Prevalence and clinical association of gene mutations through Multiplex Mutation Testing in patients with NSCLC: Results from the ETOP Lungscape Project

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Running head:

ETOP Lungscape: Multiplex Mutation Testing in patients with NSCLC

Previous presentations:

Parts of this study were previously presented at the European Cancer Congress 2015.

ABSTRACT

Background

Reported prevalence of driver gene mutations in non-small cell lung cancer (NSCLC) is highly variable and clinical correlations are emerging. Using NSCLC biomaterial and clinical data from the ETOP Lungscape iBiobank, we explore the epidemiology of mutations and association to clinicopathological features and patient outcome (relapse-free survival, time-to-relapse, overall survival).

Methods

Clinically-annotated, resected stage I-III NSCLC FFPE tissue was assessed for gene mutation using a microfluidics-based multiplex PCR platform. Mutant-allele detection sensitivity is >1% for most of the ~150 (13 genes) mutations covered in the multiplex test.

Results

Multiplex testing has been performed in 2063 (76.2%) of the 2709 Lungscape cases (median follow-up 4.8 years).

FFPE samples mostly date from 2005-8, yet recently extracted DNA quality and quantity was generally good. Average DNA yield/case was 2.63µg; 38 cases (1.4%) failed QC and were excluded from study. 95.1% of included cases allowed the complete panel of mutations to be tested.

Most common were *KRAS, MET, EGFR* and *PIK3CA* mutations with overall prevalence of 23.0%, 6.8%, 5.4% and 4.9% respectively.

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KRAS and *EGFR* mutations were significantly more frequent in adenocarcinomas: *PIK3CA* in squamous cell carcinomas. *MET* mutation prevalence did not differ between histology groups.

EGFR mutations were found predominantly in never smokers; *KRAS* in current/former smokers.

For all the above mutations, there was no difference in outcome between mutated and non-mutated cases.

Conclusion

Archival FFPE NSCLC material is adequate for multiplex mutation analysis. In this large, predominantly European, clinically-annotated stage I-III NSCLC cohort, none of the mutations characterized showed prognostic significance.

Keywords

Non-small cell lung cancer, multiplex mutation analysis, *EGFR*, *KRAS*, *PIK3CA*, prognosis molecular staging

KeyMessage

Surgically-resected, clinically annotated stage I-III NSCLC from the ETOP Lungscape database were successfully screened for ~150 gene mutations by a microfluidics-based multiplex PCR platform, using FFPE tumour. *KRAS*, *EGFR* and *PI3KCA* mutation profile in NSCLC showed expected clinicopathological relations yet none showed prognostic significance. Molecular staging will need wider genomic profiling.

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Introduction

Primary carcinoma of the lung is one of the most mutated of solid tumors [1], reflecting the importance of tobacco carcinogenesis in the development of most cases, and contributing to the generally aggressive clinical course of these tumors. Recently, there has been focus on identifying molecular drivers in advanced stage non-small cell lung carcinomas (NSCLC), primarily adenocarcinomas. Driver tyrosine kinases may be therapeutically targeted, an approach which is, in general, beneficial to patients [2]. An expanding list of probable molecular drivers and corresponding inhibitory drugs has driven interest in multiplex genomic testing in NSCLC, as a means of efficiently detecting possible targets in individual cases. Assessment of therapy outcomes may be confounded by any prognostic effect that any such molecular drivers may confer.

The prognostic effect of *EGFR* mutation in resected disease is still disputed [3]. Our previous work in Lungscape showed a prognostic significance of *ALK* gene rearrangements [4] but for many other emerging molecular drivers the effect is unclear [5]. Such data will become more relevant as disease staging systems become more sophisticated and provide better prognostication beyond tumor stage; a molecular staging system to compliment TNM is becoming a real prospect.

Data sets describing mutations in large surgically resected lung tumor cohorts, in association with clinical data, are relatively few, mainly adenocarcinomas, and mostly derived from North American or East Asian centres [6-9]. The European Thoracic Oncology Platform (ETOP) Lungscape database (iBiobank) is a virtual biobank of fully clinically annotated, surgically resected NSCLCs [10]. In this study, mutation profiles derived from a multigene, multiplex platform were generated, and

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compared with clinicopathological characteristics and post-operative patient outcomes.

Materials and Methods

The ETOP Lungscape iBiobank holds extensive clinicopathological data on over 2700 resected stage I-III NSCLCs, with ≥3 years follow-up.

