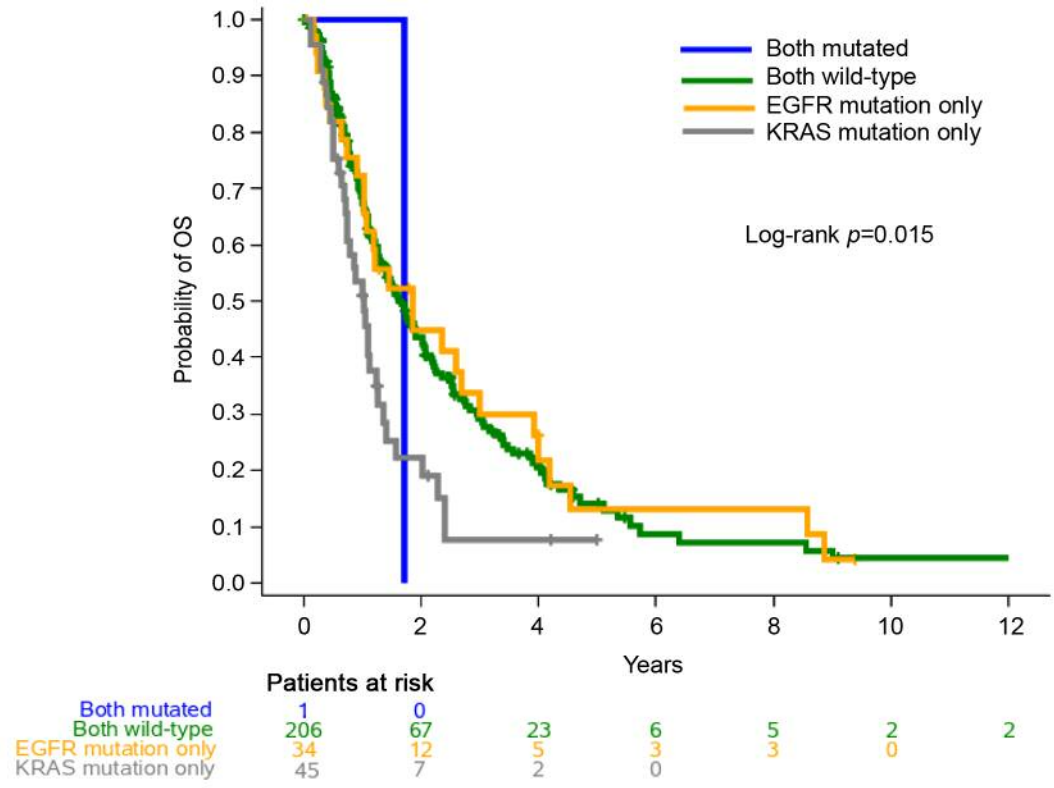

Figure 5. *Overall survival according to mutational status for all patients receiving first-line treatment.*

with first line platinum-based treatment. These results further suggest that the presence of *KRAS* mutations could indicate the need for alternative or additional therapies to platinum-based chemotherapy, strengthening the evidence that *KRAS* mutations represent a marker of resistance to platinum-based therapy.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

HL and VK designed the study, GK provided statistical analysis, HL, VK and GM contributed primarily in the writing of the manuscript, while all other authors contributed in the collection of data, completion of study, and reviewing the writing of the manuscript. All Authors approved the final manuscript.

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References

1 Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in globocan 2012. Int J Cancer *136(5)*: E359-386, 2015. PMID: 25220842. DOI: 10.1002/ijc.29210

2 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J and Haber DA: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med *350(21)*: 2129-2139, 2004. PMID: 15118073. DOI: 10.1056/NEJMoa040938

3 Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, Fujiwara S, Watanabe H, Kurashina K, Hatanaka H, Bando M, Ohno S, Ishikawa Y, Aburatani H, Niki T, Sohara Y, Sugiyama Y and Mano H: Identification of the transforming eml4-alk fusion gene in non-small-cell lung cancer. Nature *448(7153)*: 561-566, 2007. PMID: 17625570. DOI: 10.1038/nature05945

