Patterns of DNA Mutations and *ALK* Rearrangement in Resected Node Negative Lung Adenocarcinoma

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Background: Many studies have examined specific mutations in patients with resected lung adenocarcinoma across heterogeneous stages, comprising predominantly advanced/metastatic disease, but there is little data regarding the mutation profile of patients with early stage node negative disease. The aim of this study was to identify patterns of mutations in early stage node negative lung adenocarcinoma.

Methods: A total of 204 patients who underwent resection for stage IB (sixth Ed American Joint Committee on Cancer) lung adenocarcinoma and received no neoadjuvant or adjuvant treatments were identified. Tumors were genotyped using the OncoCarta v1.0 kit (Sequenom, San Diego, CA) on the Sequenom MassARRAY platform. Fluorescence in situ hybridization for *ALK* rearrangement was also performed.

Results: A total of 110 (54%) patients' tumors harbored at least one mutation. *KRAS*, *EGFR*, *PIK3CA*, *ALK*, *PDGFRA*, *AKT1*, *BRAF*, *FGFR1*, and *HRAS* mutations were detected in tumors from 77 (37.7%), 29 (14.2%), 9 (4.4%), 2 (1%), 2 (1%), 1 (0.5%), 1 (0.5%), 1 (0.5%), and 1 (0.5%) patients respectively. Synchronous mutations (either comutations or double mutations) were identified in 18 (8.8%) patients. *KRAS* and *PIK3CA* mutations were associated with poorly differentiated tumors ($p = 0.03$; $p = 0.02$), whereas *EGFR* mutations were associated with well-differentiated tumors ($p = 0.001$). Five tumours contained *EGFR* mutations (one T790M and four exon 20 insertions), which are associated with resistance to *EGFR* tyrosine kinase inhibitors (EGFR-TKIs).

Conclusions: Diverse patterns of mutations are seen in resected node-negative lung adenocarcinoma including an unexpectedly low rate of *ALK* rearrangement, *EGFR* mutations associated with resistance to EGFR-TKIs and a high rate of synchronous mutations. These data may influence the design of future adjuvant targeted therapy trials.

Key Words: Lung adenocarcinoma, *KRAS*, *EGFR*, *PIK3CA*, *ALK*.

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Lung cancer is the second most common cancer and the leading cause of cancer death worldwide, with an incidence of 1.6 million new cases annually and a mortality of 1.38 million in 2008.[1] For the past two decades, decisions regarding lung cancer treatment have been based largely on the histological distinction between non–small-cell (NSCLC) and small-cell lung carcinoma. In recent years, more definitive histological classifications and identification of somatic mutations have become an essential component in determining the management of NSCLC. Molecular driven therapeutic targets such as epidermal growth factor receptor gene (*EGFR*)[2] and abnormal fusion of echinoderm microtubule-associated protein-like 4 and anaplastic lymphoma kinase genes (*EML4-ALK*)[3] have resulted in a paradigm shift in the treatment of advanced lung adenocarcinoma. However, in early NSCLC, surgical resection remains the treatment of choice with adjuvant chemotherapy having a modest absolute survival benefit of 5% at 5 years in stage II–IIIA disease, but an equivocal effect only in stage IB

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disease.[4] Several studies have assessed the presence of specific mutations, predominantly *KRAS, EGFR*, and *PIK3CA*,[5–7] in patients with resected lung adenocarcinoma across a heterogeneous group of stages, but there is little data on the mutation profile of patients with early stage disease. Inevitably, the design of future adjuvant trials of targeted therapies in early stage lung adenocarcinoma will be based on the mutation data from advanced/metastatic disease. Therefore, our aim was to examine a mutation profile in a cohort of patients who had resected early node-negative lung adenocarcinoma to inform better the design of future clinical trials.

