Comprehensive Molecular Analysis of NSCLC; Clinicopathological Associations

Ilenia Chatziandreou*, Panagiota Tsioli, Stratigoula Sakellariou, Ioanna Mourkioti, Ioanna Giannopoulou, Georgia Levidou, Penelope Korkolopoulou, Efstratios Patsouris, Angelica A. Saetta

First Department of Pathology, Laikon General Hospital, Athens University Medical School, Athens, Greece

* hchatzian@med.uoa.gr

Abstract

Background

Selection of NSCLC patients for targeted therapy is currently based upon the presence of sensitizing mutations in EGFR and EML4/ALK translocations. The heterogeneity of molecular alterations in lung cancer has led to the ongoing discovery of potential biomarkers and targets in order to improve survival.

Aim

This study aimed to detect alterations in EGFR, KRAS, BRAF, PIK3CA, MET-gene copy number and ALK rearrangements in a large cohort of 956 NSCLC patients of Hellenic origin using highly sensitive techniques and correlations with clinicopathological characteristics.

Results

Mutations were detected in EGFR 10.6% (101 out of 956 samples), KRAS 26.5% (191 out of 720 samples), BRAF 2.5% (12 out of 471 samples), PIK3CA 3.8% (7 out of 184 samples), MET gene amplification was detected in 18% (31 out of 170) and ALK rearrangements in 3.7% (4 out of 107 samples). EGFR mutations were detected in exon 19 (61.4% of mutant cases), exon 21 p.Leu858Arg (19.8%), exon 20 (15.8%), exon 18 (2.9%) and were correlated with gender histology, smoking status and TTF1 staining. p.Thr790Met mutant cases (3.9%) displayed concurrent mutations in exons 19 or 21. Negative TTF-1 staining showed strong negative predictive value for the presence of EGFR mutations. KRAS mutations were associated with histology, the most common mutation being p.Gly12Cys (38%).

Discussion

In conclusion, only 89 patients were eligible for EGFR -TKIs and ALK inhibitors therapy, whereas 257 patients showed other alterations, highlighting the necessity for a detailed molecular profiling potentially leading to more efficient individualized therapies for NSCLC patients.

Introduction

Lung cancer remains the leading cause of cancer related mortality worldwide. Non- Small cell lung cancer (NSCLC) histology including adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and bronchioloalveolar carcinoma, accounts for approximately 85% of all lung cancers [1, 2]. NSCLC patients have a poor prognosis, often diagnosed at an advanced stage due to the fact that early disease is typically asymptomatic. The overall 5- year survival has improved over the years but still remains at approximately 16–18% [3–5, 6] despite therapeutic advances.

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein activates downstream RAS/RAF/MAPK, and PI3K/AKT signaling pathways, which cooperate to modulate several important mechanisms such as cell proliferation, adhesion, angiogenesis, migration, and survival [7]. Aberrant activation of EGFR could be triggered by mutation or amplification/over-expression causing upregulation of oncogenic cell signaling and malignant transformation [8]. Activating mutations of EGFR kinase domain clustered in exons 18–21 are well established as predictive biomarkers for treatment of patients with EGFR tyrosine kinase inhibitors (TKIs) [9]. Lung cancer patients harboring such alterations show a 70% to 80% response rate to TKIs [10–12].

Although EGFR mutations are being used as either positive or negative predictive factors, accumulating data suggest a possible predictive value for alterations in other genes (KRAS, BRAF, PIK3CA, etc) which also affect the two major signaling pathways downstream of EGFR. In order to apply an individualized approach for a more efficient treatment of lung cancer patients, a molecular characterization is now mandatory, as part of baseline diagnostic procedures.

KRAS is a well-established predictive biomarker for colorectal cancer also implicated in lung carcinogenesis. KRAS mutations are found frequently in white patients with lung adenocarcinoma and smoking history [11, 13–16] and have been associated with poor prognosis and resistance to TKIs towards EGFR [17,18].

BRAF mutations, although detected at lower frequencies in lung cancer, have emerged as an alternative important mechanism of MAPK signaling activation downstream of KRAS. To date, BRAF has been successfully utilised as a therapeutic target in melanomas. The predictive value of BRAF mutations in NSCLC has not been clarified yet, although clinical trials with BRAF and MEK inhibitors in the NSCLC setting are ongoing in order to evaluate the clinical value of this potential biomarker [18–21].

PIK3CA gene encodes for the catalytic subunit of lipid kinase PI3K involved in signaling downstream of EGFR. Mutations in a broad spectrum of tumors, such as breast, bladder, colon, gastric cancer and glioblastomas [22, 23] and at much lower frequency in NSCLC cause aberrant activation of phosphatidylinositol 3-kinase (PI3K)/AKT signaling. Such alterations are considered as potentially useful biomarkers of resistance to EGFR-targeted therapy undergoing clinical validation.

MET gene, on chromosome 7q31, encodes a transmembrane tyrosine kinase receptor for HGF/scatter factor. Aberrant MET activation may be derived from overexpression, gene amplification or gene mutations. In NSCLC it has been linked with acquired resistance to EGFR TKIs. Therefore several MET inhibitors are being developed and tested as potential therapeutic strategies for NSCLC.

The *ALK* (anaplastic lymphoma kinase) gene rearrangement was originally identified in the context of a subtype of Non-Hodgkin lymphoma where *ALK* was fused to nucleophosmin (NPM) as a result of a chromosomal translocation [24]. In 2007, Soda et al [25] found the fusion of ALK gene with Echinoderm Microtubule-associated protein-like 4(EML4), as a consequence of a small inversion within chromosome 2p, in NSCLC tumors. This rearrangement

leads to the production of a chimeric protein with constitutive ALK kinase activity, which promotes malignant growth and proliferation [26]. The incidence of EML4-ALK rearrangement ranges from 3–7% in NSCLC, depending upon the population studied and the detection methods used [27]. ALK rearrangements are more frequently observed in younger patients, light or never-smokers, adenocarcinoma histology with frequent signet ring cells [28, 29]. ALK rearrangement status is a critical biomarker to predict response to tyrosine kinase inhibitors, such as crizotinib which has been associated with significant reduction of tumor burden [30].

Taking into consideration the reported differences of mutation frequencies among various populations and ethnic groups, we aimed to examine the molecular profile of a large cohort of Greek NSCLC cancer patients, by performing mutational analysis in genes implicated in EGFR/RAS/MAPK, PI3K/AKT signaling and to search for relevant clinicopathological associations.

Materials and Methods

Ethics Statement

The present study was approved by the Laikon General Hospital University of Athens Ethics Committee (Protocol No. EΣ283/26-5-10). Since this was a retrospective study the Ethics Committee waived the need for an informed consent, and a policy of strict anonymity and confidentiality was assured. All patient data were anonymized and de-identified in a confidential manner. All information included in the data set was used exclusively for the purpose of this study, and was not shared with other individuals or organizations. Cases were randomly selected based on the availability of archival material from the database maintained in the First Department of Pathology, Laikon General Hospital, University of Athens Medical School, Greece.

Patients

This is a retrospective study of 956 cases of non-small cell lung cancer (males = 681, females = 275 median age = 65; range years 29–94) for which archival material from primary tumours was available in our institute, a major pathology centre in Greece receiving samples from hospitals across the country. Samples were classified as Adenocarcinomas (n = 716), Squamous cell carcinomas (n = 87), Adenosquamous (n = 11), NSCLC NOS (n = 110), Large cell carcinomas (n = 5), Large cell Neuroendocrine carcinomas (n = 18), Pleomorphic and other carcinomas (n = 9), according to the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society International Multidisciplinary Classification of Lung Adenocarcinoma/2011, WHO 4th edition 2015 (Tables 1 and 2). This cohort included predominantly bronchoscopic biopsies and surgical specimens, but also a small number of bone marrow biopsies and cytology specimens. Complete information regarding tumour staging was available for 291 samples, due to the analysis of a large number of incisional biopsies. The distribution of tumour stages for 291 samples according the 7th edition of lung cancer staging was as follows: 11 cases stage IA, 23 cases stage IB, 41 cases stage IIA, 24 cases stages IIB, 51 cases stage IIIA, 8 cases stage IIIB and 124 cases stage IV. Smoking status was available for 132 cases, current smokers or ex-smokers were defined as smokers whereas never smokers as non-smokers. TTF-1 staining data was available for 595 cases (Table 1).