4 Tan DS, Yom SS, Tsao MS, Pass HI, Kelly K, Peled N, Yung RC, Wistuba, II, Yatabe Y, Unger M, Mack PC, Wynes MW, Mitsudomi T, Weder W, Yankelevitz D, Herbst RS, Gandara DR, Carbone DP, Bunn PA, Jr., Mok TS and Hirsch FR: The international association for the study of lung cancer consensus statement on optimizing management of egfr mutation-positive non-small cell lung cancer: Status in 2016. J Thorac Oncol *11(7)*: 946-963, 2016. PMID: 27229180. DOI: 10.1016/j.jtho.2016.05.008

5 Shepherd FA and Tsao MS: Unraveling the mystery of prognostic and predictive factors in epidermal growth factor receptor therapy. J Clin Oncol *24(7)*: 1219-1220, 2006. PMID: 16505443. DOI: 10.1200/jco.2005.04.4420

6 Zhang Z, Wang T, Zhang J, Cai X, Pan C, Long Y, Chen J, Zhou C and Yin X: Prognostic value of epidermal growth factor receptor mutations in resected non-small cell lung cancer: A systematic review with meta-analysis. PLoS One *9(8)*: e106053, 2014. PMID: 25162713. DOI: 10.1371/journal.pone.0106053

7 Pesek M, Benesova L, Belsanova B, Mukensnabl P, Bruha F and Minarik M: Dominance of egfr and insignificant kras mutations in prediction of tyrosine-kinase therapy for nsclc patients stratified by tumor subtype and smoking status. Anticancer Res *29(7)*: 2767-2773, 2009. PMID: 19596959.

8 Satoh T, Nakafuku M and Kaziro Y: Function of ras as a molecular switch in signal transduction. J Biol Chem *267(34)*: 24149-24152, 1992. PMID: 1447166.

9 Wood K, Hensing T, Malik R and Salgia R: Prognostic and predictive value in kras in non-small-cell lung cancer: A review. JAMA Oncol *2(6)*: 805-812, 2016. PMID: 27100819. DOI: 10.1001/jamaoncol.2016.0405

10 Garassino MC, Marabese M, Rusconi P, Rulli E, Martelli O, Farina G, Scanni A and Broggini M: Different types of k-ras mutations could affect drug sensitivity and tumour behaviour in non-small-cell lung cancer. Ann Oncol *22(1)*: 235-237, 2011. PMID: 21169473. DOI: 10.1093/annonc/mdq680

11 Ihle NT, Byers LA, Kim ES, Saintigny P, Lee JJ, Blumenschein GR, Tsao A, Liu S, Larsen JE, Wang J, Diao L, Coombes KR, Chen L, Zhang S, Abdelmelek MF, Tang X, Papadimitrakopoulou V, Minna JD, Lippman SM, Hong WK, Herbst RS, Wistuba, II, Heymach JV and Powis G: Effect of kras oncogene substitutions on protein behavior: Implications for signaling and clinical outcome. J Natl Cancer Inst *104(3)*: 228-239, 2012. PMID: 22247021. DOI: 10.1093/jnci/djr523

12 Lee T, Lee B, Choi YL, Han J, Ahn MJ and Um SW: Non-small cell lung cancer with concomitant egfr, kras, and alk mutation: Clinicopathologic features of 12 cases. J Pathol Transl Med *50(3)*: 197-203, 2016. PMID: 27086595. DOI: 10.4132/jptm.2016.03.09

13 Lee B, Lee T, Lee SH, Choi YL and Han J: Clinicopathologic characteristics of egfr, kras, and alk alterations in 6,595 lung cancers. Oncotarget *7(17)*: 23874-23884, 2016. PMID: 26992209. DOI: 10.18632/oncotarget.8074

14 Riely GJ, Kris MG, Rosenbaum D, Marks J, Li A, Chitale DA, Nafa K, Riedel ER, Hsu M, Pao W, Miller VA and Ladanyi M: Frequency and distinctive spectrum of kras mutations in never smokers with lung adenocarcinoma. Clin Cancer Res *14(18)*: 5731-5734, 2008. PMID: 18794081. DOI: 10.1158/1078-0432.ccr-08-0646