PATIENTS AND METHODS

We retrospectively reviewed 204 patients with stage IB primary lung adenocarcinoma according to the American Joint Commission on Cancer, (AJCC) sixth edition tumor-node-metastasis (TNM) staging system,[8] who underwent surgical resection between January 1990 and May 2008. Patients who received neoadjuvant or adjuvant treatments were excluded. Pathology reports were reviewed and pathologic characteristics (tumor size, histopathologic type, grade, visceral pleural, vessel, and perineural invasion) were extracted. Two representative formalin fixed paraffin embedded (FFPE) tumor blocks from each case were retrieved from the anatomical and tissue pathology archives. They were sectioned and stained with hematoxylin and eosin. An experienced pulmonary pathologist (W.A.C.) reviewed the slides and marked representative areas. Two 1-mm cores of the marked representative areas from FFPE tissue were collected for DNA extraction. This study was approved by the Human Research Ethics Committee of Royal Prince Alfred Hospital. (X10-0278; HREC/10/RPAH/491)

Mutation Detection

DNA was extracted from the FFPE tissue using NucleoSpin FFPE DNA Kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instruction with an overnight proteinase digestion. The quality and quantity of the extracted DNA was assessed using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). A minimum of 480 ng of DNA was required for successful mutational analysis. Samples were amplified for 238 variant targets in a 24-multiplex PCRs using the OncoCarta Panel v1.0 Kit (*ABL1, AKT1, AKT2, BRAF, CDK, EGFR, ERBB2, FGFR1, FGFR3, FLT3, JAK2, KIT, MET, HRAS, KRAS, NRAS, PDGFR, PIK3CA,* and *RET*) and analyzed based on the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) technology on the Sequenom MassArray platform.[9,10] The targeted mutations in the 19 oncogenes comprising the OncoCarta v1.0 Panel are reported to be biologically significant in carcinogenesis or progression in a range of malignancies. These mutational analyses and Fluorescence in situ hybridization (FISH) for *ALK* rearrangement described below were performed at an Australian National Association of Testing Authorities (NATA) accredited laboratory to the ISO15189 standard, which is comparable to the Clinical Laboratory Improvement Amendments (CLIA) in the United States. NATA accreditation provides a mechanism to determine, formally recognize and promote the competence of facilities in performing specific types of testing. It also ensures that the accredited laboratory operates at a high standard with internal and external quality assurance measures.

FISH for ALK Rearrangement

Interphase fluorescence in situ hybridisation for *ALK* rearrangement was performed on tissue microarrays constructed from all cases for *ALK* rearrangement using the Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe (Abbott Molecular, Abbott Park, IL). This probe detects rearrangements in chromosome 2p23 encompassing the *ALK* gene, which includes a SpectrumOrange labeled 250 kb DNA fragment telomeric to *ALK* (3′ end) and a SpectrumGreen labeled 300 kb DNA fragment centromeric to *ALK* (5′ end). The probe set does not identify the specific rearrangement gene partner. Tissue microarray tissue sections of 4 μm were baked at 60 °C overnight and stained for *ALK* FISH according to the manufacturers instructions, except that Invitrogen Pretreatment Solution (Life Technologies, Grand Island, NY) was used at 98–102 °C for 20 minutes.

Signals were counted in at least 100 tumor nuclei per case using an epifluorescence microscope (Zeiss , Oberkochen, Germany). FISH for *ALK* locus rearrangement was considered positive if at least 15% of cells analyzed showed either a split of one set of red and green signals greater than one signal width apart, and/or if loss of one green signal had occurred.[3,11–14]

A positive control consisting of an independently validated lung tumor confirmed by FISH to be positive for *ALK* rearrangement was included. Negative controls included an independently validated lung tumor confirmed by FISH to be negative for rearrangement and normal lung tissue.

Statistical Analysis

Visceral pleural invasion was consistently reported, but more than 50% of cases had missing data for vessel and perineural invasion. Therefore, vessel and perineural invasion were not included in the subsequent analysis.