Genomic DNA isolation

DNA extraction from paraffin embedded tissues.   Sections 10 μm thick were cut from— paraffin-embedded tissue blocks after macrodissection under the Light Microscope by an

Table 1. Clinicopathological and demographic characteristics of NSCLC patients.

Characteristics	Patients (n)	Characteristics	Patients (n)
All patients	956		
Gender		**Histology**	
Female	275	AdCa	716
Male	681	Squamous	87
Age		Adenosquamous	11
≤50	77	NSCLC NOS	110
51–60	181	Large cell carcinoma	5
61–70	279	Lage cell neuroendocrine carcinoma	18
>70	240	Pleomorphic carcinoma	8
Median age, year (range)	65 (29–94)	poorly differentiated NSCLC with spindle cell carcinoma	1
Smoking		**Stage**	
smokers	105	IA	11
non-smokers	27	IB	23
TTF-1 staining		IIA	41
Positive	419	IIB	24
Negative	176	IIIA	51
Grade		IIIB	8
high-intermediate	181	IV	124
low	325		

doi:10.1371/journal.pone.0133859.t001

experienced pathologist for tumor enrichment. DNA was extracted from the selected tissue areas following a standard DNA extraction kit protocol (NucleoSpin tissue, Macherey–Nagel, Duren, Germany). The extracted DNA was quantitated on a Picodrop Microliter spectrophotometer. Laser Microdissection was applied in 68 cases on which macrodissection could not be performed due to low content of tumour cells, or vast dispersion of tumour cells, and high infiltration with normal or inflammatory cells. Microdissection was performed using a Leica LMD6000B laser capture system (Leica Microsystems CMS GmbH, Wetzlar, Germany). DNA was extracted using the Qiagen DNA Micro extraction kit.

Molecular Analysis

High Resolution Melting Analysis (HRMA) and Cobas Method. KRAS, BRAF and PIK3CA mutations were screened using HRMA on a Light Cycler 480 (Roche Diagnostics, GmbH, Germany) in duplicate and further identified by Pyrosequencing and/or sequencing analysis. Each PCR reaction consisted of 20ng DNA, 0.3μM of each primer, 10μl LightCycler 480 HRM Master Mix (Roche Diagnostics, GmbH, Germany), 3.5mM $MgCl_2$ in a total volume of 20μl. The thermal profile used in the Light Cycler was: 95°C for 10 min, followed by 50 cycles of 95°C for 10 sec, with annealing temperatures at 56°C–KRAS, 60°C–EGFR/BRAF and 62°C-PIK3CA for 15 sec, 72°C for 7 sec. The sequences of the primers for EGFR, BRAF, KRAS and PIK3CA have been published previously [24, 25]. EGFR mutations were examined using HRMA/pyrosequencing as described above or by the Cobas method ("Cobas EGFR test", Roche Molecular Systems, Inc, Branchburg, NJ, USA) according to the manufacturer's protocol. The Cobas EGFR test is designed to detect 41 mutations displaying maximum clinical significance in exons 18, 19, 20, and 21. Samples with limited material (biopsies) were selected for analysis with the cobas test, in order to minimize the DNA input requirements. In these cases an additional examination of exon 21 using HRMA was performed for p.Leu861Gln mutation detection.

Table 2. Histological Classification of 956 NSCLC samples A. Classification of Resection Samples B. Classification of Small Biopsies/cytology samples and C. Metastasis Samples according to IASLC/ATS/ERS 2011, WHO 2015.

A. RESECTION SAMPLES	(n)	B. SMALL BIOPSIES/CYTOLOGY	(n)	SMALL BIOPSIES/CYTOLOGY	(n)
Preinvasive Lessions		**Adenocarcinoma**			
Adenocarcinoma in situ Non mucinous	4	with solid pattern	293	with acinar pattern and clear cell features	1
Adenocarcinoma in situ Mucinous	1	with acinar pattern	57	with solid or colloid pattern and signet ring features	7
Invasive adenocarcinoma		with papillary pattern	13	**Squamous**	
Lepidic predominant	9	with micropapillary pattern	1	squamous and NSCLC favor squamous	51
Acinar predominant	83	with solid and acinar pattern	21	**Other categories**	
Solid predominant	113	with solid or acinar and micropapillary pattern	6	NSCLC with squamous cell and adenocarcinoma patterns	10
Papillary predominant	18	with acinar and papillary pattern	3	NSCLC, NOS	65
Invasive mucinous adenocarcinoma	14	with lepidic pattern (an invasive component cannot be excluded)	10	NSCLC with neuroendocrine (NE) morphology, possible LCNEC	13
Squamous cell carcinoma		mucinous adenocarcinoma with lepidic pattern	1	poorly differentiated NSCLC with spindle cell carcinoma	1
Squamous	32	AdCa with colloid pattern	3	**Total**	**556**
Keratinizing squamous carcinoma	1				
Other categories					
Adenosquamous	2				
Combined large cell neuroendocrine carcinoma	2				
Large cell neuroendocrine carcinoma	2				
Large cell	5				
NSCLC NOS	39				
Pleomorphic	6				
Total	**331**				
C. METASTASIS	**(n)**				
Adenocarcinoma					
with solid pattern	45				
with acinar pattern	7				
with papillary pattern	1				
with solid and acinar pattern	4				
with solid or acinar and micropapillary pattern	1				
squamous	3				
Metastatic NOS	5				
NSCLC with neuroendocrine (NE) morphology, possible LCNEC	3				
Total	**69**				

doi:10.1371/journal.pone.0133859.t002

Pyrosequencing/Sequencing. Alterations in KRAS exon 2, BRAF exon 15 and EGFR exons 18–21 observed by HRMA, were identified by Pyrosequencing using the Pyromark Gold Q24 Reagent kit with the Q24 Pyrosequencer (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol as previously described [31,32]. Sanger Sequencing was used to identify mutations in BRAF (except Val600Glu) and PIK3CA gene exons 9 and 20. Briefly PCR products positive by HRMA were sequenced using the BigDye terminator cycle sequencing kit (Applied Biosystems, CA, USA) in order to confirm the presence of mutations. The sequencing products were analysed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). PCR

primers were also used for sequencing analysis. Results were verified by sequencing analysis of at least two independent PCR products. PIK3CA gene exon 9 sequence analysis did not show amplification of the pseudogene.

Relative copy number analysis. MET relative copy number was determined for 170 samples by Real Time PCR. GADPH was used as a reference gene. The primer sequences used for each gene were as follows: MET gene Forward *5'-ACGGTCCAAAGGGAAACTCT-3'*, Reverse *5'-CTCCAGAGGCATTTCCATGT-3'*, GADPH gene Forward 5'-CAATTCCCCATCTCAGTC GT-3', Reverse 5'- GCAGCAGGACACTAGGGAGT-3'. Results are expressed as normalized ratio calculated using a calibrator sample consisting of a pool of normal DNAs extracted from blood from healthy subjects. A standard curve from 5 consecutive serial dilutions of normal human genomic DNA was used to determine the reaction Efficiency (E). The data were analysed using the efficiency corrected calculation models, based on one sample [33]:

$$\text{Ratio} = \left(\text{E target}\right)^{\Delta Cp\ target(calibrator-sample)} \div \left(\text{E ref}\right)^{\Delta Cp\ ref(calibrator-sample)} \tag{1}$$

The cut off value was established as previously described [34]. Briefly the mean of normalized ratio values of 20 normal lung DNA samples was calculated and a lung tumor sample was considered as amplified if its normalized ratio was over Mean+2SD. All specimens were analysed in duplicate.

Immunohistochemistry- IHC. IHC was applied on 4μm thick formalin-fixed paraffin-embedded tissue sections with the prediluted rabbit monoclonal IVD anti-ALK antibody D5F3 (Ventana Medical Systems, Tuscon, AZ) on the Ventana Benchmark XT system, using the Optiview DAB detection/ Amplification kit according to the manufacturer's instructions. IHC was evaluated by two experienced pathologists, using the recommended scoring algorithm. IHC detects ALK rearrangements independently of the fusion partner and represents a reliable screening approach [35].

