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***PTEN, ATM, IDH1* mutations and MAPK pathway alterations are prognostic markers of PFS and OS in patients treated by first line EGFR TKI, an ancillary study of the French cooperative thoracic intergroup (IFCT) biomarkers France**

H. Blons^{ab}, J.-B. Oudart^a, J.-P. Merlio, D. Debieuvre, F. de Fraipont, C. Audigier-Valette, F. Escande, S. Hominal, P.-P. Bringuier, S. Fraboulet-Moreau, L. Ouafik, D. Moro-Sibilot^e, A. Lemoine, A. Langlais^c, P. Missy^d, F. Morin^d, P.-J. Souquet, F. Barlesi^f, J. Cadranel^g, M. Beau-Faller^{h,i}

On behalf of the French Cooperative Thoracic Intergroup(IFCT)

^aHEGP, Biochimie UF de Pharmacogénétique et Oncologie Moléculaire, Paris, France

^bCentre de Recherche des Cordeliers, INSERM, Sorbonne Université, Université de Paris,

^cDepartment of Biostatistics, French Cooperative Thoracic Intergroup, Paris, France

^dClinical Research Unit, French Cooperative Thoracic Intergroup, Paris, France

^eUnité d'Oncologie Thoracique, Service Hospitalier Universitaire Pneumologie Physiologie Pôle Thorax et Vaisseaux, CHU Grenoble Alpes, Grenoble, France

^fAix Marseille University, CNRS, INSERM, CRCM, Assistance Publique Hôpitaux de Marseille, Marseille, France

^gAP-HP, Hôpital Tenon, Service de Pneumologie, GRC 04 Theranoscan, Sorbonne Université, Paris, France

^hLaboratory of Biochemistry and Molecular Biology, Centre Hospitalier Universitaire de Strasbourg, Hôpital de Hautepierre, Strasbourg, France

ⁱIRFAC UMR-S1113, Inserm, Université de Strasbourg, Strasbourg, France

Corresponding Author

Pr M. Beau-Faller; Michele.FALLER@chru-strasbourg.fr

Laboratory of Biochemistry and Molecular Biology, Centre Hospitalier Universitaire de Strasbourg, Hôpital de Hautepierre, Strasbourg, France

IRFAC UMR-S1113, Inserm, Université de Strasbourg, Strasbourg, France

Highlights

- Response to EGFR TKI is heterogeneous among patients with *EGFR* mutated NSCLC
- Routine use of NGS enables co-mutations detection that may impact response to treatment
- Complex *EGFR* mutations are linked to reduced PFS and OS in patients with NSCLC
- MAPK activation is linked to reduced OS in patients with *EGFR* mutated NSCLC
- *PTEN*, *ATM* and *IDH1* mutations are linked to low PFS and OS in patients with *EGFR* mutated NSCLC

ABSTRACT

Tumor mutation screening is standard of care for patients with stage IV NSCLC. Since a couple of years, widespread NGS approaches used in routine diagnostics to detect driver mutations such as *EGFR*, *KRAS*, *BRAF* or *MET* allows the identification of other alterations that could modulated the intensity or duration of response to targeted therapies. The prevalence of co-occurring alterations that could affect response or prognosis as not been largely analyzed in clinical settings and large cohorts of patients. Thanks to the IFCT program “Biomarkers France”, a collection of samples and data at a nation-wide level was available to test the impact of co-mutations on first line EGFR TKI in patients with *EGFR* mutated cancers. Targeted NGS was assessed on available (n=208) samples using the Ion AmpliSeq™ Cancer Hotspot Panel v2 to screen for mutations in 50 different cancer genes. This study showed that *PTEN* inactivating mutations, *ATM* alterations, *IDH1* mutations and complex *EGFR* mutations were predictors of short PFS in patients with a stage 4 lung adenocarcinoma receiving first line EGFR TKI and that *PTEN*, *ATM*, *IDH1* and *KRAS* mutations as well as alterations in the MAPK pathway were related to shorter OS. These findings may lead to new treatment options in patients with unfavorable genotypes to optimize first line responses.