To examine the relationship between categorical variables, Pearson χ^2 test with continuity correction and Fisher's exact test were performed where appropriate. Linear-by-linear association χ^2 test was used to evaluate trend for ordinal variable. All p values were two-sided and a p value less than 0.05 was considered significant. Kaplan–Meier analysis was used to determine the overall survival and differences between genotypes were compared using the log-rank test. All statistical analyses were performed using PASW Statistics version18.0 (SPSS Inc., Chicago, IL).

Terminology

DNA variants refer to any DNA change while mutations imply pathogenicity. Double mutation refers to synchronous mutations within a single oncogene while comutation refers to synchronous mutations within two different oncogenes.

TABLE 1. Clinicopathologic Characteristics of 204 Patients with Resected Stage IB Lung Adenocarcinoma

Clinicopathologic factors ($N = 204$)	Number (%)
Median age	69 (range 40–87)
Gender	
Male	120 (59)
Female	84 (41)
Type of operation	
Lobectomy	164 (80)
Pneumonectomy	13 (7)
Wedge resections/segmentectomy	27 (13)
Tumor size	
Less than or equal to 5 cm	155 (76)
5.1 to 7 cm	30 (15)
Greater than 7 cm	18 (9)
Unknown	1
Histopathological grade	
Well differentiated	26 (13)
Moderately differentiated	116 (56)
Poorly differentiated	62 (30)
Visceral pleural invasion	
Yes	85 (42)
No	103 (50)
Not reported	16 (8)

RESULTS

The clinical characteristics of 204 patients with resected node-negative primary lung adenocarcinoma are described in Table 1. The majority of patients (80.4%) underwent lobectomy because of the early stage of the disease.

There were 110 (54%) patients whose tumors harbored at least one mutation with 18 patients having two or more mutations (Fig. 1*A*). *KRAS, EGFR, PIK3CA, MET* variants, and *PDGFRA* mutations/variants were detected in tumors from 77 (37.7%), 29 (14.2%), 9 (4.4%), 7 (3.4%), and 2 (1%) patients respectively (Table 2). *AKT1, BRAF, FGFR1,* and *HRAS* mutations were uncommon, and all except for the *BRAF* mutation coexisted with another oncogene mutation (Table 2; Fig. 1*B*). There were only two patients whose tumors had *ALK* rearrangement. The median survival was 6.5 years and 5-year survival rate was 55%. There was a trend to suggest that patients whose tumors harbored *KRAS* ($p = 0.055$) and *PIK3CA* ($p = 0.046$) mutations had a poor prognosis but such trend was not observed in those with *EGFR* mutations ($p = 0.5$). (Fig. 2*A–C*)

The relationship between clinicopathologic factors and mutation patterns were assessed. *EGFR* mutations were more common in women than men (23.8% versus 7.5%). However, no sex difference was observed in tumors with *KRAS* and *PIK3CA* mutations. Tumors containing *KRAS* mutations were more likely to be poorly differentiated ($p = 0.03$) as were tumors containing *PIK3CA* mutations ($p = 0.02$). Conversely, *EGFR* mutations were associated with well-differentiated tumors ($p = 0.001$). There were no associations between mutation patterns and visceral pleural invasion or tumor size ($p > 0.05$).