The research was conducted according to Lungscape master and MULTIPLEX sub-study protocols; with adherence to country specific ethics, regulatory requirements and REMARK recommendations.

For each case, a paraffin block containing ≥~30% tumor was cut, using measures to avoid cross-case contamination, and resulting 4-5µm thick paraffin sections were used for DNA extraction from tumor rich regions (usually >50%) using the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, FRG). Extracted DNA samples were analysed for quality by Spectrophotometric analysis and shipped to Laboratory of Oncology, Quiron Dexeus University Hospital, Barcelona. All samples had ratios A260/A280 in the interval 1.8±0.1 and standard UV spectra (220nm-320nm). Samples were anonymized, diluted to 50ng/µL and a minimum of 30µL (corresponding to 1500ng) was shipped to Genentech Inc., South San Francisco for mutation testing.

Testing was carried out on a high-throughput microfluidics-based PCR platform running an allele-specific multiplex test (further details in Supplementary data). A previously described validated panel was updated to include 13 genes (*AKT1, BRAF, EGFR, ERBB2, FLT3, HRAS, JAK2, KIT, KRAS, MET, MYD88, NRAS and PIK3CA*) incorporating 130 hot-spot mutations found in various tumor types [11-13]

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(Table S1). The mutant-allele detection sensitivity is >1% with a minimum requirement of 2-100ng DNA.

Clinicopathological characteristics were compared between groups of patients, by mutation status or histology type, using Fisher's exact, Mantel-Haenszel or Mann-Whitney test.

Cases with mutation detected (MD) or mutation not detected (MND) were considered in prevalence estimation and 95% exact binomial confidence interval (95%CI), for each available gene. Further analyses were limited to genes with a sufficient number of detected mutations.

Correlation of mutations with MET (clone SP44) and ALK (clone 5A4) expression, available from previous Lungscape studies [4,5,14], was evaluated through Fisher's exact tests.

Clinical outcome is presented by overall survival (OS, time from surgery date to death from any cause); relapse-free survival (RFS, time from surgery date to first relapse or death from any cause), and time-to-relapse (TTR, time from surgery date to first relapse) [4,10]. Median follow-up time was estimated using the reverse censoring method for OS.

The effect of gene mutations on outcome was explored through Cox proportional hazards regression models, adjusted for a series of clinicopathological characteristics: Gender, Ethnicity, Smoking history, Age, Adjuvant chemotherapy, Adjuvant radiotherapy, Previous history of cancer, Performance status at diagnosis, Stage, Primary tumor localization, Tumor size, Histology, Surgery year, technique

and anatomy. Backwards elimination method (removal $P \geq 0.10$) was used to identify the final models with significant outcome prognostic factors. Hazard Ratios (HRs), along with 95%CIs, were estimated and differences in hazard were depicted graphically via Kaplan-Meier curves.

In all exploratory analyses, results with two-sided p-value≤0.05 were considered significant. Analyses were performed overall and separately for the two primary histology groups: adenocarcinomas and squamous cell carcinomas (SCCs).
Statistical analyses were carried out in SAS 9.3 and R 3.2.2.

Results

Description of multiplex cohort

Current analysis was based on 2063 cases from 16 centres (multiplex cohort), 76.2% of the overall Lungscape population [10], consisting of 1017 adenocarcinomas (49.3%), 888 SCCs (43.0%) and 158 (7.7%) undifferentiated or adenosquamous/combined-mixed carcinomas. Clinicopathological characteristics, overall, and for the two primary histology groups are presented in Table 1, where some significant differences between the histology groups were detected.

Prevalence of gene mutations

Overall, *KRAS* mutations were the most frequently encountered (23.0%; 95%CI: 21.2-24.9), followed by mutations in *MET* (6.8%; 95%CI: 5.8-8.0), *EGFR* (5.4%; 95%CI: 4.5-6.5) and *PIK3CA* (4.9%; 95%CI: 4.0-6.0). Only 0.7% of cases showed *BRAF* mutations whilst mutations in *NRAS, HRAS, MYD88, AKT1* and *KIT* were very uncommon. There were no mutations found in *ERBB2, FLT3* and *JAK2*. (Table 2)

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In the adenocarcinomas, *KRAS* mutations remained dominant (38.0%; 95%CI: 35.0-41.0), while *EGFR* mutations were the second most frequent (9.7%; 95%CI: 7.9-11.7). Both *KRAS* and *EGFR* mutations were significantly more frequent in adenocarcinomas compared to SCCs (both *P*<0.001; Table 2).