Fluorescent In Situ Hybridisation–FISH. FISH was used in order to verify positive IHC results according to recent guidelines [36, 37]. FISH was performed on 4 μm–thick paraffin slides from FFPE tumor tissues, using an established break-apart probe (IVD) specific to the ALK locus (Vysis ALK Break Apart FISH Probe Kit, Abbott Molecular, Abbott Park, Illinois, USA) according to the manufacturer's instructions. Stained slides were evaluated by two trained pathologists under a 100x oil immersion objective with a fluorescence microscope using the scoring criteria defined by the manufacturer. Only tumor cells of which the nuclei had one or more FISH signals of each color were enumerated. A positive cell was defined as one displaying split signals (two or more signal diameters apart), or a single orange signal (deleted green signal) in addition to fused and/or split signals. A sample was considered positive if >25 cells out of 50 were positive. If a sample had 5 to 25 positive cells (10 to 50%) another 50 tumor cells were counted and the sample was considered positive if the average percentage of positive cells was >15% [37].

Statistical analysis

Statistical analysis was performed in order to correlate mutational status with other parameters such as gender, histological type, grade and stage using Pearson's Chi square and Fisher's exact test where appropriate. Statistical calculations were performed using the statistical package SPSSv21.0 for Windows. All results with a two-sided p-value <0.05 were considered significant. Associations of mutational status with TNM stage were limited to samples with available clinical data.

Results

Patient characteristics

All patients were of Hellenic origin and the available clinicopathological characteristics are presented in Table 1. Median age was 65 years (range 29–94 years) and the analysed cohort exhibited a male predominance (71%), with a 2.4:1 male to female ratio. Regarding histological classification specimens were mainly adenocarcinomas at 75% followed by squamous cell carcinomas at 9%. There was statistical significant correlation between tumor stage and age of the patients (p = 0.01), in older patients ≥65 years old (75%), higher stage (III/IV) tumors were more common than earlier stage (I/II) tumors. The age of the patients was also associated with gender (p = 0.003), 63% of female patients were under 65 years of age and 49% of male patients were over 65 years of age. In the subset of patients with known smoking status, gender was related with smoking history as males were more frequently smokers (p<0.0001). Furthermore, it was observed that SCC histology was associated with gender (p = 0.002) as SCC tumors were more common in men (75 of 87 squamous cases) than women (12 of 87 squamous cases). There was also a marginal correlation of grade and stage of tumors (p = 0.054) where, well and moderately differentiated cases associated with stages I/II while low differentiated tumors with stages III/IV. TTF-1 staining was associated with adenocarcinoma histology (p<0.0001).

EGFR Mutational analysis and correlation to clinico-pathological data

956 tumor samples from patients with NSCL cancer were tested for the presence of activating mutations in exons 18, 19, 20 and 21 of the EGFR gene. It is important to note that mutations were detected in several cases with low tumor or cell content, indicating the high sensitivity of the methods used. Mutational analysis was feasible even in cases with tumor content as low as 10% or low cellular content.

EGFR mutations were detected in 10.6% (101 out of 956 samples) of the examined cases (Table 3). Most identified mutations were sensitizing, namely deletions in exon 19 (61.4%, 62 cases out of 101, Fig 1) the majority of which were identified as deletion of 15 bases starting at nucleotides 2236 or 2235 leading to a deletion of Glu-Leu-Arg-Glu-Ala (p. Glu746_Ala750delELREA). The rest of exon 19 mutations were identified as: p.Leu747_Glu749delLRE, p. Leu747_Thr751delLREAT, p.Glu746_Thr751delELREAT, p.Lys745_Ala750delKELREA, p. Ile744Met and also a rare point mutation, p.Leu747Pro. Exon 21 was found mutated at lower frequency (21.7%, 22 out of 101 cases). A well-known G to T transversion at nucleotide 2573 leading to p.Leu858Arg amino acid substitution was seen in 20 cases. Furthermore, one sample displayed a mutation p.Gly863Val and another sample displayed two point mutations, namely p.Val834Leu along with p.Leu858Arg. In addition, sixteen samples (15.8%) were mutated in exon 20, the majority of which displayed insertions. The resistance mutation p.Thr790Met in exon 20 was found in 4 cases all of which showed also sensitizing mutations (3 cases in exon 19 and one in exon 21). Exon 18 mutations, namely p.Gly719Ala, were found in three samples (2.9%) in our cohort (Fig 1).

The percentage of mutated adenocarcinomas was 12.8% (92 out of 716 adenocarcinomas), whilst in all other histological types the percentage of EGFR mutations did not surpass 5% (9 out of 240 samples), and no mutation was detected in squamous cell carcinomas (Fig 2, Table 3). As far as adenocarcinoma groups are concerned, EGFR mutations were more frequent in lepidic (4 out of 19 samples, 21%), micropapillary (either pure or mixed) (2 out of 6 samples 33%), non-mucinous in situ AdCa (2 out of 4 samples 50%), while in all other groups the percentage ranged from 8 to 16% (Table 4), but no statistical correlations were elicited. The

Table 3. Mutational analysis of NSCLC samples. This table shows the percentage of mutations in this cohort (All NSCLC) and the two major histological subtypes; adenocarcinomas (AdCa) and squamous cell carcinomas (Squamous).

	All NSCLC		AdCa		Squamous	
	(n)	(%)	(n)	(%)	(n)	(%)
EGFR						
Wild type	855	89.4	624	87.2	87	100
Mutant	101	10.6	92	12.8	0	0
KRAS						
Wild type	529	73.5	385	70.1	64	97
Mutant	191	26.5	164	29.9	2	3
BRAF						
Wild type	459	97.5	369	97.4	37	95
Mutant	12	2.5	10	2.6	2	5
PIK3CA						
Wild type	177	96.7	139	96.5	25	96.2
Mutant	7	3.8	5	3.5	1	3.8
MET Amplification						
Absent	139	82	85	85	34	72.4
Present	31	18	15	15	13	27.6
ALK rearrangement						
Absent	103	96.3	72	94.7	12	100
Present	4	3.7	4	5.3	0	0

doi:10.1371/journal.pone.0133859.t003

presence of EGFR mutations correlated with adenocarcinoma histology (p<0.001), with gender (p<0.001) as it was encountered at higher rates in females (21.3%) than in males (7%), and
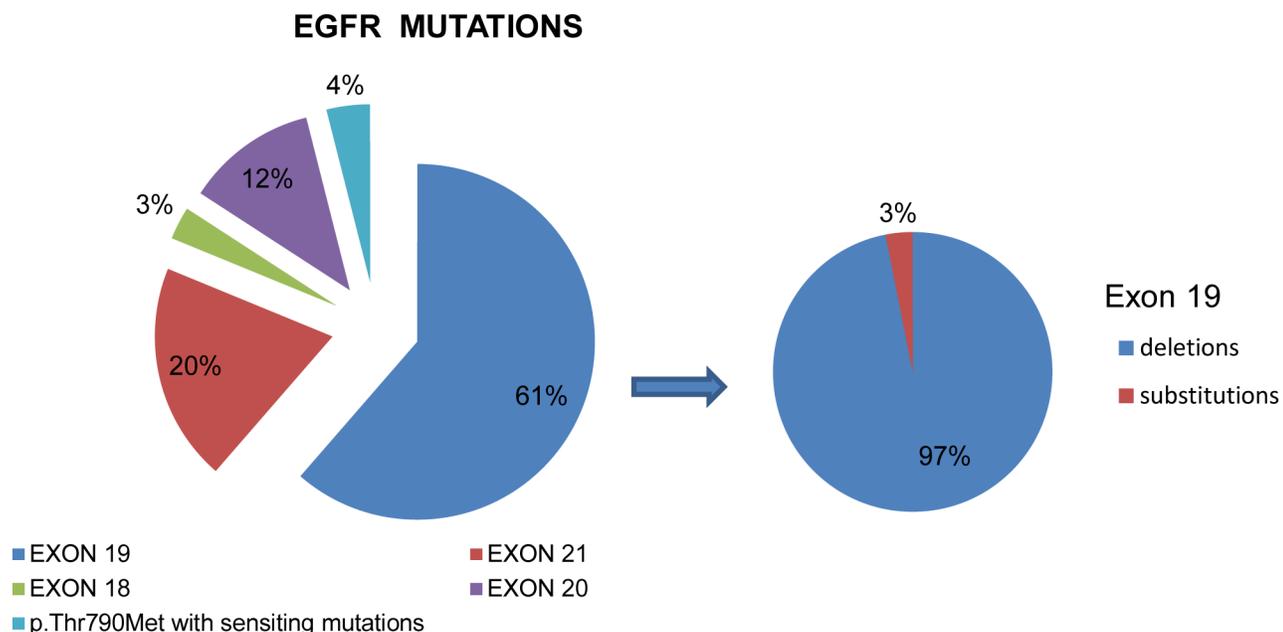
Fig 1. Frequency of mutations (%) in exons 18, 19, 20 and 21 of EGFR gene.