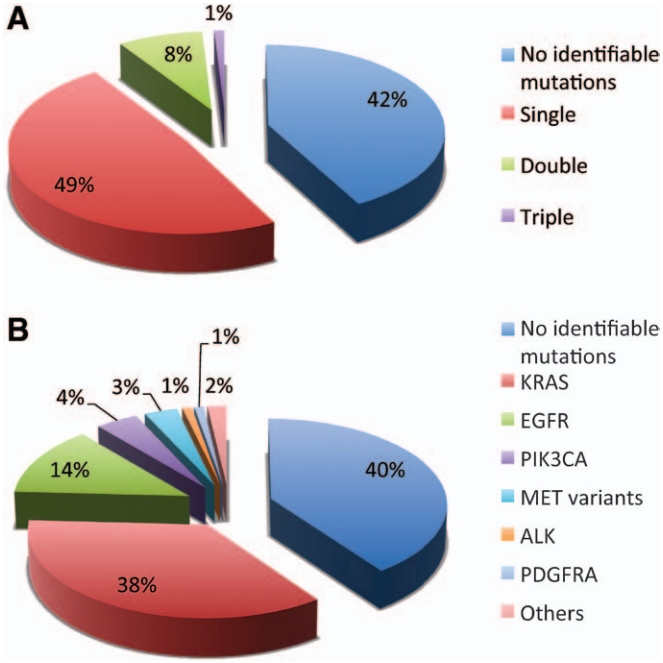

FIGURE 1. (*A*) Distribution of number of mutations/variants present. (*B*) Frequency of different mutations/variants and *ALK* rearrangement.

KRAS

The most common mutation identified was in the *KRAS* gene. All *KRAS* mutations were missense changes and were identified at codons 12, 13, or 61. The mutations were mutually exclusive from *EGFR* mutations ($p < 0.001$). Ten patients who had *KRAS*-mutant tumors had comutations; five (6.5%) had *PIK3CA* mutations, three (3.9%) had *MET* variants, one had a *PDGFRA* mutation, and one had an *AKT1* mutation (Table 2).

EGFR

The second most common mutation identified was in the *EGFR* gene (Table 2). Of the 29 patients with *EGFR* mutations, 12 (43%) of tumors harbored common classic mutations in exon 19 (in-frame simple deletions) and only 7 (24%) contained the L858R mutation in exon 21, whereas 14% of *EGFR* mutations were seen in exon 18. Unexpectedly, five tumors contained *EGFR* mutations associated with resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) therapy. One patient had a tumor with a T790M mutation and four had tumors containing exon 20 insertions. Furthermore, two patients had comutations with other oncogenes (*PIK3CA* and *FGFR1*) in addition to *EGFR* mutations (Table 2). There were four patients whose tumors harbored double *EGFR* mutations, two different *EGFR* mutations (one G719C + E709A, one G719A + S768I, and two G719S + S768I) found simultaneously in a single tumor (Table 2).

PIK3CA

Only nine of the 204 patients had mutations in the *PIK3CA* gene. Mutations were not only detected at the hotspot locations of exon 9 (helical domain) and exon 20 (kinase

TABLE 2. DNA Mutations and Variants identified in 204 Patients with Resected Stage IB Adenocarcinoma

	***KRAS* mutation** ***N* = 77 (37.7%)**	***EGFR* mutation** ***N* = 29 (14.2%)**	***PIK3CA* mutation** ***N* = 9 (4.4%)**	***MET* variant** ***N* = 7 (3.4%)**
Gender				
Male	47 (61%)	9 (31%)	6 (67%)	5 (71%)
Female	30 (39%)	20 (69%)	3 (33%)	2 (29%)
Mutation Subtypes	Exon 2 (92%)	Exon 18 (14%)	Exon 9 (56%)	R970C 1 (14%)
	G12A 3	G719A 1	E545K 5	T992I 6 (86%)
	G12C 26	G719S 2		
	G12D 13	G719C + E709A 1	Exon 20 (11%)	
	G12R 2		H1047R 1	
	G12S 1	Exon 19 (43%)		
	G12V 23	Deletions 12	Others (33%)	
	G13D 3		R38H 2	
		Exon 20 (29%)	E545K + M1043I 1	
	Exon 3 (8%)	T790M 1		
	Q61H 6	Insertions 4		
		S768I 3		
		Exon 21 (29%)		
		L858R 7		
		L861Q 1		
Comutations	PIK3CA 5	PIK3CA 1	KRAS 5	KRAS 3
	MET 3	FGFR1 1	HRAS 1	
	PDGFRA 1		EGFR 1	
	AKT1 1			
Double mutations in a single oncogene	0	4	1	0

domain), but also two cases had an R38H mutation, which was located at the p85 binding domain away from the helical and kinase domains. Of the nine patients, seven had comutations with another oncogenes predominantly *KRAS*, although there was one case of a *PIK3CA* mutation coexisting with an *EGFR* (L858R) mutation. There was also one case in which two *PIK3CA* mutations (E545K + M1043I) were identified coexisting with a *HRAS* mutation. (Table 2)