PIK3CA mutation was reported in 7.1% of SCCs (95%CI: 5.5-9.1) and was significantly more frequent compared to adenocarcinomas (*P*<0.001; Table 2).

Table S2 presents the mutations reported for the most frequent genes, overall and for adenocarcinomas/SCCs.

<u>Co-existence of mutations and associations with MET and ALK IHC expression</u>

Co-existence of *KRAS* and either *EGFR* or *PIK3CA* mutations were exceptionally rare (Figures S1.A-S1.C). Only two adenocarcinomas showed dual *KRAS*/*EGFR* mutation (Eg_19del/Kr_G12S; Eg_S768I/Kr_G12V), and 9 cases dual *KRAS*/*PIK3CA* mutation (7 adenocarcinomas, 2 large cell carcinomas). There were 3 adenocarcinomas and one SCC with *EGFR/PIK3CA* mutation co-existence.

There was one adenocarcinoma with dual *EGFR* mutation (G719X/S768I), and one SCC with two *PIK3CA* mutations (H1047R/E545K).

KRAS and *EGFR* mutations were significantly more frequent in the MET IHC positive group (*KRAS*: 34.2% in MET IHC positive vs 19.8% in negative, *P*<0.001; *EGFR*: 10.4% in positive vs 4.0% in negative: *P*<0.001). There was no difference in MET IHC status by *PIK3CA* or *MET* gene mutations (Table S3).

For adenocarcinomas, only the association of MET IHC overexpression with *EGFR* mutation remains significant (*P*=0.0014), while for SCC, only the association with *KRAS* mutation (*P*<0.001) (Table S3).

There was no significant association between ALK IHC expression, evaluated in adenocarcinomas, and *KRAS, MET, EGFR* or *PIK3CA* mutations (Table S4).

Associations between mutations and smoking status

A significant association was detected between *KRAS* and *EGFR* genes and smoking status overall (*P*=0.0065 and *P*<0.001), and for adenocarcinomas (both *P*<0.001), but not for SCCs (*P*>0.99 and *P*=0.37) (Table 3). Overall, *KRAS* mutations were significantly higher in the current/former smokers (24.1%; 95%CI: 22.1-26.2) vs never smokers (15.9%; 95%CI: 11.3-21.4), with the opposite true for *EGFR* mutations (current/former smokers: 3.2%; 95%CI: 2.4-4.2; never smokers: 20.3%; 95%CI: 15.1-26.3). Analogous were the results in the adenocarcinoma cohort (Table 3). *KRAS* mutation type by smoking status is presented in Table S5. Regarding *MET* and *PIK3CA* mutations, a significant association was observed only between *MET* mutation status and smoking history (*P*=0.032) in SCCs, with *MET* mutations more frequently observed in never smokers (15.0%; 95%CI: 7.1-26.6) (Table 3).

Time-to-event outcome and mutation status

RFS, TTR and OS were evaluated at a median follow-up of 4.75 years. An RFS event was experienced by 51.6% of patients, with 5-year RFS 47.3% (95%CI 44.9-49.6). TTR events were experienced by 40.0% of patients, with 5-year TTR 56.6% (95%CI: 54.2-59.0). The total number of deaths recorded was 925 (44.8%), with 5-yr OS 53.7% (95%CI: 51.3-56.1). Median OS was 72.0 months, and no difference was detected by mutation status for any gene (all p-values non-significant, un-stratified or stratified by histology or stage); this also holds for RFS and TTR. No effect of gene mutation and adjuvant therapy on outcome was detected (their effect on all Cox models was non-significant, Tables S6-S8).

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Analysing patient outcome by the 3 codon groups of *KRAS* mutations (12/13/61/MND), in the corresponding adjusted models, a significant effect of codon 61 mutation is observed for TTR, but not for RFS (marginally) and OS (*P*=0.039, *P*=0.057 and *P*=0.16, respectively; Table S9). Patients with codon 61 mutation had an HR of 0.39 for a TTR event compared to non-mutated patients (95%CI: 0.16-0.96) and half the hazard of an RFS event (HR: 0.51; 95%CI: 0.25-1.02).

Kaplan-Meier plots for all endpoints by *EGFR*/*KRAS* mutation status, for adenocarcinomas are presented in Figures S2-7.