KEY WORDS

non-small cell lung cancer, next-generation sequencing, EGFR mutations, molecular profiles, prognosis

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1. INTRODUCTION

In non-small cell lung cancer (NSCLC) different targeted treatment strategies can be offered in first line for patients with advanced diseases depending on either the presence of molecular targets or the existence of a high PDL1 expression. Although the identification of a targetable driver has improved patients' outcome, responses are heterogeneous and a better tumor classification is mandatory to optimize treatment. Epidermal growth factor receptor (*EGFR*) tumor mutations are validated markers of response to *EGFR* tyrosine kinase inhibitors. In patients with advanced non-small cell lung cancer (NSCLC) harboring *EGFR* mutations the expected response rate in first line ranges from 56 to 83% with mean progression free survival (PFS) of 9 to 14 months [1–4]. However despite clear clinical benefits for most patients, time to progression is heterogeneous and some patients may experience primary resistance. Patients with a smoking history have a shorter overall survival (OS) [5], progression free survival (PFS) [6] and overall response rate (ORR) [7], at the opposite, women have a better OS. Molecular factors may also contribute to modulate response to *EGFR*-TKI. Previous works have suggested that co-occurring genomic alterations delineate different biological subgroups of patients with *EGFR* mutated cancers suggesting that a more comprehensive interpretation of genetic profiles could help identify biomarkers that impinge on response to treatment [8-12]. The IFCT program "Biomarkers France" (BMF), founded by the French National Cancer Institute (INCa) collected at a nation-wide level clinical and molecular data during a 1-year period. A total of 17 632 patients with advanced NSCLC, were screened for *EGFR*, *HER2 (ERBB2)*, *KRAS*, *BRAF*, *PIK3CA* mutations and *ALK* rearrangements, corresponding to 18645 molecular tests [13]. Focused on the *EGFR* subgroup an ancillary study based on this project was programmed to analyze whether extending molecular analysis to a 50 genes panel in a nationwide real life context impacts response prediction.

2 PATIENTS AND METHODS

2.1 Patients

Between April 2012 and April 2013, 17,664 NSCLC patients (median age, 64.5 years; male, 64.6%; smokers or former smokers, 81.2%; adenocarcinoma, 76%) were recruited and analyzed in the initial study. Clinical data were collected in a dedicated 'Biomarkers France' secured Web CRF as previously described (13). Among *EGFR* mutated tumors (11% of all samples), 204 had available material for NGS testing and clinical data fully filed in the e-CRF and were selected for subsequent analyses. This study was approved by a national ethics committee for observational studies (Comité d'Evaluation des Protocoles de Recherche Observationnelle, CEPRO) on 09/28/2011, by the French Advisory Committee on Information Processing in Material Research in the Field of Health (Comité Consultatif sur le Traitement de l'Information en Matière de Recherche dans le Domaine de la Santé, CCTIRS) on 09/22/2011 and by the National Commission of Informatics and Liberty (CNIL) on 12/18/2011, according to French laws, and was registered on the ClinicalTrials.gov website (NCT01700582).

2.2 NGS analyses

DNA (targeted NGS): tumor DNAs obtained using various extraction methods were collected from 21 INCa platforms and sent to one INCa laboratory to centralize NGS sequencing. Sequencing was done on the Ion Proton™ System using the Ion AmpliSeq™ Cancer Hotspot Panel v2 (Thermo Fisher Scientific). Detailed method is available as supplementary information (supplementary data 1). Co-mutations were analyzed as pathways; MAPK pathway defines samples with *EGFR* and associated *KRAS*, *BRAF*, *NRAS* or *HRAS* mutations; *PI3K-AKT* pathway defines samples with *EGFR* and *PIK3CA*, *PTEN* or *AKT1* mutations; cell cycle pathway samples with *EGFR* and *RB1* or *CDKN2A* mutations and WNT pathway samples with *EGFR* and *APC* or *CTNNB1* mutations.

2.3 Statistical methods

Results were expressed as medians for continuous variables and percentages for categorical variables, with comparisons made using chi-squared or Fisher's exact tests for categorical variables, and Student's t-test or ANOVA for continuous variables, with a significance level at $p < 0.05$. Survival

curves were estimated using the Kaplan–Meier method. Overall survival (OS) and progression-free survival (PFS) were previously defined [13]. Disease control rate (DCR) was defined as the percentage of patients with stable disease, partial response, or complete response, and overall response rate (ORR) as that of patients with partial and complete response. A Cox model was applied to estimate hazard ratios (HR) and 95% confidence intervals (CI). SAS software, Version 9.4 (SAS Institute, Cary, NC), was employed.