15 Shepherd FA, Domerg C, Hainaut P, Janne PA, Pignon JP, Graziano S, Douillard JY, Brambilla E, Le Chevalier T, Seymour L, Bourredjem A, Le Teuff G, Pirker R, Filipits M, Rosell R, Kratzke R, Bandarchi B, Ma X, Capelletti M, Soria JC and Tsao MS: Pooled analysis of the prognostic and predictive effects of kras mutation status and kras mutation subtype in early-stage resected non-small-cell lung cancer in four trials of adjuvant chemotherapy. J Clin Oncol *31(17)*: 2173-2181, 2013. PMID: 23630215. DOI: 10.1200/jco.2012.48.1390

16 Steuer CE, Behera M, Berry L, Kim S, Rossi M, Sica G, Owonikoko TK, Johnson BE, Kris MG, Bunn PA, Khuri FR, Garon EB and Ramalingam SS: Role of race in oncogenic driver prevalence and outcomes in lung adenocarcinoma: Results from the lung cancer mutation consortium. Cancer *122(5)*: 766-772, 2016. PMID: 26695526. DOI: 10.1002/cncr.29812

17 Kotoula V, Charalambous E, Biesmans B, Malousi A, Vrettou E, Fountzilas G and Karkavelas G: Targeted kras mutation assessment on patient tumor histologic material in real time diagnostics. PLoS One *4(11)*: e7746, 2009. PMID: 19888477. DOI: 10.1371/journal.pone.0007746

18 Altman DG, McShane LM, Sauerbrei W and Taube SE: Reporting recommendations for tumor marker prognostic studies (remark): Explanation and elaboration. PLoS Med *9(5)*: e1001216, 2012. PMID: 22675273. DOI: 10.1371/journal.pmed.1001216

19 Travis WD, Brambilla E, Nicholson AG, Yatabe Y, Austin JHM, Beasley MB, Chirieac LR, Dacic S, Duhig E, Flieder DB, Geisinger K, Hirsch FR, Ishikawa Y, Kerr KM, Noguchi M, Pelosi G, Powell CA, Tsao MS and Wistuba I: The 2015 world health organization classification of lung tumors: Impact of genetic, clinical and radiologic advances since the 2004 classification. J Thorac Oncol *10(9)*: 1243-1260, 2015. PMID: 26291008. DOI: 10.1097/jto.0000000000000630

20 Zhang YL, Yuan JQ, Wang KF, Fu XH, Han XR, Threapleton D, Yang ZY, Mao C and Tang JL: The prevalence of egfr mutation in patients with non-small cell lung cancer: A systematic review and meta-analysis. Oncotarget *7(48)*: 78985-78993, 2016. PMID: 27738317. DOI: 10.18632/oncotarget.12587

21 Barlesi F, Mazieres J, Merlio JP, Debieuvre D, Mosser J, Lena H, Ouafik L, Besse B, Rouquette I, Westeel V, Escande F, Monnet I, Lemoine A, Veillon R, Blons H, Audigier-Valette C, Bringuier PP, Lamy R, Beau-Faller M, Pujol JL, Sabourin JC, Penault-Llorca F, Denis MG, Lantuejoul S, Morin F, Tran Q, Missy P, Langlais A, Milleron B, Cadranel J, Soria JC and Zalcman G: Routine molecular profiling of patients with advanced non-small-cell lung cancer: Results of a 1-year nationwide programme of the french cooperative thoracic intergroup (ifct). Lancet *387(10026)*: 1415-1426, 2016. PMID: 26777916. DOI: 10.1016/s0140-6736(16)00004-0

22 Szumera-Cieckiewicz A, Olszewski WT, Tysarowski A, Kowalski DM, Glogowski M, Krzakowski M, Siedlecki JA, Wagrodzki M and Prochorec-Sobieszek M: Egfr mutation testing on cytological and histological samples in non-small cell lung cancer: A polish, single institution study and systematic review of european incidence. Int J Clin Exp Pathol *6(12)*: 2800-2812, 2013. PMID: 24294366.

23 Papadopoulou E, Tsoulos N, Tsirigoti A, Apessos A, Agiannitopoulos K, Metaxa-Mariatou V, Zarogoulidis K, Zarogoulidis P, Kasarakis D, Kakolyris S, Dahabreh J, Vlastos F, Zoublios C, Rapti A, Papageorgiou NG, Veldekis D, Gaga M, Aravantinos G, Karavasilis V, Karagiannidis N and Nasioulas G: Determination of egfr and kras mutational status in greek non-small-cell lung cancer patients. Oncol Lett *10(4)*: 2176-2184, 2015. PMID: 26622815. DOI: 10.3892/ol.2015.3600

24 Mountzios G and Linardou HSA (ed.): Epidemiology and incidence of EGFR mutation in Greek patients with advanced NSCLC. National Hellenic Oncology Meeting; 2016.