MET

Like *PIK3CA* mutations, *MET* variants were relatively uncommon (Table 2). The majority of *MET* variants were the T992I, whereas one tumor contained a R970C variant. These variants are located within the coding region for the juxtamembrane domain. Further analysis demonstrated that the *MET* variants were also present in the matched non-neoplastic lung tissue, indicating that these were germline variants rather than somatic mutations.

DISCUSSION

This study demonstrates that mutations are common in resected early node-negative, treatment-naive lung adenocarcinoma with 54% of patients having tumors which harbor at least one biologically relevant mutation. Although mutations in *KRAS, EGFR* and *ALK* were mutually exclusive, some tumors harbored synchronous mutations within a single oncogene such as double *EGFR* mutations or within two different oncogenes in the case of comutations. Unexpectedly, there were also mutations associated with drug resistance despite the fact that this was a cohort of early stage disease patients who had not been treated with chemotherapy or targeted agents.

The baseline characteristics of our cohort are similar to other reported studies, which have sought to identify patterns of gene mutations in NSCLC.[5–7] Most of these studies have analyzed the pattern of specific somatic mutations (*EGFR* and *KRAS* or *PIK3CA*) in cohorts of adenocarcinoma with heterogeneous disease stage including locally advanced/metastatic disease and patients who had been exposed to various treatments.[5–7] In contrast, we performed a wider mutational analysis encompassing *EGFR, KRAS, PIK3CA,* and *ALK, PDGFRA, FGFR1, AKT1* and *HRAS*. In addition, the cohort in this study was homogeneous in stage (node negative adenocarcinoma) and treatment (surgery only). Our rate of *EGFR, KRAS,* and *PIK3CA* mutations was comparable to published cohorts of NSCLC in western population,[2,7,15–18] but our rate of *ALK* rearrangement (1%) was lower than generally reported in advanced NSCLC (5%).[3] Furthermore, we identified a higher rate of multiple mutations than previously reported in other studies.[19,20] This is of considerable importance as preclinical and clinical studies demonstrate that tumors with comutations may have different responses to targeted therapy depending on the combinations of oncogenes.[21–23] Although we interrogated a more extensive set of mutations in a larger range of oncogenes than other studies, the panel still has its