Discussion

This is one of the largest cohorts of surgically-resected European NSCLC patients exploring associations of driver mutations with clinicopathological features. The patient demographics are in line with what would be expected for such a cohort in terms of stage distribution and post-operative outcomes [10]. The histological distribution is slightly biased against undifferentiated tumors, comprising 4.7% of the cohort versus an expected prevalence of around 12% in an unselected cohort using WHO 2004 criteria. A 43% prevalence of SCC is representative for a European patient cohort, at the time of surgical samples collection between 2003-2009.

The microfluidics-based PCR platform used for mutation testing was originally developed for testing multiple tumor types, with a corresponding range of clinically relevant genes and mutations. Test sensitivity and sample requirements were within parameters met by the material used in this study. This platform provides an allele-specific range of mutations to be tested but is limited in terms of more recent developments in mutations of interest. The range of *BRAF* mutations, for example, was limited to V600E and V600K. The *MET* mutations found are polymorphisms of

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questionable clinical significance [15-17]; *MET* exon14 skipping mutations were not tested [5,18,19], which is a limitation of our study.

The mutations found and their relation to tumor histology and smoking status are again, largely in line with previous reports [6-9], bearing in mind that the allele-specific technique used in this study risks under-estimating gene mutation prevalence compared to studies where whole exons are sequenced. *KRAS* and *EGFR* would be the expected dominant findings in adenocarcinomas, as would *PIK3CA* in SCCs. The prevalence of *KRAS* mutation subtype is in line with expectations [6,9], predominantly represented by G12C change (G12D commonest in never smokers). European cohorts have also previously shown a dominance of exon 19 *EGFR* deletions over L858R substitution mutations [20]. *BRAF* mutations, all V600E, were found in only 1.1% of our adenocarcinomas. Marchetti et al [21], reported a 4.7% prevalence in 739 resected adenocarcinomas after screening *BRAF* exons 11 and 15, but only 2.8% for V600E mutations. Marchetti et al [21] and the current study found a very low prevalence of *BRAF* mutation in SCC. The rare *EGFR* mutations found in SCC in this study were largely confined to never or former smokers, a trait reflected in testing recommendations [22,23]. Similarly, *KRAS* mutations which were smoking-associated in adenocarcinomas, showed no such correlation in SCC. The association between MET IHC expression and *EGFR* mutation in adenocarcinoma, and *KRAS* mutation in SCC, has not been previously reported; the biological significance of this is unclear. Only rare co-existence of *KRAS* and *EGFR* mutations is as expected; equally, *PIK3CA* mutations are known to co-exist with *KRAS* or *EGFR* mutations [24], albeit this is not frequently observed.

The lack of any association between *KRAS*, *EGFR* or *PIK3CA* mutation and post-operative outcome is, perhaps, surprising. *KRAS* mutations have previously been

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shown to be associated with poor post-operative survival [25-27], but more recently, this has been challenged [28-30]. Shepherd et al reported a deleterious effect of codon 13 *KRAS* mutations in patients receiving adjuvant chemotherapy [28]. This *KRAS* mutation subgroup did not have an inferior post-operative survival in the absence of adjuvant therapy. Our finding of better outcomes in rare codon 61 mutations is of uncertain significance. Most studies have suggested that *EGFR* mutations are associated with a good prognosis although again, this is not a universal finding [3,27,29,31]. The observed prevalence of *PIK3CA* mutations of 4.9% overall, higher in SCC (7.1%) and lower in adenocarcinomas (3.3%) is in line with previous report [24], which also included stage 4 patients but, like us, described no prognostic significance for *PIK3CA* mutation.

The data in our study derive from one of the largest, well-annotated database, focusing on early NSCLC. This series suggest that at least the three most frequently found mutations (*KRAS, EGFR* and *PIK3CA*) would have little role in a molecular staging system. We do note, however, that the prevalence of *EGFR* and *PIK3CA* gene mutations is low, though not unusually so, such that prognostication is based on relatively few patients. This illustrates the challenges of studying single genes as prognostic indicators, even in large studies such as this. Mutations of even lower prevalence would have no value in this regard, unless of very strong prognostic significance; mutations of high prevalence, such as *TP53* (not available in this study)*,* may be a more likely candidate. It is more likely that, should there be a relevant mutational profile related to prognosis, it would involve several genes. This is akin to the plethora of historical data on gene expression profiles relating to prognosis in resected NSCLC [32-36]. This study found relatively few cases with co-existent mutations but this is a reflection of the panel of genes examined, focusing

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on possible drug targets. Wider genome profiling will have a greater chance of identifying prognostically significant panel of genes or gene signatures. These findings also emphasize the potential benefits of merging large databases, to identify and describe rare, but potentially clinically useful, mutation profiles.