25 Linardou H, Dahabreh IJ, Bafaloukos D, Kosmidis P and Murray S: Somatic egfr mutations and efficacy of tyrosine kinase inhibitors in nsclc. Nat Rev Clin Oncol *6(6)*: 352-366, 2009. PMID: 19483740. DOI: 10.1038/nrclinonc.2009.62

26 Murray S, Timotheadou E, Linardou H, Vrettou AV, Kostopoulos I, Skrickova J, Papakostantinou C, Christodoulou C, Pectasides D, Samantas E, Papakostas P, Skarlos DV, Kosmidis P and Fountzilas G: Mutations of the epidermal growth factor receptor tyrosine kinase domain and associations with clinicopathological features in non-small cell lung cancer patients. Lung Cancer *52(2)*: 225-233, 2006. PMID: 16567021. DOI: 10.1016/j.lungcan.2005.12.015

27 Linardou H, Briasoulis E, Dahabreh IJ, Mountzios G, Papadimitriou C, Papadopoulos S, Bafaloukos D, Kosmidis P and Murray S: All about kras for clinical oncology practice: Gene profile, clinical implications and laboratory recommendations for somatic mutational testing in colorectal cancer. Cancer Treat Rev *37(3)*: 221-233, 2011. PMID: 20817364. DOI: 10.1016/j.ctrv.2010.07.008

28 Jung KJ, Jeon C and Jee SH: The effect of smoking on lung cancer: Ethnic differences and the smoking paradox. Epidemiol Health *38*: e2016060, 2016. PMID: 28092929. DOI: 10.4178/epih.e2016060

29 Rulli E, Marabese M, Torri V, Farina G, Veronese S, Bettini A, Longo F, Moscetti L, Ganzinelli M, Lauricella C, Copreni E, Labianca R, Martelli O, Marsoni S, Broggini M and Garassino MC: Value of kras as prognostic or predictive marker in nsclc: Results from the tailor trial. Ann Oncol *26(10)*: 2079-2084, 2015. PMID: 26209642. DOI: 10.1093/annonc/mdv318

30 Mascaux C, Iannino N, Martin B, Paesmans M, Berghmans T, Dusart M, Haller A, Lothaire P, Meert AP, Noel S, Lafitte JJ and Sculier JP: The role of ras oncogene in survival of patients with lung cancer: A systematic review of the literature with meta-analysis. Br J Cancer *92(1)*: 131-139, 2005. PMID: 15597105. DOI: 10.1038/sj.bjc.6602258

31 Sun JM, Hwang DW, Ahn JS, Ahn MJ and Park K: Prognostic and predictive value of kras mutations in advanced non-small cell lung cancer. PLoS One *8(5)*: e64816, 2013. PMID: 26799652. DOI: 10.1371/journal.pone.0064816

32 Linardou H, Dahabreh IJ, Kanaloupiti D, Siannis F, Bafaloukos D, Kosmidis P, Papadimitriou CA and Murray S: Assessment of somatic k-ras mutations as a mechanism associated with resistance to egfr-targeted agents: A systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. Lancet Oncol *9(10)*: 962-972, 2008. PMID: 18804418. DOI: 10.1016/s1470-2045(08)70206-7

33 Svaton M, Fiala O, Pesek M, Bortlicek Z, Minarik M, Benesova L and Topolcan O: The prognostic role of kras mutation in patients with advanced nsclc treated with second- or third-line chemotherapy. Anticancer Res *36(3)*: 1077-1082, 2016. PMID: 26977001.

34 Bonanno L, Schiavon M, Nardo G, Bertorelle R, Bonaldi L, Galligioni A, Indraccolo S, Pasello G, Rea F and Favaretto A: Prognostic and predictive implications of egfr mutations, egfr copy number and kras mutations in advanced stage lung adenocarcinoma. Anticancer Res *30(12)*: 5121-5128, 2010. PMID: 21187500.