3 RESULTS

3.1 Patients

A total of 204 NSCLC patients with *EGFR* mutated tumors treated by EGFR TKI with available DNAs were collected from the biomarker France cohort. Among those, 1 was not *EGFR* mutated, 4 were DNA duplicates, 24 could not be amplified and 17 were not first line patients. Characteristics of patients analyzed in this ancillary study (n=158) were compared to the biomarker France patients with *EGFR* mutated tumors (n=1559). No statistical differences were observed for sex, age ethnicity, smoking, PS, personal history of cancer and histology. For this study, only BMF patients with stage IV cancer (n=138) or relapses (n=20) that had received first line TKI were analyzed (supplementary Table 1)

3.2 Co-occurring mutations identified by targeted NGS in *EGFR* mutated NSCLC

EGFR mutations were grouped as follow: DEL19, L858R, complex (DEL19 or L858R with a second mutation) and uncommon (no DEL19 or L858R) (Table 1). *EGFR* mutations detected by NGS were consistent with those identified at diagnosis except for 3 uncommon mutations, (*EGFR* p.Pro848Leu) detected at diagnosis but not by NGS due to the panel coverage design. *EGFR* mutant allele ratios ranged from 4 to 98%. Ten tumors (6%) had more than one *EGFR* mutation including 2 samples with a p.Thr790Met (less than 2%) primary sub-clonal co-occurring alteration. Among the 158 samples with NGS data, low coverage impaired full analysis for 13 samples (8%) that were properly

characterized for *EGFR* but inconclusive for co-alterations or copy number. Considering the 145 cases with full NGS data, gene amplifications were detected in 22, 6 and 6 samples for *EGFR*, *ERBB2* and *MET*, respectively. *EGFR*, *ERBB2* and *MET* amplifications were mutually exclusive and all samples with *EGFR* amplifications had a mutant allele ratio > 50% suggesting that the mutant copy was amplified (Table 1, Supplementary Table 2).

We identified 0, 1, 2, 3, 4 and 5 additional mutations in 28 (20%), 63 (43%), 30 (20.5%), 17(12%), 5 (3%) and 2 (1.5%) tumors, respectively. The most frequent association was *EGFR* and *TP53* mutations in 82 samples (57%) (Supplementary Table 2). Other recurrent alterations were found in *PIK3CA* (n=15; 10.5%), *CTNNB1* (n=13; 9%), *PTEN* (n=8; 5.5%), *ATM* (n=7; 4.8%), *CDKN2A* (n=4; 3%), *RB1* (n=8; 5.5%), *KRAS* (n=5; 3.5%), *STK11* (n=5; 3.5%) and *BRAF* (2; 1.4%) (Figure 1). MAPK activation was found in samples (n=8) with uncommon (n=4), complex (n=2) or L858R (n=2) mutations and was exclusive of DEL19 alterations (p<0.0001). *KRAS* (n=5) mutations were also more frequently associated to uncommon mutations (n=4) (Supplementary Table 3). No other association was identified.

3.3 Clinical correlations

Uncommon *EGFR* mutations (p=0.02), *PTEN* (p=0.006), *PI3K-AKT* pathway (p=0.02) and *MAPK* (p=0.058) alterations were more frequent in smokers (Table 2). PFS was correlated with *EGFR* mutation types (p<0.001) and the existence of more than one *EGFR* mutation after exclusion of the pThr790Met mutation as the secondary event (p<0.0001); however no correlation was found with OS (supplementary figures 1). No difference in terms of OS or PSF was found between samples with *EGFR* mutations only and samples with non-*EGFR* additional mutations. When looking at alterations individually; *PTEN*, *ATM* and *IDH1* mutations (p=0.03; p=0.05; p=0.045) were associated to shorter PFS (Figure 2). Multivariate analysis showed that *IDH1* HR=5.1[1.2-21.7] (p=0.03), *PTEN* HR=2.4 [1.1-5.0] (p=0.02) and a complex *EGFR* mutational status HR=6.1 [2.4-15.8] (p=0.0002) were independent predictors of shorter PFS. *IDH1*, *KRAS*, *PTEN* and *ATM* mutations (p=0.006, p=0.02; p=0.02; p=0.008) as well as MAPK alterations p=0.017 were associated with lower OS (Figure 3). In samples with *TP53* mutations, no significant association with PFS or OS was found. We tested gain of function versus

loss of function mutations and DNA binding domain versus non-DNA binding domain mutations; it did not permit the identification of any association.