doi:10.1371/journal.pone.0133859.g001
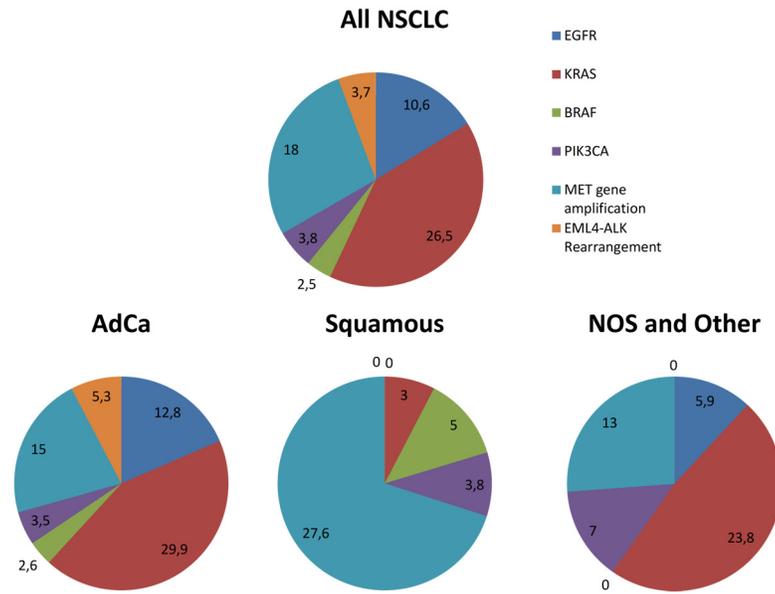
Fig 2. Pie charts representing the frequencies (%) of alterations of the examined genes in this cohort.
Percentage of alterations for all the samples of the cohort (All NSCLC) and between the different histological
types adenocarcinoma (AdCa), squamous cell carcinomas (Squamous), and other types (NOS and Other).

were also associated with smoking status (p<0.001), in particular EGFR mutations were pres-
ent in 8 of 105 (7.6%) smokers and 12 of 27 (44%) non-smokers. Additionally, EGFR muta-
tions were mutually exclusive with KRAS mutations (p<0.001). Statistical significant
correlations were not found with other clinicopathological characteristics such as age
(p = 0.137), grade (p = 0.587) or stage (p = 0.097). It has to be noted that cases with advanced
stage (20 of 183 cases with Stage III/IV) were found more frequently mutated (11%) than those
with early stage (5%) (5 of 99 cases with Stage I/II) but the association was not statistically
significant.

Of the cases with available TTF-1 data, only 2 of 74 EGFR mutant cases were TTF1-negative
therefore EGFR mutations were also associated (p<0.0001) with TTF-1 positivity (Negative
predictive value, 98.8%).

Table 4. EGFR mutations distributed amongst different Adenocarcinoma groups.

	EGFR		
	NL	**MT**	**(%)**
In situ Non mucinous AdCa	2	2	50
lepidic	15	4	21
acinar	124	23	15.6
solid	399	52	11.5
papillary	26	5	16.1
Invasive mucinous AdCa	12	2	14.3
More than one growth pattern present	22	2	8.3
AdCa with micropapillary pattern (either pure or mixed)	4	2	33

Alterations in KRAS, BRAF, PIK3CA and correlations to clinicopathological data

In order to further examine the mutational profile of the patients, as far as MAPK and PI3K/AKT signaling pathways are concerned, HRM analysis and Pyrosequencing/sanger sequencing were applied to detect mutations in KRAS (exon 2), BRAF (exon 15) and PIK3CA (exons 9 and 20) genes. KRAS mutational analysis was performed for 720 samples and 26.5% (191 out of 720) of the cases were mutated, mainly adenocarcinomas (29.9%, 164 out of 549 adenocarcinomas). Regarding the adenocarcinoma groups the presence of KRAS mutations was more common in Invasive mucinous AdCa (5 out of 11 samples, Table 5), but no statistical correlations were elicited. In the other NSCLC histological types KRAS mutation frequency was 3% in squamous cell carcinomas (2 out of 66, Table 3) and 19% in NOS and other histologies (25 out of 105 Fig 2). Interestingly, a statistically significant relation ($p<0.0001$) depicting the association of KRAS mutations with tumors' histological type in NSCLC patients was determined. In contrast with EGFR, KRAS mutations were equally distributed between the two sexes, observed in 26.4% of males and 25.9% of females. KRAS mutations mainly affected codon 12 (92%) and the distribution of mutations was the following: the most frequent mutation was p.Gly12Cys (38% of mutant cases) which has been reported to be smoking related and p.Gly12Val (24%). In detail, ten different mutations were identified in total namely: p.Gly12Cys, p.Gly12Val, p.Gly12A, p.Gly12Asp, p.Gly12Arg. p.Gly12Ser, p.Gly12Phe, p.Gly12Gly, p.Gly13Asp and p.Gly13Cys (Fig 3). From the cases analysed for KRAS, smoking status was available for only 84 patients. KRAS was mutated in 21 of 70 smokers (30%) and 1 of 14 (7.1%) non- smokers; although 21 out of 22 mutations were found in smokers, this finding was not statistical significant ($p = 0.1$), probably due to the small number of cases and in particular the limited number of non-smoking patients (14 patients). No clinicopathological associations were found between KRAS mutations and stage ($p = 0.586$), grade ($p = 0.582$) or age ($p = 0.294$).

BRAF mutational screening was performed for 471 samples. Analysis of exon 15 (activation segment) revealed mutations in 2.5% of the cases (12 out of 471) mainly adenocarcinomas of different groups as shown in Table 6 (10 out of 12 mutant samples) and only two squamous cell carcinomas were found mutated (Fig 2, Table 3). Mutations were identified as T to A transition at nucleotide 1799 leading to a Valine to Glutamine substitution at codon 600, (p.Val600Glu) in 8 cases, and 4 cases exhibited non- p.Val600Glu, namely p.Ser614Ser, p.Thr589Ala, p.Ala598Val, and pVal600>TyrMet. BRAF mutations were not correlated with clinicopathological data such as histological type ($p = 0.667$), stage ($p = 1.0$), grade ($p = 1.0$), age ($p = 0.310$) or gender ($p = 0.762$).

Table 5. KRAS mutations distributed amongst different Adenocarcinoma groups.

	KRAS		
	NL	MT	(%)
In situ Non mucinous AdCa	1	1	50
lepidic	12	3	20
acinar	80	34	29.8
solid	248	102	29.1
papillary	16	8	33.3
Invasive mucinous AdCa	6	5	45.5
More than one growth pattern present	11	7	38.9
AdCa with micropapillary pattern (either pure or mixed)	5	1	16.7
Colloid	5	2	28.6

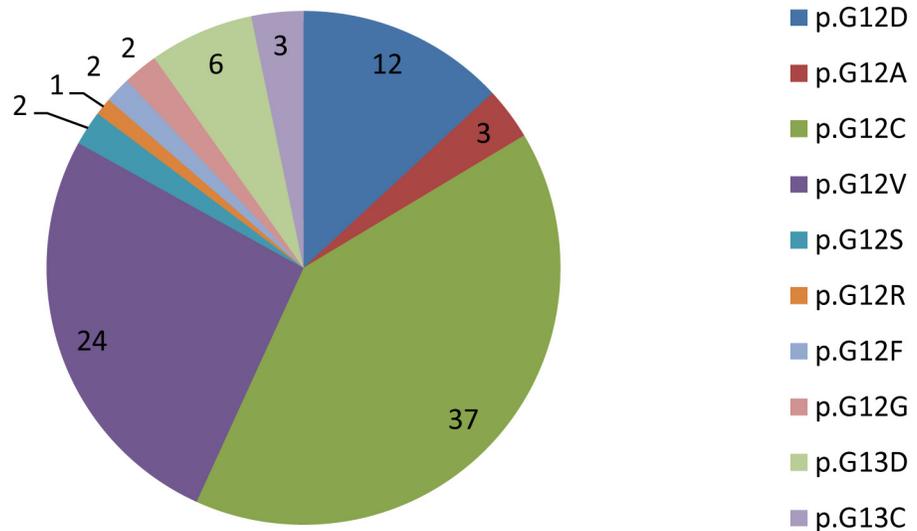doi:10.1371/journal.pone.0133859.t005

Fig 3. Schematic representation of the distribution (%) of different KRAS mutations.

doi:10.1371/journal.pone.0133859.g003

As far as PIK3CA gene alterations are concerned, 184 samples were analysed for PIK3CA mutations in both exons 9 and 20, showing a mutation frequency of 3.8% (7 out of 184) (Fig 2, Tables 3 and 6). The mutations detected were mainly clustered in exon 20. The only PI3KCA exon 9 mutation, namely p.Glu545Lys, was present in a squamous cell carcinoma. Interestingly, most of the PIK3CA mutations in exon 20 coexisted with other mutations. More specifically they were found simultaneously with EGFR (p.Lys745_Ala750 del KELREA), KRAS (p. Gly13Cys, p.Gly12Val) or BRAF (p.Val600Glu) mutations. PIK3CA mutations were not associated with any of the clinicopathological characteristics in this cohort.