35 Fan G, Zhang K, Ding J and Li J: Prognostic value of egfr and kras in circulating tumor DNA in patients with advanced non-small cell lung cancer: A systematic review and meta-analysis. Oncotarget *8(20)*: 33922-33932, 2017. PMID: 28430611. DOI: 10.18632/oncotarget.15412

36 Shen H, Che K, Cong L, Dong W, Zhang T, Liu Q and Du J: Diagnostic and prognostic value of blood samples for kras mutation identification in lung cancer: A meta-analysis. Oncotarget *8(22)*: 36812-36823, 2017. PMID: 28415658. DOI: 10.18632/oncotarget.15972

37 Lohinai Z, Klikovits T, Moldvay J, Ostoros G, Raso E, Timar J, Fabian K, Kovalszky I, Kenessey I, Aigner C, Renyi-Vamos F, Klepetko W, Dome B and Hegedus B: Kras-mutation incidence and prognostic value are metastatic site-specific in lung adenocarcinoma: Poor prognosis in patients with kras mutation and bone metastasis. Sci Rep *7*: 39721, 2017. PMID: 28051122. DOI: 10.1038/srep39721

38 Zhao N, Wilkerson MD, Shah U, Yin X, Wang A, Hayward MC, Roberts P, Lee CB, Parsons AM, Thorne LB, Haithcock BE, Grilley-Olson JE, Stinchcombe TE, Funkhouser WK, Wong KK, Sharpless NE and Hayes DN: Alterations of lkb1 and kras and risk of brain metastasis: Comprehensive characterization by mutation analysis, copy number, and gene expression in non-small-cell lung carcinoma. Lung Cancer *86(2)*: 255-261, 2014. PMID: 25224251. DOI: 10.1016/j.lungcan.2014.08.013

39 Mellema WW, Dingemans AM, Thunnissen E, Snijders PJ, Derks J, Heideman DA, Van Suylen R and Smit EF: Kras mutations in advanced nonsquamous non-small-cell lung cancer patients treated with first-line platinum-based chemotherapy have no predictive value. J Thorac Oncol *8(9)*: 1190-1195, 2013. PMID: 23787801. DOI: 10.1097/JTO.0b013e318298764e

40 Dong X, Zhao X, Hao Y, Wei Y, Yin Q and Du J: Response to first-line chemotherapy in patients with non-small-cell lung cancer according to epidermal growth factor receptor and k-ras mutation status. Clin Lung Cancer *14(6)*: 680-687, 2013. PMID: 23910066. DOI: 10.1016/j.cllc.2013.05.004

41 Marabese M, Ganzinelli M, Garassino MC, Shepherd FA, Piva S, Caiola E, Macerelli M, Bettini A, Lauricella C, Floriani I, Farina G, Longo F, Bonomi L, Fabbri MA, Veronese S, Marsoni S, Broggini M and Rulli E: Kras mutations affect prognosis of non-small-cell lung cancer patients treated with first-line platinum containing chemotherapy. Oncotarget *6(32)*: 34014-34022, 2015. PMID: 26416458. DOI: 10.18632/oncotarget.5607

42 Zhang Y, Fang W, Yan Y, Wang M, Kang S, Sheng J, Zhan J, Chen N, Hong S, Yang Y, Ma Y, He D, Qin T, Zhou T, Tang Y, He X, Liang W and Zhang L: The efficacy of first-line chemotherapy is associated with kras mutation status in patients with advanced non-small cell lung cancer: A meta-analysis. Med Oncol *32(3)*: 61, 2015. PMID: 25663066. DOI: 10.1007/s12032-015-0489-y

43 Kim JH, Kim HS and Kim BJ: Prognostic value of kras mutation in advanced non-small-cell lung cancer treated with immune checkpoint inhibitors: A meta-analysis and review. Oncotarget *8(29)*: 48248-48252, 2017. PMID: 26799652. DOI: 10.18632/oncotarget.17594

44 Arrieta O, Cardona AF, Corrales L, Campos-Parra AD, Sanchez-Reyes R, Amieva-Rivera E, Rodriguez J, Vargas C, Carranza H, Otero J, Karachaliou N, Astudillo H and Rosell R: The impact of common and rare egfr mutations in response to egfr tyrosine kinase inhibitors and platinum-based chemotherapy in patients with non-small cell lung cancer. Lung Cancer *87(2)*: 169-175, 2015. PMID: 25558790. DOI: 10.1016/j.lungcan.2014.12.009

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