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Disclaimers:

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Table Legends

Table 1: Clinicopathological characteristics, by histology cohort[#]

(*) Chi-square or Fisher's exact test, (§) Mann-Whitney test, (!) Mantel-Haenszel test, (~) Category 'Missing' or 'Unknown' excluded, (f) Categories Ia & Ib to I, IIa & IIb to II and IIIa & IIIb to III

[#]The Multiplex cohort of 2063 patients consists primarily of Adenocarcinoma histology patients: 1017 (49.3%), followed by Squamous cell: 888 (43.0%), Large cell: 91(4.4%), Adeno-squamous: 33 (1.6%), Combined-mixed: 27 (1.3%) and Sarcomatoid: 7 (0.3%).

Table 2: Prevalence of cancer related mutations, overall and by histology cohort

MD: Mutation detected, MND: Mutation not detected, (*) Fisher's exact test of the comparison of % mutated between adenocarcinoma and squamous cell patients, (f) "No call" or "Repeat" results are excluded from the calculation

Note: Two mutations were detected for MYD88 gene, one for KIT & AKT1, while none for ERBB2, FLT3 & JAK2 genes

Table 3: Association of cancer related mutations with smoking status

MD: Mutation detected, MND: Mutation not detected, (*) Fisher's exact test of the comparison of % mutated between Current/Former and Never smokers, 95% exact binomial confidence interval of %MD is provided for Current/Former and Never smokers groups

Appendix:

Lungscape Consortium

Appendix Lungscape 003 Multiplex Mutation Testing

European Thoracic Oncology Platform (ETOP) Lungscape Contributors

European Thoracic Oncology Platform Lungscape Consortium:

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Table 1: Clinicopathological characteristics, by histology cohort[#]

(*) Chi-square or Fisher's exact test, ($) Mann-Whitney test, (!) Mantel-Haenszel test, (~) Category 'Missing' or 'Unknown' excluded, (f) Categories Ia & Ib to I, IIa & IIb to II and IIIa & IIIb to III

Characteristic	All patients (N=2063)	Adenocarcinoma patients (N=1017)	Squamous cell patients (N=888)	p-value
Gender – *N* (%)				
Male	1361 (66.0)	551 (54.2)	711 (80.1)	<0.001*
Female	702 (34.0)	466 (45.8)	177 (19.9)	
Ethnicity – *N* (%)				
Caucasian	2044 (99.1)	1004 (98.7)	883 (99.4)	0.15*
Other	19 (0.9)	13 (1.3)	5 (0.6)	
Smoking history – *N* (%)				
Current	640 (31.0)	314 (30.9)	275 (31.0)	<0.001*, ~
Former	1100 (53.3)	511 (50.2)	504 (56.8)	
Never	220 (10.7)	148 (14.6)	60 (6.8)	
Unknown	103 (5.0)	44 (4.3)	49 (5.5)	
Age at surgery (yrs)				

	2062	1017	887	
N	2062	1017	887	
Mean (95% CI)	65.5 (65.1, 65.9)	64.8(64.2,65.4)	66.7(66.1,67.3)	<0.001[§]
Median (Min-Max)	66.4 (22.6-89.5)	65.6(23.1-88.7)	67.5(38.3-89.5)	
BMI (kg/m^2)				
N	938	446	401	
Mean (95% CI)	26.0 (25.7,26.3)	25.9(25.5,26.3)	26.1(25.6,26.5)	0.56[§]
Median (Min-Max)	25.5(15.2-59.1)	25.3(15.2-47.6)	25.5(15.4-59.1)	
Adjuvant chemotherapy – *N* (%)				
Yes	470 (22.8)	232 (22.8)	198 (22.3)	0.74*,~
No	1322 (64.1)	647 (63.6)	576 (64.9)	
Unknown/Missing	271 (13.1)	138 (13.6)	114 (12.8)	
Adjuvant radiotherapy – *N* (%)				
Yes	110 (5.3)	53 (5.2)	51 (5.7)	0.61*,~
No	1666 (80.8)	824 (81.0)	711 (80.1)	
Unknown/Missing	287 (13.9)	140 (13.8)	126 (14.2)	
Previous history of cancer – *N* (%)				
Yes	339 (16.4)	173 (17.0)	136 (15.3)	0.23*,~

No	1441 (69.8)	693 (68.1)	638 (71.8)	
Missing	283 (13.7)	151 (14.8)	114 (12.8)	