MET gene copy number amplification and associations with clinicopathological features

MET gene was found amplified in 18% (31 out of 170 cases)(Fig 2, Tables 3 and 6). More specifically MET amplification was found in 15 of 100 (15%) ADC and 13 of 47 (27.6%) SCC; 8 of 38 (21%) women and 23 of 132 (17.4%) men. MET amplification was detected at similar frequencies in younger (under 65 years) and older ages (over 65 years), 19% and 17% respectively. Regarding tumor grade, MET amplification was observed in 18% of well/moderately differentiated tumors (7 of 39) and 19.3% (11 of 57) in low differentiated tumors. MET was found amplified in both EGFR wild type and mutant samples at similar percentages 18% (29 of 158) and

Table 6. Frequencies of alterations between different groups of adenocarcinomas (NL = Normal, MT = Mutant, AMPL = gene amplification).

AdCa	BRAF			AdCa	PIK			Adca	MET		
	NL	MT	(%)		NL	MT	(%)		NL	AMPL	(%)
acinar	28	1	3.4	acinar	11	1	8.3	acinar	16	3	15.8
solid	170	7	3.9	solid	45	4	8.2	solid	48	9	15.8
AdCa with micropapillary pattern (either pure or mixed)	2	1	33.3					More than one pattern	4	1	20
colloid	2	1	33.3					lepidic	2	1	33.3
								colloid	0	1	100

doi:10.1371/journal.pone.0133859.t006

17% (2 of 12) respectively. In detail, one MET amplified sample displayed a double EGFR mutation, namely a deletion in exon 19 and p.Thr790Met in exon 20 and the other sample exhibited an insertion in exon 20. In addition, MET amplification was more common in KRAS wild type than KRAS mutant samples as it was detected in 16 of 84 (19%) and in 2 of 26 (7.7%) of KRAS wild type and mutant samples respectively. MET gene amplification was not correlated with any clinicopathological features.

ALK rearrangement status

Immunohistochemical analysis was performed on 107 cases in order to evaluate ALK status. 3.7% of the samples (4 out of 107) were identified as ALK positive by IHC and validated by FISH. More specifically *ALK* rearrangement was found in 4 adenocarcinomas (1 female, 3 male patients) all with advanced stage disease. ALK rearrangement was mutually exclusive with the other examined alterations.

Discussion

The characterization of EGFR activating mutations which predict sensitivity or resistance to anti-EGFR therapies has provided a basis for selecting lung cancer patients for targeted therapies. Currently in clinical practice patients with specific *EGFR* mutations or echinoderm microtubule-associated protein-like 4/anaplastic lymphoma kinase *(EML4/ ALK)* fusion gene could be treated with EGFR or ALK tyrosine kinase inhibitors (TKIs) respectively. However these biomarkers are clinically relevant for a limited subset of patients, approximately 15% in populations of European descent, and consequently the search for additional predictive biomarkers is ongoing in NSCLC.

In the present study we illustrate the mutation spectrum of NSCLC patients of Hellenic origin. NSCLC samples were examined following either macro or Laser micro dissection for the presence of driver mutations in EGFR (n = 956), KRAS (n = 720), BRAF (n = 472), PIK3CA (n = 184) genes, MET gene copy number amplification (n = 170), ALK gene rearrangement (n = 107) in an effort to define the occurrence of molecular alterations in NSCLC and their possible contribution to clinical decision making. The median age of NSCLC in our cohort (65 years) is similar to the median age worldwide (70 years) [6]. We report 85 sensitizing EGFR mutations among which four coexisted with resistant mutation p.Thr790Met, and 12 non sensitizing mutations in exon 20. In our study 10.6% of the examined NSCLC samples displayed EGFR mutations and the respective frequency reached 12.8% in adenocarcinomas in accordance with current results on populations of European descent [38–42]. There is a remarkable ethnical variation of EGFR mutation frequency, ranging from 9 to 12% in European populations [11, 39, 40, 42, 43] and reaching 40% in populations of South East Asia [9, 44–46]. It has been reported that more than 80% of the detected mutations are deletions clustered between codons 746 and 753 in exon 19 and point mutations affecting codon 858 in exon 21 [11, 47, 48]. Similarly, exon 19 alterations detected in this cohort accounted for 60% of the mutations identified with the most frequent being deletion p.Glu746-Ala750del. The second most common mutation detected (20%) was p.Leu858Arg in exon 21, followed by insertions in exon 20 (12%). Only three samples were mutants in exon 18 (p.Gly719Ala). Exon 20 point mutation p. Thr790Met was present at a frequency of 4% of mutant cases. Mutations were statistically correlated with adenocarcinoma histological type, non-smoking patients, female gender and TTF-1 positive staining, findings which are in line with the existing literature. TTF-1 staining showed a correlation with EGFR mutation status, displaying a high negative predictive value of 98.8% reinforcing previous data that bring out TTF-1 IHC as a clinical feature that may reliably estimate the absence of mutations with a great potential for the clinician [41, 42, 49].

KRAS mutations were the most common alteration detected in our cohort accounting for 26.5% of the examined cases and correlated with adenocarcinoma histology. In total, ten different alterations were identified the smoking related mutation p.Gly12Cys at codon 12 represented 38% of all KRAS alterations, followed by p.Gly12Val (24%), in accordance with results from previous studies [50, 51]. Interestingly, p.Gly12Asp which is the most common mutation in colon cancer adenocarcinomas was present in 12% of the mutant cases in our NSCLC cohort. The significance of KRAS mutations as a biomarker for NSCLC, remains elusive and although it has been linked with resistance to anti-EGFR therapy, recent bibliography suggests a possible clinical significance amongst the various KRAS mutations [50, 52]. It has been hypothesized that due to non-identical biological activities of different RAS alleles, different signaling outputs could be induced subsequently leading to variation of sensitivity to drugs. Recently a MEK inhibitor, selumetinib, in combination with docetaxel has been used in patients with KRAS-mutant tumors with promising efficacy [52–54].

Analyzing downstream the MAPK signaling pathway, at the level of B-RAF gene, low prevalence of BRAF mutations was encountered (2.5% of the cases) in our cohort in accordance with recent investigations in which 2–5% incidence is reported [20, 55, 56]. Albeit the low incidence of BRAF mutations, it has been proposed that NSCLC patients carrying Val600Glu mutation may potentially benefit from treatment with selective inhibitors, currently in clinical trials, highlighting the importance of prospective genotyping of NSCLC patients for BRAF mutations. BRAF mutations in this cohort were not confined to samples displaying adenocarcinoma histology but were also found in two SCC cases. In addition, a high percentage (33%) of the mutant cases exhibited non-pVal600Glu mutations, observations which are in line with recent studies [55–57]. BRAF mutation has been related with smoking status in recent reports [55–57], an analysis not feasible in this cohort due to the lack of data for smoking status for BRAF mutant cases. The detected frequency of Val600Glu mutation is in agreement with previous studies of populations of European descent [55–57], but it differs from the results of a cohort of 5125 Chinese NSCLC patients, in which it was present in only 0.5% of the cases, associated with gender, but not with smoking status [58].