There was no impact of *EGFR* allelic ratio or gene amplification on PFS or OS. Similar observations were made for *ERBB2* (OS: 0.83 [0.26-2.63]; PFS: 1.04 [0.33-3.28]) and *MET* (OS: 1.00 [0.41-2.47]; PFS: 1.01 [0.41-2.51]) amplifications.

4 Discussion

Current management of lung cancer is based on molecular screening and targeted therapies for patients with oncogene drivers. Patients with *EGFR* mutated cancers will experience different levels of response to EGFR-TKI. As NGS gene panels are now part of routine testing, the clinical impact of co-occurring molecular events has been addressed. Different studies have reported links between concomitant molecular changes and response to EGFR TKI suggesting that not all *EGFR* mutated tumors are equal. Co-occurring alterations impact response rates and duration suggesting that specific treatment options could be evaluated in patients with co-drivers [8,14,15]. Here, we had the opportunity to test this hypothesis and analyze *EGFR* mutated samples from the biomarker France cohort to identify important modulators of EGFR response in real life settings.

We show that co-occurring mutations frequencies for the set of genes analyzed are in accordance with previous series [8,17]. In line with previous publications, a few co-occurrences were identified in *KRAS* and *BRAF* [18-19]. Here, RAS-RAF alterations are not due to treatment selection of resistant clones as all were first line TKI patients. *BRAF* mutations were sub-clonal in both case, indeed *BRAF* VAFs were lower than *EGFR* VAFs. In this situation, *BRAF* mutated cells might drive primary or secondary resistance in patients receiving EGFR TKI. Concerning *KRAS*, VAFs were high (> 25%) in 3 out of 5 cases; however *KRAS* mutations co-occurred with uncommon *EGFR* mutations suggesting that, in those cases the main driver might be *KRAS*. RAS-RAF co-mutations were analyzed as MAPK pathway alterations and shown to lower OS. As underline previously, WNT-CTNNB1 pathway alterations are enriched in *EGFR* mutated lung cancers here, in accordance with previous findings, we identified 20/158 (12.5%) samples with WNT-CTNNB1 alterations [8].

Our data confirm that *EGFR* mutated cancers have different mutational backgrounds and raise the question of the clinical impact of inter-tumor heterogeneity to predict first line response and secondary resistance mechanisms. Previous works suggested links between *TP53* mutations [15] or sub-groups of *TP53* mutations and low OS or PFS [20-22]. Here no association between *TP53* mutations (or mutation subgroups) and clinical data was identified, contrasting with results published by Griesinger et al that suggested an impact of non-disruptive mutations on PFS. Different patient populations could explain this discrepancy. In line with our results, a recent Chinese series of patients with *EGFR* mutated tumors showed no impact of *TP53* mutations between short (< 6 months) versus long (>24 months) PFS [14].

Here, *PTEN* mutations dramatically decrease PFS and OS suggesting that patients with *PTEN* mutated tumors are poor responders to EGFR-TKIs. *PTEN* mutations were either known in cancer or loss of function suggesting that all were deleterious alterations. A recent work, using *PTEN*-small interfering RNA showed that *PTEN* down-regulation led to decreased sensitivity of HCC827 cells to icotinib [23]. Similar observations were made in other cell lines that confirmed that *PTEN* loss impacts response to first generation EGFR-TKI in lung cancer [24-25]. Although less documented, *PTEN* loss may also be associated with osimertinib resistance suggesting that it could be a pan-EGFR-TKI resistance mechanism [26]. Because *PTEN* loss is associated with high level of AKT activity dual blockade of EGFR and PI3K-AKT pathway should be considered as a therapeutic approach. *IDH1* or *IDH2* mutations are rare events in lung cancers. Here we identified 3 tumors with *EGFR* (*DEL19* or *L858R*) and *IDH* mutations. *IDH* mutations were either “known in cancer” or “driver” (Cancer Genome Interpreter) that dramatically impacted PFS and OS. Multiple *IDH* inhibitors have been developed over the last several years and could represent new treatment options for patients with *EGFR/IDH* mutated tumors.