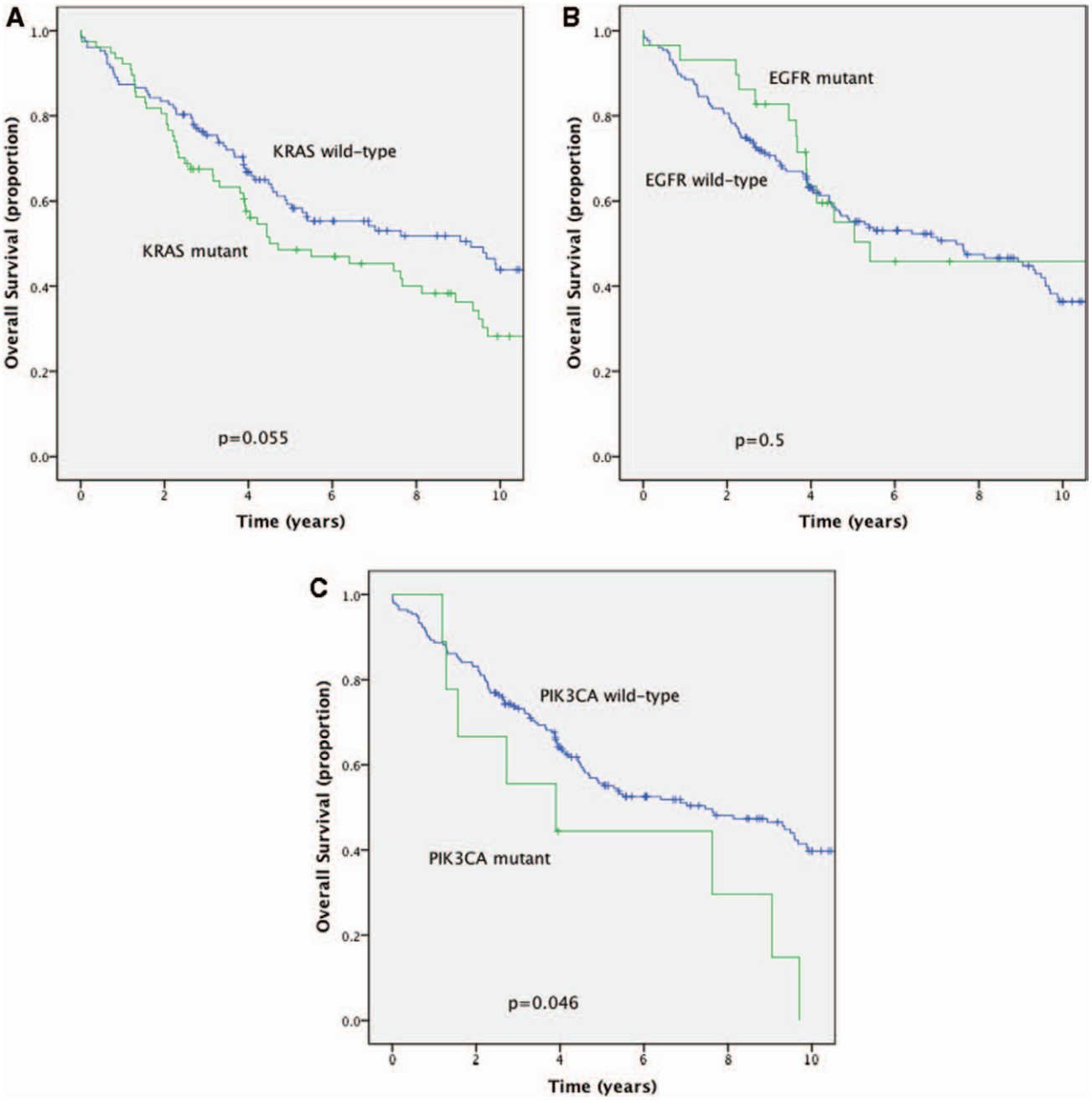

FIGURE 2. Kaplan–Meier overall survival curves of *KRAS*, *EGFR*, and *PIK3CA* wild-type and mutant populations censored at 10 years.

limitations. Naturally, there are potentially other significant oncogenic mutations which may be found on more detailed sequencing.

In a western population, approximately 20% of lung adenocarcinomas contain *EGFR* mutations. The commonest classic *EGFR* mutations at exon 19 (in-frame deletions) and exon 21 (missense mutation L858R) together account for approximately 90% of *EGFR* mutant tumors in published studies.[24,25] However, they are less prevalent, accounting for only 66% of *EGFR* mutations in our cohort. On the other hand, *EGFR* mutations associated with EGFR-TKIs resistance were identified, including the T790M mutation and exon 20 insertions. In particular, *EGFR* exon 20 insertion mutations were more common in our study (14%) than reported by Yasuda et al.[26] (5%). This could be explained by the use of the more sensitive multiplexed system array technology as Su et al.[27] demonstrated that the MALDI-TOF method is as sensitive as next generation sequencing, and more sensitive than Sanger sequencing. Furthermore, one of the four double *EGFR* mutation combinations identified in our study, G719C