As far as PIK3CA gene mutations are concerned we detected a low frequency of mutations (3.3%) by analyzing a subset of 184 NSCLC samples, which is in accordance with previous studies [51, 59–61]. Interestingly, similar rates of PIK3CA point mutations have been observed between East Asian [44, 62] and patients of European descent [22, 51, 61] although a difference on the type of mutations identified is noted; there seems to be a higher incidence of PIK3CA exon 9 mutations in East Asian patients [34, 60, 62, 63]. In this cohort, PIK3CA point mutations coexisted with mutations in other examined genes such as EGFR, KRAS and BRAF and no significant correlation with clinicopathological factors was elicited, in line with current literature [43–49]. An association of PIK3CA mutations with TKI resistance and poor survival in NSCLC patients treated with EGFR-TKIs has been reported [61]. Recently, it has been suggested that patients presenting with advanced cancer showing p.His1047Arg mutation (exon 20) may be more sensitive to PI3K/AKT/mTOR pathway inhibitors [64], although preclinical and early clinical studies imply that KRAS and PIK3CA concurrent mutations may induce resistance to such inhibitors [64, 65].

MET relative copy number variation analysis by qRT-PCR showed gene amplification in 18% of the NSCLC cases examined. This percentage is in accordance with similar investigations in which MET gene copy amplification in TKI-chemo naïve patients ranges from 5–20% [27, 66–70], although higher incidence (45%) has been reported in one study [66]. This variability could be attributed to different methods of analysis and cut off values as well as to population differences. Although MET amplification has been linked to acquired resistance to TKI treatment, the present study as well as previous investigations on chemo-TKI naïve patients,

are in favor of a pre-existing nature of this alteration. MET amplification has been associated with poor prognosis, increased proliferation, tumor invasiveness and angiogenesis [71, 72]. Conflicting data regarding clinicopathological associations of MET alterations with gender, smoking and histology exist [67, 70, 72]. In the present study no clinicopathological associations with MET amplification were defined, in accordance with other reports [56, 68].

Regarding ALK rearrangement, in our study ALK positive cases analysed by IHC and confirmed by FISH were found in 3.7% of the cases. This frequency is consistent with previous reports demonstrating ALK positivity at a range of 3.0% to 7.0% [25,30,73]. No statistically significant associations with the clinicopathological parameters were found which is in accordance with previous studies [74]. Recently, two large meta-analysis studies have elicited correlations with never/light smoking history, female gender, adenocarcinoma histology, as well as non-Asian patients with advanced stage [40,75]. It is worth noting that in our study although no significant statistical correlations were found, possibly due to the limited number of cases, the positive samples were advanced stage adenocarcinomas.

To conclude, in this study we report the frequency of EGFR mutations in a cohort of 956 lung cancer patients as well as the frequency of alterations in KRAS, BRAF, MET, PIK3CA and ALK genes in a subset of these patients. The selection of NSCLC patients for targeted therapies to date is exclusively based on EGFR and ALK mutational status, accounting for a small percentage of patients. Accordingly, in this cohort only 89 patients were eligible for therapy with EGFR TKIs or ALK inhibitors. On the other hand, a subset of 207 patients displayed non sensitizing EGFR and KRAS mutations, and further 50 patients showed alterations in BRAF, PIK3CA or MET genes. New targeted drugs currently in development and clinical trials would increase the number of patients eligible for targeted therapeutic approaches that may overcome acquired resistance to TKIs. Our findings in this large cohort of NSCLC patients, add to a growing body of data concerning NSCLC molecular profiling, highlighting the necessity of a more detailed molecular analysis potentially leading to more efficient individualized therapies.

Author Contributions

Conceived and designed the experiments: AAS IC. Performed the experiments: IC PT IM IG. Analyzed the data: IC AAS SS GL PK. Contributed reagents/materials/analysis tools: PK EP AAS. Wrote the paper: IC AAS. Clinicopathological assessment: SS GL PK EP.

References

1. Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med. 2008; 359: 1367–1380. doi: 10.1056/NEJMra0802714 PMID: 18815398

2. Ramalingam SS, Owonikoko TK, Khuri FR. Lung Cancer : New Biological Insights and Recent Therapeutic Advances. Cancer. 2011; 61: 91–112. doi: 10.3322/caac.20102 Available

3. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin. 2013; 63: 11–30. doi: 10.3322/caac.21166 PMID: 23335087

4. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin. 2014; 64: 9–29. doi: 10.3322/caac.21208 PMID: 24399786

5. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global Cancer Statistics: 2011. CA Cancer J Clin. 2011; 61: 69–90. doi: 10.3322/caac.20107 PMID: 21296855

6. DeSantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, et al. Cancer treatment and survivorship statistics, 2014. CA Cancer J Clin. 2014; doi: 10.3322/caac.21235

7. Cheng L, Li Y, Zhang SB, Teng XD. Molecular pathology of lung cancer: Key to personalized medicine. Chinese J Pathol. Nature Publishing Group; 2012; 41: 715–720. doi: 10.1038/modpathol.2011.215

8. Soria J-C, Mok TS, Cappuzzo F, Jänne PA. EGFR-mutated oncogene-addicted non-small cell lung cancer: current trends and future prospects. Cancer Treat Rev. Elsevier Ltd; 2012; 38: 416–430. doi: 10.1016/j.ctrv.2011.10.003 PMID: 22119437

9. Wu J-Y, Shih J-Y, Chen K-Y, Yang C-H, Yu C-J, Yang P-C. Gefitinib therapy in patients with advanced non-small cell lung cancer with or without testing for epidermal growth factor receptor (EGFR) mutations. Medicine (Baltimore). 2011; 90: 159–167. doi: 10.1097/MD.0b013e31821a16f4

10. Sequist LV, Bell DW, Lynch TJ, Haber DA. Molecular predictors of response to epidermal growth factor receptor antagonists in non-small-cell lung cancer. J Clin Oncol. 2007; 25: 587–595. doi: 10.1200/JCO. 2006.07.3585 PMID: 17290067

11. Munfus-McCray D, Harada S, Adams C, Askin F, Clark D, Gabrielson E, et al. EGFR and KRAS mutations in metastatic lung adenocarcinomas. Hum Pathol. Elsevier Inc.; 2011; 42: 1447–1453. doi: 10. 1016/j.humpath.2010.12.011 PMID: 21497370

12. Hirsch FR, Jänne PA, Eberhardt WE, Cappuzzo F, Thatcher N, Pirker R, et al. Epidermal growth factor receptor inhibition in lung cancer: status 2012. J Thorac Oncol. 2013; 8: 373–84. doi: 10.1097/JTO. 0b013e31827ed0ff PMID: 23370315

13. Reungwetwattana T, Weroha SJ, Molina JR. Oncogenic pathways, molecularly targeted therapies, and highlighted clinical trials in non-small-cell lung cancer (NSCLC). Clin Lung Cancer. Elsevier; 2012; 13: 252–66. doi: 10.1016/j.cllc.2011.09.004 PMID: 22154278

14. Langer C. Roles of EGFR and KRAS Mutations in the Treatment Of Patients With Non–Small-Cell Lung Cancer. Pharm Ther. 2011; 36.

15. Riely GJ, Marks J, Pao W. KRAS mutations in non-small cell lung cancer. Proc Am Thorac Soc. 2009; 6: 201–5. doi: 10.1513/pats.200809-107LC PMID: 19349489

16. Roberts PJ, Stinchcombe TE, Der CJ, Socinski MA. Personalized medicine in non-small-cell lung cancer: Is KRAS a useful marker in selecting patients for epidermal growth factor receptor-targeted therapy? J Clin Oncol. 2010; 28: 4769–4777. doi: 10.1200/JCO.2009.27.4365 PMID: 20921461

17. Mascaux C, Iannino N, Martin B, Berghmans T, Paesmans M, Berghmans T, et al. The role of RAS oncogene in survival of patients with lung cancer: a systematic review of the literature with meta-analysis. Br J Cancer. 2005; 92: 131–139. PMID: 15597105

18. Mitsudomi T, Yatabe Y. Mutations of the epidermal growth factor receptor gene and related genes as determinants of epidermal growth factor receptor tyrosine kinase inhibitors sensitivity in lung cancer. Cancer Science. 2007. pp. 1817–1824. doi: 10.1111/j.1349-7006.2007.00607.x PMID: 17888036

19. Pao W, Girard N. New driver mutations in non-small-cell lung cancer. Lancet Oncol. Elsevier Ltd; 2011; 12: 175–180. doi: 10.1016/S1470-2045(10)70087-5 PMID: 21277552

20. Cardarella S, Ogino A, Nishino M, Butaney M, Shen J, Lydon C, et al. Clinical, pathological and biological features associated with BRAF mutations in non-small cell lung cancer. Clin Cancer Res. 2013; 4532–4540. doi: 10.1158/1078-0432.CCR-13-0657 PMID: 23833300