ATM mutations have not been largely documented in *EGFR* lung cancer. Here *ATM* mutations were linked to low PFS and OS. It is somehow difficult to understand these associations as some identified variants have conflicting interpretation of pathogenicity. *ATM* is a master regulator of DNA damage

responses but has many other effects and modulates cell cycle activation. It acts as an activator of the G1/S checkpoint and prevents damaged cells from entering in S-phase. It was shown that *EGFR* could translocate to the nucleus where it interacts with DNA strand breaks repair proteins including ATM and that ATM itself could phosphorylate AKT a downstream effector in the EGFR pathway [27]. ATM is a tumor suppressor that is recurrently mutated in lung cancer and in other cancer types. It was described in colorectal cancer that *ATM* mutations were associated to an absence of response to cetuximab in *RAS* wild type samples [28]. In a paper exploring cross talks between the EGFR pathway and ATM, authors observed a synergistic cell growth inhibition when cells were co-treated with gefitinib and an ATM inhibitor [27]. This shows how complex interactions can be. In our series we identified missense *ATM* mutations and showed that they were related to low PFS in patients with *EGFR* mutation receiving first line EGFR TKI. Although this association needs to be confirmed it suggests that ATM alterations might, as PTEN, be a PAN-EGFR TKI resistance marker.

Finally, cell cycle alterations were linked to *DEL19* mutations. It was shown for all EGFR-TKI types that *RB1* mutations were predictive of secondary resistance through phenotypic changes and small cell lung cancer transformation [29]. Unfortunately we could not explore further, as patients were not biopsied at relapse. However, here, the presence of a *RB1* mutation at diagnostic had no impact on first line EGFR-TKI response.

Our study has some limitations that we need to underline. NGS testing was only possible for a subset of BMF samples due to either a lack of available DNA or registered clinical data. For samples with available DNAs, some could not be amplified or were only partially conclusive especially when centers used microdissection techniques before DNA extraction. Even though no significant differences were identified between groups, this study is based on a retrospective subgroup analysis. And finally all patients had first generation EGFR-TKI only so our results would need validation for second or third generation drugs.

6 Conclusions

This is, to our knowledge the first study to explore the impact of co-occurring genetic events in first line TKI Caucasian patients with *EGFR* mutated lung cancer based on a nationwide data collection in real life clinical settings. It shows that *PTEN* inactivating mutations, *ATM* alterations, *IDH* mutations and complex *EGFR* mutations are predictors of short PFS in patients with a stage 4, lung adenocarcinoma receiving first line EGFR TKI. This may lead to new treatment options in patients with unfavorable genotypes to optimize first line response such as combinations with antiangiogenic drugs, other targeted therapies or chemotherapy.

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Figure legends

Figure 1

OncoPrint plots for frequent mutations in 158 *EGFR* mutated lung cancers analyzed by a 50 genes NGS panel. *EGFR* mutations are split into *EGFR*exon19del for inframe deletion in exon 19, p.Leu858Arg and uncommon mutations. Pathways alterations are shown and defined in the material and method section.

Figure 2

Impact of the presence of a co-mutation on progression free survival (PFS) in patients with *EGFR* mutated NSCLC treated in first line by an *EGFR* TKI (A) PFS according to the presence of a *PTEN* mutation (B) PFS according to the presence of an *ATM* mutation (C) PFS according to the presence of an *IDH1* mutation.

Figure 3

Impact of the presence of a co-mutation on overall survival (OS) in patients with *EGFR* mutated NSCLC treated in first line by an *EGFR* TKI (A) OS according to the presence of a *PTEN* mutation (B) OS according to the presence of a *KRAS* mutation (C) OS according to the presence of a *IDH1* mutation (D) OS according to the presence of an *ATM* mutation and (E) OS according to the presence of a *MAPK* alteration as define in material and methods.

Table 1

Frequency of *EGFR* mutation types grouped as complex, DEL19, L858R and uncommon. Complex mutations consist of one DEL19 or L858R with a rare alteration and uncommon consist of rare alterations only, including mutations at codons 861, 709, 719, INS20 and other rare changes.