Performance Status at diagnosis - N (%)

0	749 (36.3)	384 (37.8)	312 (35.1)	0.0017[!, ~]
1	445 (21.6)	192 (18.9)	223 (25.1)	
2	27 (1.3)	10 (1.0)	14 (1.6)	
3	7 (0.3)	2 (0.2)	4 (0.5)	
Missing	835 (40.5)	429 (42.2)	335 (37.7)	

Stage (TNM 7) - N (%)

Ia	481 (23.3)	280 (27.5)	171 (19.3)	0.080[!]
Ib	518 (25.1)	263 (25.9)	222 (25.0)	0.017[!, ƒ]
IIa	343 (16.6)	140 (13.8)	172 (19.4)	
IIb	262 (12.7)	108 (10.6)	129 (14.5)	
IIIa	422 (20.5)	208 (20.5)	180 (20.3)	
IIIb	37 (1.8)	18 (1.8)	14 (1.6)	

Localization of primary tumor - N (%)

Upper Lobe R	633 (30.7)	358 (35.2)	222 (25.0)	<0.001*

Upper Lobe L	582 (28.2)	292 (28.7)	245 (27.6)	
Lower Lobe R	287 (13.9)	131 (12.9)	138 (15.5)	
Lower Lobe L	313 (15.2)	131 (12.9)	155 (17.5)	
Middle Lobe R	103 (5.0)	57 (5.6)	42 (4.7)	
Overlapping	91 (4.4)	41 (4.0)	41 (4.6)	
Central Tumor	54 (2.6)	7 (0.7)	45 (5.1)	
Tumor Size - *N* (%)				
≤4	1296 (62.8)	736 (72.4)	485 (54.6)	<0.001*,~
>4	765 (37.1)	279 (27.4)	403 (45.4)	
Missing	2 (0.1)	2 (0.2)	0 (0.0)	
Tumor size (cm)				
N	2061	1015	888	
Mean (95% CI)	4.1(4.0,4.2)	3.6(3.5,3.7)	4.5(4.3,4.7)	<0.001§
Median (Min-Max)	3.5(0.2-16.0)	3.0(0.2-15.0)	4.0(0.6-16.0)	
Surgery Anatomy - *N* (%)				
Lobectomy	1499 (72.7)	809 (79.5)	574 (64.6)	<0.001*,~
Pneumonectomy	287 (13.9)	75 (7.4)	190 (21.4)	
Bilobectomy	125 (6.1)	47 (4.6)	72 (8.1)	

Wedge Resection	86 (4.2)	46 (4.5)	31 (3.5)	
Segmentectomy	38 (1.8)	23 (2.3)	15 (1.7)	
Other/Missing	28 (1.4)	17 (1.7)	6 (0.7)	
Surgery technique – N (%)				
Open Thoracotomy	1816 (88.0)	886 (87.1)	788 (88.7)	0.047*,~
Thoracoscopy	170 (8.2)	98 (9.6)	62 (7.0)	
Missing	77 (3.7)	33 (3.2)	38 (4.3)	
Surgery Year - N (%)				
<2006	667 (32.3)	343 (33.7)	268 (30.2)	0.11*
≥2006	1396 (67.7)	674 (66.3)	620 (69.8)	

#The Multiplex cohort of 2063 patients consists primarily of Adenocarcinoma histology patients 1017 (49.3%), followed by Squamous cell 888 (43.0%), Large cell 91(4.4%), Adeno-squamous 33 (1.6%), Combined-mixed 27 (1.3%) and Sarcomatoid 7 (0.3%).

Table 2: Prevalence of cancer related mutations, overall and by histology cohort

MD: Mutation detected, MND: Mutation not detected, (*) Fisher's exact test of the comparison of % mutated between adenocarcinoma and squamous cell patients, (ƒ) "No call" or "Repeat" results are excluded from the calculation

Gene	All patients (N=2063)		Adenocarcinoma patients (N=1017)		Squamous cell patients (N=888)		p-value*
	N (%)ƒ	95% CI	N (%)ƒ	95% CI	N (%)ƒ	95% CI	
KRAS							
N successful cases	2055		1014		883		
MD	473 (23.0)	(21.2, 24.9)	385 (38.0)	(35.0, 41.0)	54 (6.1)	(4.6, 7.9)	<0.001
MND	1582 (77.0)		629 (62.0)		829(93.9)		
No call	8		3		5		
MET							
N successful cases	2056		1014		884		
MD	140 (6.8)	(5.8, 8.0)	71 (7.0)	(5.5, 8.8)	61 (6.9)	(5.3, 8.8)	>0.99
MND	1916 (93.2)		943 (93.0)		823 (93.1)		
No call	7		3		4		
EGFR							
N successful cases	1976		973		850		
MD	107 (5.4)	(4.5, 6.5)	94 (9.7)	(7.9, 11.7)	8 (0.9)	(0.4, 1.9)	<0.001
MND	1869 (94.6)		879 (90.3)		842 (99.1)		