21. Sánchez-Torres J, Viteri S. BRAF mutant non-small cell lung cancer and treatment with BRAF inhibitors. Transl Lung Cancer Res. 2013; 1: 244–250. doi: 10.3978/j.issn.2218-6751.2013.04.01

22. Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, et al. High frequency of mutations of the PIK3CA gene in human cancers. Science. 2004; 304: 554. doi: 10.1126/science.1096502 PMID: 15016963

23. Korkolopoulou P, Levidou G, Trigka EA, Prekete N, Karlou M, Thymara I, et al. A comprehensive immunohistochemical and molecular approach to the PI3K/AKT/mTOR (phosphoinositide 3-kinase/v-akt murine thymoma viral oncogene/mammalian target of rapamycin) pathway in bladder urothelial carcinoma. BJU Int. 2012; 110. doi: 10.1111/j.1464-410X.2012.11569.x

24. Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL, et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science. 1994; 263: 1281–1284. doi: 10.1126/science.8122112 PMID: 8122112

25. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature. 2007; 448: 561–566. doi: 10.1038/nature05945 PMID: 17625570

26. Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med. 2010; 363: 1693–1703. doi: 10.1056/NEJMoa1006448 PMID: 20979469

27. Thunnissen E, Bubendorf L, Dietel M, Elmberger G, Kerr K, Lopez-Rios F, et al. EML4-ALK testing in non-small cell carcinomas of the lung: A review with recommendations. Virchows Arch. 2012; 461: 245–257. doi: 10.1007/s00428-012-1281-4 PMID: 22825000

28. Chan BA, Hughes BGM. Targeted therapy for non-small cell lung cancer : current standards and the promise of the future. Transl Lung Cancer Res. 2015; 4: 36–54. doi: 10.3978/j.issn.2218-6751.2014. 05.01 PMID: 25806345

29. Shaw AT, Solomon B, Mino-Kenudson M. Crizotinib and testing for ALK. JNCCN Journal of the National Comprehensive Cancer Network. 2011. pp. 1335–1341.

30. Zhao F, Xu M, Lei H, Zhou Z, Wang L, Li P, et al. Clinicopathological Characteristics of Patients with Non-Small-Cell Lung Cancer Who Harbor EML4-ALK Fusion Gene: A Meta-Analysis. PLoS One. 2015; 10: e0117333. doi: 10.1371/journal.pone.0117333 PMID: 25706305

31. Levidou G, Saetta AA, Gigelou F, Karlou M, Papanastasiou P, Stamatelli A, et al. ERK/pERK expression, B-raf and K-ras mutations in colon adenocarcinomas: correlation with clinicopathological characteristics and expression of hMLH1 and hMSH2. World Journal of Surgical Oncology. 2012. p. 47. doi: 10.1186/PREACCEPT-1032136556616351 PMID: 22376079

32. Tasioudi KE, Saetta AA, Sakellariou S, Levidou G, Michalopoulos NV, Theodorou D, et al. pERK activation in esophageal carcinomas: clinicopathological associations. Pathol Res Pract. Elsevier GmbH.; 2012; 208: 398–404. doi: 10.1016/j.prp.2012.05.009 PMID: 22658382

33. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29: e45. doi: 10.1093/nar/29.9.e45 PMID: 11328886

34. Beau-Faller M, Ruppert A-M, Voegeli A-C, Neuville A, Meyer N, Guerin E, et al. MET gene copy number in non-small cell lung cancer: molecular analysis in a targeted tyrosine kinase inhibitor naïve cohort. J Thorac Oncol. 2008; 3: 331–9. doi: 10.1097/JTO.0b013e318168d9d4 PMID: 18379349

35. Gruber K, Kohlhäufl M, Friedel G, Ott G, Kalla C. A Novel, Highly Sensitive ALK Antibody 1A4 Facilitates Effective Screening for ALK Rearrangements in Lung Adenocarcinomas by Standard Immunohistochemistry. J Thorac Oncol. 2015; 10: 713–716. doi: 10.1097/JTO.0000000000000427 PMID: 25789835

36. Leighl NB, Rekhtman N, Biermann WA, Huang J, Mino-Kenudson M, Ramalingam SS, et al. Molecular Testing for Selection of Patients With Lung Cancer for Epidermal Growth Factor Receptor and Anaplastic Lymphoma Kinase Tyrosine Kinase Inhibitors: American Society of Clinical Oncology Endorsement of the College of American Pathologists/Internat. J Clin Oncol. 2014; 32: 3673–3679. doi: 10.1200/JCO.2014.57.3055 PMID: 25311215

37. Shan L, Lian F, Guo L, Yang X, Ying J, Lin D. Combination of conventional immunohistochemistry and qRT-PCR to detect ALK rearrangement. Diagn Pathol. Diagnostic Pathology; 2014; 9: 3. doi: 10.1186/1746-1596-9-3

38. Dimou A, Harrington K, Syrigos KN. From the bench to bedside: biological and methodology considerations for the future of companion diagnostics in nonsmall cell lung cancer. Patholog Res Int. 2011; 2011: 312346. doi: 10.4061/2011/312346 PMID: 21785682

39. Cortes-Funes H, Gomez C, Rosell R, Valero P, Garcia-Giron C, Velasco A, et al. Epidermal growth factor receptor activating mutations in Spanish gefitinib-treated non-small-cell lung cancer patients. Ann Oncol. 2005; 16: 1081–1086. doi: 10.1093/annonc/mdi221 PMID: 15851406

40. Rosell R, Moran T, Queralt C, Porta R, Cardenal F, Camps C, et al. Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med. 2009; 361: 958–967. doi: 10.1056/NEJMoa0904554 PMID: 19692684

41. Leary AF, De Castro DG, Nicholson AG, Ashley S, Wotherspoon A, O'Brien MER, et al. Establishing an EGFR mutation screening service for non-small cell lung cancer—Sample quality criteria and candidate histological predictors. Eur J Cancer. 2012; 48: 61–67. doi: 10.1016/j.ejca.2011.09.022 PMID: 22036089

42. Hantson I, Dooms C, Verbeken E, Vandenberghe P, Vliegen L, Roskams T, et al. Performance of standard procedures in detection of EGFR mutations in daily practice in advanced NSCLC patients selected according to the ESMO guideline: a large Caucasian cohort study. Transl Respir Med. 2014; 2: 9. doi: 10.1186/s40247-014-0009-0 PMID: 25264519

43. Herreros-Villanueva M, Rodrigo M, Claver M, Muñiz P, Lastra E, García-Girón C, et al. KRAS, BRAF, EGFR and HER2 gene status in a Spanish population of colorectal cancer. Mol Biol Rep. 2011; 38: 1315–1320. doi: 10.1007/s11033-010-0232-x PMID: 20563851

44. Li C, Hao L, Li Y, Wang S, Chen H, Zhang L, et al. Prognostic value analysis of mutational and clinicopathological factors in non-small cell lung cancer. PLoS One. 2014; 9: e107276. doi: 10.1371/journal.pone.0107276 PMID: 25198510

45. Zhang X, Chang A. Molecular predictors of EGFR-TKI sensitivity in advanced non-small cell lung cancer. Int J Med Sci. 2008; 5: 209–217. PMID: 18645621

46. Sun P-L, Seol H, Lee HJ, Yoo SB, Kim H, Xu X, et al. High incidence of EGFR mutations in Korean men smokers with no intratumoral heterogeneity of lung adenocarcinomas: correlation with histologic subtypes, EGFR/TTF-1 expressions, and clinical features. J Thorac Oncol. 2012; 7: 323–30. doi: 10.1097/JTO.0b013e3182381515 PMID: 22237264

47. Lynch T, Bell D, Sordella R, Gurubhagavatula S, Okimoto R, Brannigan B, et al. Activating Mutations in the Epidermal Growth Factor Receptor Underlying Responsiveness of Non–Small-Cell Lung Cancer to Gefitinib. N Engl J Med. 2004; 350: 2129–2139. PMID: 15118073

48. Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science. 2004; 304: 1497–1500. doi: 10.1126/science.1099314 PMID: 15118125

49. Vincenten J, Smit EF, Vos W, Grünberg K, Postmus PE, Heideman DAM, et al. Negative NKX2-1 (TTF-1) as temporary surrogate marker for treatment selection during EGFR-mutation analysis in patients with non-small-cell lung cancer. J Thorac Oncol. 2012; 7: 1522–7. doi: 10.1097/JTO.0b013e3182635a91 PMID: 22982653