+ E709A has been associated with EGFR-TKI resistance.[28] An in vitro study demonstrated that the combination of G719C and E709A *EGFR* mutations attenuated the response to gefitinib as compared with the individual mutations alone.[28] The rest of the tumors, which contained double *EGFR* mutations (G719A/S + S768I), were the combination of a sensitive mutation and a resistant mutation to EGFR-TKIs. It is unclear how these mutation patterns will affect the response to EGFR-TKIs. Therefore, it is important to examine the entire *EGFR* kinase domain from exon 18 to 21 for *EGFR* mutations and their specific subtypes to avoid missing nonclassic yet clinically relevant *EGFR* mutations.

Mutations in the *KRAS* oncogene are the most common somatic mutation in lung adenocarcinoma. Meta-analysis[29] suggests that *KRAS* mutant cancers have a poorer prognosis regarding overall survival and a poor response to EGFR-TKIs. However, these data are contradictory and independent validation studies have failed to produce a consensus view. There is increasing evidence that the subtype of *KRAS* mutations may be important with *KRAS* codon 13 mutations (G13D) predicting for significantly poorer survival with adjuvant chemotherapy in the four large clinical trials of adjuvant chemotherapy in early NSCLC.[30] More recently, a phase II study has demonstrated that selumetinib, a MEK inhibitor, combined with docetaxel improves overall survival, response rate and progression-free survival in patients with advanced NSCLC whose tumor harbored *KRAS* mutations.[31]

In our cohort, *PIK3CA* comutations were much more common than isolated *PIK3CA* mutations suggesting these mutations may modulate tumor biology in concert with other genetic mutations rather than being the primary driver. Halilovic et al.[32] speculated that comutations provide a fail-safe mechanism for cancer cells to grow and proliferate when facing environmental stress such as hypoxia. Our study demonstrated a 78% rate of *PIK3CA* mutations coexisting with mutations in other genes, almost all being *RAS* mutations, upstream from the *PIK3CA* signaling cascade. Little is known about the clinical implications of *PIK3CA* mutations in NSCLC, although data from other cancer types have suggested that *PIK3CA* mutations may predict for a poor prognosis in breast cancer and are associated with resistance to cetuximab in colorectal cancer. It is unclear what functional implication these comutations may have on response to potential treatments.

MET is a proto-oncogene located at chromosome 7 (7q31) and has been implicated in metastases and invasion in a variety of cancers.[33] Although *MET* nonsynonymous variants (R970C and T992I) in NSCLC are reported to be germline variants without transforming ability, they may still play a role in tumorgenesis particularly when combined with an oncogene that drives cellular proliferation such as *KRAS*.[34,35] Germline variants can influence tumor biology, for instance an *FGFR4* germline variant Arg388 is associated with resistance to adjuvant therapy in primary breast cancer.[36] The biological significance of germline *MET* variants remains to be seen.

We found a trend towards poor prognosis among patients whose tumors contained *KRAS* or *PIK3CA* mutations but not in those with *EGFR* mutations. However, the number of patients examined within these subgroups was small. Therefore, the prognostic value of presence of specific mutations remains to be validated in larger studies.

In summary, we have demonstrated diverse patterns of mutations in resected early stage, node negative lung adenocarcinoma which can be used to inform future clinical research. For example, the low rate of *ALK* rearrangement suggests that adjuvant trials with crizotinib are unlikely to be successful. Furthermore, the presence of *EGFR* mutations associated with resistance to EGFR-TKIs may influence the results of adjuvant trials of erlotinib or gefitinib, whereas comutations associated with signaling pathways such as *PIK3CA* may also affect response to therapy. Our data provide compelling evidence for comprehensive tumor mutation profiling as an essential element of adjuvant trial design as DNA changes present in advanced cancer cohorts do not necessarily match those from early stage disease.

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