Gene	All patients (N=2063)		Adenocarcinoma patients (N=1017)		Squamous cell patients (N=888)		p-value*
	N (%)ƒ	95% CI	N (%)ƒ	95% CI	N (%)ƒ	95% CI	
No call	87		44		38		
PIK3CA							
N successful cases	1970		962		855		
MD	97 (4.9)	(4.0, 6.0)	32 (3.3)	(2.3, 4.7)	61 (7.1)	(5.5, 9.1)	<0.001
MND	1873 (95.1)		930 (96.7)		794 (92.9)		
No call	93		55		33		
BRAF							
N successful cases	2060		1015		887		
MD	15 (0.7)	(0.4, 1.2)	11 (1.1)	(0.5, 1.9)	3 (0.3)	(0.07, 1.0)	0.064
MND	2045 (99.3)		1004 (98.9)		884 (99.7)		
No call	3		2		1		
NRAS							
N successful cases	2061		1015		888		
MD	13 (0.6)	(0.3, 1.1)	7 (0.7)	(0.3, 1.4)	4 (0.5)	(0.1, 1.2)	0.56
MND	2048 (99.4)		1008 (99.3)		884 (99.5)		
No call	2		2		0		
HRAS							

Gene	All patients (N=2063)		Adenocarcinoma patients (N=1017)		Squamous cell patients (N=888)		p-value*
	N (%)ƒ	95% CI	N (%)ƒ	95% CI	N (%)ƒ	95% CI	
N successful cases	2043		1009		877		
MD	10 (0.5)	(0.2, 0.9)	1 (0.1)	(0.0, 0.6)	8 (0.9)	(0.4, 1.8)	0.015
MND	2033 (99.5)		1008 (99.9)		869 (99.1)		
No call	19		7		11		
Repeat	1		1		0		

Note: Two mutations were detected for MYD88 gene, one for KIT & AKT1, while none for ERBB2, FLT3 & JAK2 genes

Table 3: Association of cancer related mutations with smoking status

MD: Mutation detected, MND: Mutation not detected, (*) Fisher's exact test of the comparison of % mutated between Current/Former and Never smokers, 95% exact binomial confidence interval of %MD is provided for Current/Former and Never smokers groups

	All patients		Adenocarcinoma patients		Squamous cell patients	
	KRAS gene					
	KRAS MD (*N*=473)	**KRAS MND** (*N*=1582)	**KRAS MD** (*N*=385)	**KRAS MND** (*N*=629)	**KRAS MD** (*N*=54)	**KRAS MND** (*N*=829)
Smoking history - n (%)	(*P*=0.0065*)		(*P*<0.001*)		(*P*>0.99*)	
Current/Former	417 (24.1) (22.1, 26.2)	1315 (75.9)	339 (41.2) (37.9, 44.7)	483 (58.6)	49 (6.3) (4.7, 8.3)	725 (93.7)
Never	35 (15.9) (11.3, 21.4)	185 (84.1)	28 (18.9) (13.0, 26.2)	120 (81.1)	3 (5.0) (1.0, 13.9)	57 (95.0)
Unknown	21 (20.4)	82 (79.6)	18 (40.9)	26 (59.1)	2 (4.1)	47 (95.9)
	MET Gene					
	MET MD (*N*=140)	**MET MND** (*N*=1916)	**MET MD** (*N*=71)	**MET MND** (*N*=943)	**MET MD** (*N*=61)	**MET MND** (*N*=823)
Smoking history - n (%)	(*P*=0.20*)		(*P*=0.48*)		(*P*=0.032*)	

Current/Former	117 (6.7)	1616 (93.3)	61 (7.4)	761 (92.6)	51 (6.6)	724 (93.4)
	(5.6, 8.0)		(5.7, 9.4)		(4.9, 8.6)	
Never	20 (9.1)	200 (90.9)	8 (5.4)	140 (94.6)	9 (15.0)	51 (85.0)
	(5.6, 13.7)		(2.4, 10.4)		(7.1, 26.6)	
Unknown	3 (2.9)	100 (97.1)	2 (4.5)	42 (95.5)	1 (2.0)	48 (98.0)