50. Garassino MC, Marabese M, Rusconi P, Rulli E, Martelli O, Farina G, et al. Different types of K-Ras mutations could affect drug sensitivity and tumour behaviour in non-small-cell lung cancer. Ann Oncol. 2011; 22: 235–7. doi: 10.1093/annonc/mdq680 PMID: 21169473

51. Cardarella S, Ortiz TM, Joshi VA, Butaney M, Jackman DM, Kwiatkowski DJ, et al. The Introduction of Systematic Genomic Testing for Patients with Non-Small-Cell Lung Cancer. J Thorac Oncol. 2012; 7: 1767–1774. doi: 10.1097/JTO.0b013e3182745bcb PMID: 23154547

52. Ihle NT, Byers LA, Kim ES, Saintigny P, Lee JJ, Blumenschein GR, et al. Effect of KRAS oncogene substitutions on protein behavior: Implications for signaling and clinical outcome. J Natl Cancer Inst. 2012; 104: 228–239. doi: 10.1093/jnci/djr523 PMID: 22247021

53. Karachaliou N, Mayo C, Costa C, Magrí I, Gimenez-Capitan A, Molina-Vila MA, et al. KRAS mutations in lung cancer. Clin Lung Cancer. 2013; 14: 205–14. doi: 10.1016/j.cllc.2012.09.007 PMID: 23122493

54. Carpeño JDC, Belda-Iniesta C. KRAS mutant NSCLC, a new opportunity for the synthetic lethality therapeutic approach. Transl Lung Cancer Res 2013; 2: 142–151. doi: 10.3978/j.issn.2218-6751.2013.02.07 PMID: 25806225

55. Paik PK, Arcila ME, Fara M, Sima CS, Miller VA, Kris MG, et al. Clinical characteristics of patients with lung adenocarcinomas harboring BRAF mutations. J Clin Oncol. 2011; 29: 2046–2051. doi: 10.1200/JCO.2010.33.1280 PMID: 21483012

56. Marchetti A, Felicioni L, Malatesta S, Grazia Sciarrotta M, Guetti L, Chella A, et al. Clinical Features and Outcome of Patients With Non-Small-Cell Lung Cancer Harboring BRAF Mutations. J Clin Oncol. 2011; 29: 3574–3579. doi: 10.1200/JCO.2011.35.9638 PMID: 21825258

57. Cardarella S, Ogino A, Nishino M, Butaney M, Shen J, Lydon C, et al. Clinical, pathologic, and biologic features associated with BRAF mutations in non-small cell lung cancer. Clin Cancer Res. 2013; 19: 4532–40. doi: 10.1158/1078-0432.CCR-13-0657 PMID: 23833300

58. Li S, Li L, Zhu Y, Huang C, Qin Y, Liu H, et al. Coexistence of EGFR with KRAS, or BRAF, or PIK3CA somatic mutations in lung cancer: a comprehensive mutation profiling from 5125 Chinese cohorts. Br J Cancer. 2014; 110: 2812–20. doi: 10.1038/bjc.2014.210 PMID: 24743704

59. Ohashi K, Sequist LV, Arcila ME, Lovly CM, Chen X, Rudin CM, et al. NIH Public Access. 2013; 19: 2584–2591. doi: 10.1158/1078-0432

60. Kawano O, Sasaki H, Endo K, Suzuki E, Haneda H, Yukiue H, et al. PIK3CA mutation status in Japanese lung cancer patients. Lung Cancer. 2006; 54: 209–215. doi: 10.1016/j.lungcan.2006.07.006 PMID: 16930767

61. Ludovini V, Bianconi F, Pistola L, Minotti V, Chiari R, Colella R, et al. 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. Cancer Chemother Pharmacol. 2012; 69: 1289–1299. doi: 10.1007/s00280-012-1829-7 PMID: 22302407

62. Serizawa M, Koh Y, Kenmotsu H, Isaka M, Murakami H, Akamatsu H, et al. Assessment of mutational profile of Japanese lung adenocarcinoma patients by multitarget assays: a prospective, single-institute study. Cancer. 2014; 120: 1471–81. doi: 10.1002/cncr.28604 PMID: 24700479

63. Yamamoto H, Shigematsu H, Nomura M, Lockwood WW, Sato M, Okumura N, et al. PIK3CA mutations and copy number gains in human lung cancers. Cancer Res. 2008; 68: 6913–6921. doi: 10.1158/0008-5472.CAN-07-5084 PMID: 18757405

64. Janku F, Wheler JJ, Naing A, Falchook GS, Hong DS, Stepanek VM, et al. PIK3CA mutation H1047R is associated with response to PI3K/AKT/mTOR signaling pathway inhibitors in early-phase clinical trials. Cancer Res. 2013; 73: 276–84. doi: 10.1158/0008-5472.CAN-12-1726 PMID: 23066039

65. Engelman JA, Chen L, Tan X, Crosby K, Guimaraes AR, Upadhyay R, et al. Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. Nat Med. 2008; 14: 1351–1356. doi: 10.1038/nm.1890 PMID: 19029981

66. Han C-B, Ma J-T, Li F, Zhao J-Z, Jing W, Zhou Y, et al. EGFR and KRAS mutations and altered c-Met gene copy numbers in primary non-small cell lung cancer and associated stage N2 lymph node-metastasis. Cancer Lett. Elsevier Ireland Ltd; 2012; 314: 63–72. doi: 10.1016/j.canlet.2011.09.012 PMID: 21982684

67. Cai Y-R, Zhang H-Q, Zhang Z-D, Mu J, Li Z-H. Detection of MET and SOX2 amplification by quantitative real-time PCR in non-small cell lung carcinoma. Oncol Lett. 2011; 2: 257–264. doi: 10.3892/ol.2010.229 PMID: 22866074

68. Bean J, Brennan C, Shih J-Y, Riely G, Viale A, Wang L, et al. MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. Proc Natl Acad Sci U S A. 2007; 104: 20932–7. doi: 10.1073/pnas.0710370104 PMID: 18093943

69. Kubo T, Yamamoto H, Lockwood WW, Valencia I, Soh J, Peyton M, et al. MET gene amplification or EGFR mutation activate MET in lung cancers untreated with EGFR tyrosine kinase inhibitors. Int J Cancer. 2009; 124: 1778–84. doi: 10.1002/ijc.24150 PMID: 19117057

70. Okuda K, Sasaki H, Yukiue H, Yano M, Fujii Y. Met gene copy number predicts the prognosis for completely resected non-small cell lung cancer. Cancer Sci. 2008; 99: 2280–5. doi: 10.1111/j.1349-7006.2008.00916.x PMID: 19037978

71. Cappuzzo F, Marchetti A, Skokan M, Rossi E, Gajapathy S, Felicioni L, et al. Increased MET gene copy number negatively affects survival of surgically resected non-small-cell lung cancer patients. J Clin Oncol. 2009; 27: 1667–1674. doi: 10.1200/JCO.2008.19.1635 PMID: 19255323

72. Go H, Jeon YK, Park HJ, Sung S-W, Seo J-W, Chung DH. High MET gene copy number leads to shorter survival in patients with non-small cell lung cancer. J Thorac Oncol. 2010; 5: 305–313. doi: 10.1097/JTO.0b013e3181ce3d1d PMID: 20107422

73. Shaw AT, Yeap BY, Mino-Kenudson M, Digumarthy SR, Costa DB, Heist RS, et al. Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. J Clin Oncol. 2009; 27: 4247–4253. doi:JCO.2009.22.6993 [pii]\r doi: 10.1200/JCO.2009.22.6993 PMID: 19667264

74. Li Y, Pan Y, Wang R, Sun Y, Hu H, Shen X, et al. ALK-Rearranged Lung Cancer in Chinese: A Comprehensive Assessment of Clinicopathology, IHC, FISH and RT-PCR. PLoS One. 2013; 8: 1–8. doi: 10.1371/journal.pone.0069016

75. Wang Y, Wang S, Xu S, Qu J, Liu B. Clinicopathologic Features of Patients with Non-Small Cell Lung Cancer Harboring the EML4-ALK Fusion Gene: A Meta-Analysis. PLoS One. 2014; 9: e110617. doi: 10.1371/journal.pone.0110617 PMID: 25360721