Table 2

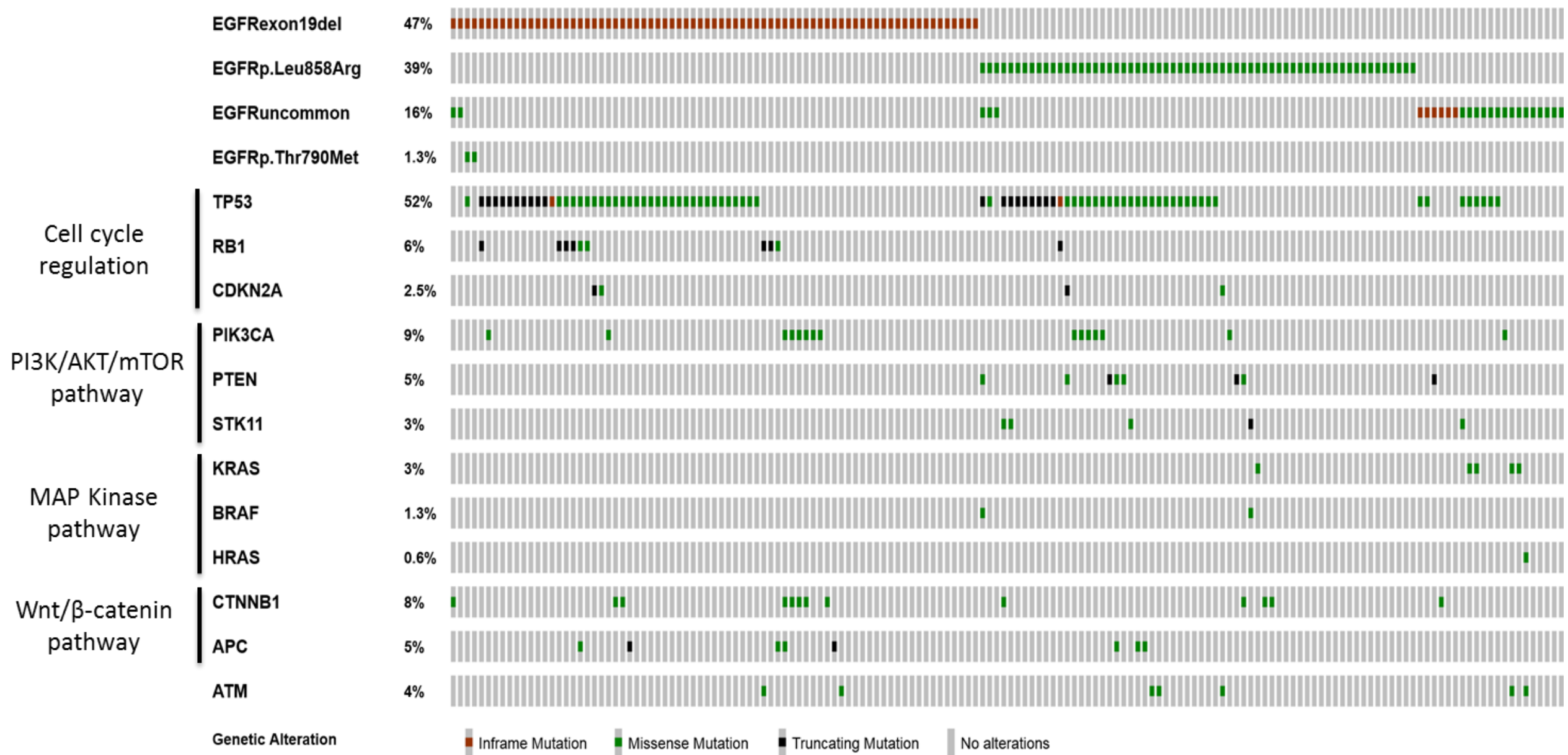
Correlations between tobacco exposure and molecular alterations. PI3K/AKT, MAPK pathway alterations and PTEN mutations are linked to tobacco exposure.