EGFR gene					
	EGFR MD (*N*=107)	EGFR MND (*N*=1869)	EGFR MD (*N*=94)	EGFR MND (*N*=879)	EGFR MD (*N*=8)	EGFR MND (*N*=842)
Smoking history - n (%)	(*P*<0.001*)		(*P*<0.001*)		(*P*=0.37*)	
Current/ Former	54 (3.2)	1612 (96.8)	47 (6.0)	742 (94.0)	5 (0.7)	739 (99.3)
	(2.4, 4.2)		(4.4, 7.8)		(0.2, 1.6)	
Never	43 (20.3)	169 (79.7)	40 (28.4)	101 (71.6)	1 (1.7)	59 (98.3)
	(15.1, 26.3)		(21.1, 36.6)		(0.4, 8.9)	
Unknown	10 (10.2)	88 (89.8)	7 (16.3)	36 (83.7)	2 (4.4)	44 (95.6)

PIK3CA gene					
	PIK3CA MD (*N*=97)	PIK3CA MND (*N*=1873)	PIK3CA MD (*N*=32)	PIK3CA MND (*N*=930)	PIK3CA MD (*N*=61)	PIK3CA MND (*N*=794)
Smoking history - n (%)	(*P*=0.49*)		(*P*=0.31*)		(*P*=0.59*)	

Current/Former	78 (4.7) (3.7, 5.8)	1584 (95.3)	25 (3.2) (2.1, 4.7)	755 (96.8)	50 (6.7) (5.0, 8.7)	700 (93.3)
Never	12 (5.7) (3.0, 9.7)	200 (94.3)	7 (5.0) (2.0, 10.0)	133 (95.0)	5 (8.3) (2.8, 18.4)	55 (91.7)
Unknown	7 (7.3)	89 (92.7)	0 (0.0)	42 (100.0)	6 (13,3)	39 (86.7)

Supplement – Multiplex Manuscript

Methods

Mutation detection

Testing was performed on a high-throughput microfluidics-based PCR platform running an allele-specific multiplex mutation test (MUT-MAP). The MUT-MAP panel was run on the BioMark platform (Fluidigm Corp.) using a 96.96 dynamic array as described previously [1,2,3] with a few modifications. Briefly, the updated panel includes 13 genes (AKT1, BRAF, EGFR, ERBB2, FLT3, HRAS, JAK2, KIT, KRAS, MET, MYD88, NRAS and PIK3CA) incorporating 130 hot spot mutations found in various tumor types [1,2]. Preamplified DNA combined with qPCR reagents and 10X assays mixed with the Fluidigm 20X sample loading reagent (Fluidigm Corp.) were loaded onto the chip as per the manufacturer's protocol. All newly added assays were allele-specific PCR (AS-PCR) assays which utilized an engineered *Thermus specie* Z05 DNA polymerase (AS1) and primers to allow for allelic discrimination between the wild-type and mutant sequence [4,5]. An exon specific probe was used in all assays. The mutant allele detection sensitivity is >1% with a minimum requirement of 2-100 ng of gDNA.

DNA Preamplification

DNA preamplification procedures were performed as described previously [1-3]. Briefly, DNA was preamplified in a 10 µl reaction for 20 cycles in the presence of a preamplification primer cocktail mix (Table S1 shows sequences of newly added primers) and 1x ABI Preamp Master Mix (Applied Biosystems; Foster City, CA). All samples were exonuclease treated after PCR amplification to remove the remaining primers before being loaded onto the chip. Exonuclease I (16 U) (New England Biolabs; Ipswitch, MA) in exonuclease reaction buffer and nuclease-free water were added to each 10 µl PCR amplification and incubated at 37ºC for 30 min followed by a 15 min incubation at 80ºC for enzyme inactivation. Samples were then diluted four-fold in nuclease-free water and stored at 4ºC or -20ºC until needed.

A positive control was prepared in bulk by amplification of a cocktail of relevant mutant plasmids for all eleven genes in the presence of a wild-type human genomic DNA background; this positive control was run in triplicate on every chip for quality control purposes.

Data was analyzed and cycle threshold (C_T) values were determined using the BioMark real-time PCR analysis software (Fluidigm Corp.) and automated mutation calls were determined using an algorithm based on the difference in C_T (ΔC_T) values between wild-type and mutant assays for all AS-PCR assays.

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