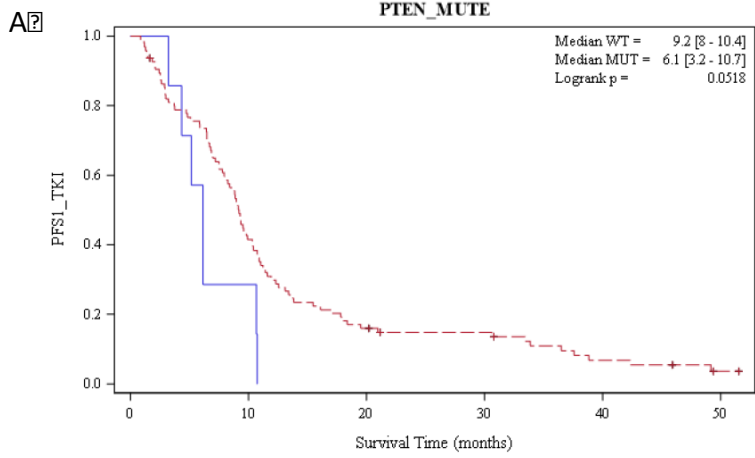
TABLE 1

			Total (N=158)
Type of <i>EGFR</i> mutation	DEL19	N(%)	72(45.6)
	L858R	N(%)	59(37.3)
	Complex	N(%)	7(4.4)
	Uncommon	N(%)	20(12.7)
EGFR amplification			
<i>EGFR</i> amplification	NO	N(%)	123(84.8)
	YES	N(%)	22(15.2)
	Missing	N	13
<i>EGFR</i> amplification level			
<i>EGFR</i> amplification level	High	N(%)	9(6.2)
	Low	N(%)	13(9)
	NO	N(%)	123(84.8)
	Missing	N	13
Number of <i>EGFR</i> mutation			
Number of <i>EGFR</i> mutation	1	N(%)	148(93.7)
	> 1	N(%)	10(6.3)

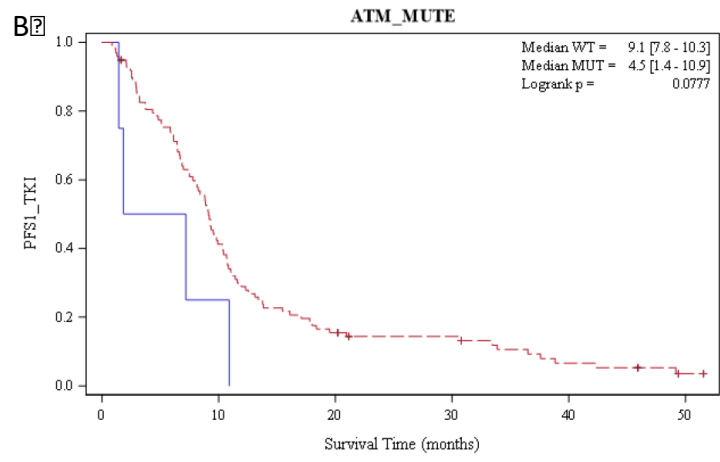
TABLE 2

		TOBACCO		
		yes	no	p
EGFR	COMPLEX	1	6	0.02
	DEL	22	49	
	L858R	25	34	
	UNCOMMON	13	7	
MAPK	M	6	2	0.056
	WT	51	85	
PI3K/AKT	M	14	9	0.03
	WT	43	78	
PTEN	M	7	1	0.006
	WT	50	86	

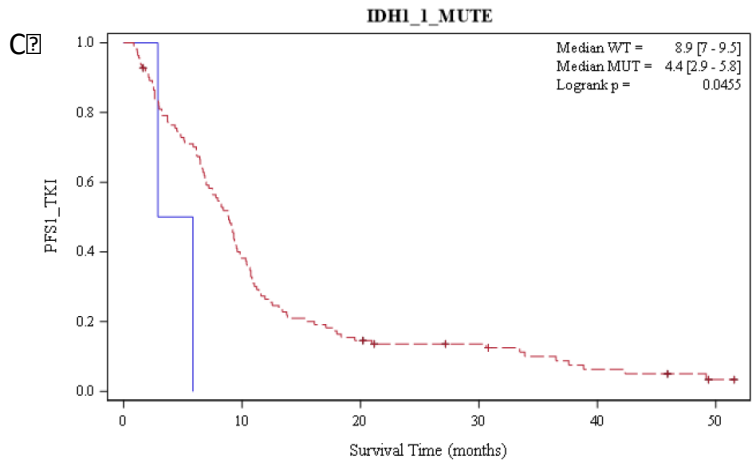




PTEN_MUTE														
		Survival Time (months)												
MUT	7	5	2	0										
WT	95	72	39	22	15	12	12	8	5	4	1			



ATM_MUTE														
		Survival Time (months)												
MUT	4	2	1	0										
WT	98	75	40	22	15	12	12	8	5	4	1			



IDH1_1_MUTE														
		Survival Time (months)												
MUT	2	1	0											
WT	111	80	42	23	16	13	12	8	5	4	1			

