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ALK immunohistochemistry is highly sensitive and specific for the detection of ALK translocated lung adenocarcinomas: lessons from an audit of lung cancer molecular testing in South-East of Scotland

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ABSTRACT

Background: The approval of novel targeted treatments for EGFR-positive and ALK-positive non-small cell lung cancer (NSCLC) has led to the increased requirement for mutation testing.

Results: We report our experience of ALK testing with immunohistochemistry (IHC) and fluorescence in-situ hybridization (FISH) and present the prevalence of EGFR, KRAS and ALK mutations. From January 2011 to May 2014, we found mutation rates of EGFR, KRAS and ALK to be 10.4% (67/643), 35.8% (86/240) and 2.3% (7/304) respectively. ALK-rearrangements were found to be associated with never smokers (p<0.001) and younger patients (≤50 years old) (p<0.001). ALK IHC protein expression in tumour cells is 100% sensitive (7 IHC+/7 FISH+) and 96.6% specific (113 IHC-/117 FISH-) for ALK-rearrangements by FISH. ALK-rearranged tumours were wild-type for EGFR and KRAS.

Conclusion: Our findings support the use of ALK protein expression and KRAS mutation testing as part of the molecular diagnostic algorithm for lung adenocarcinomas.

INTRODUCTION

Non-small cell lung cancer accounts for 87% of all lung cancers diagnosed in the UK (1). Lung adenocarcinomas can be further stratified according to the mutation status (2). Epidermal growth factor receptor (EGFR), Kirsten rat sarcoma 2 viral oncogene homolog (KRAS) and anaplastic lymphoma kinase (ALK) are currently the mutations most commonly tested for in lung adenocarcinomas. The stratification of lung adenocarcinomas according to these molecular subtypes has important clinical implications, informing the first-line treatment offered to each individual patient (2).

Activating EGFR mutations were first described in 2004 as patients with mutations in EGFR gene were found to show clinical response to EGFR tyrosine kinase inhibitors (TKIs) (3). This discovery revolutionized the molecular diagnostic for lung cancer patients. Two of the most commonly occurring mutations, exon 19 deletions and exon 21 L858R missense mutations confer sensitivity to EGFR TKIs (3). In contrast, exon 20 T790M mutations are associated with resistance to EGFR TKIs (4).

The identification of echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase (EML4-ALK) fusion gene in a subgroup of NSCLC (5) as an oncogenic driver has led to the development of ALK inhibitors that show a dramatic and long-lived response in ALK-translocated tumours (6). ALK abnormalities are typically associated with younger age and never smokers (7). KRAS mutations in NSCLC occur more frequently in Caucasian populations and are associated with smoking (8). G12C and G12V subtypes are commonly found in patients with a smoking history whereas G12D is more likely to be found in non-smokers (9).

The aim of this study was to evaluate the utility of ALK immunohistochemistry (IHC) and fluorescence in-situ hybridization (FISH) and to determine the prevalence of EGFR, KRAS and ALK mutations.

METHODS

Specimens, demographics and clinical information

From January 2011 to May 2014, a total of 682 cases in SE Scotland were tested for the presence of EGFR, KRAS mutations and/or ALK rearrangements. This comprised 586 adenocarcinomas, 75 non-small cell lung carcinomas and 21 other tumours (including 6 cases of large cell neuroendocrine carcinoma, 4 cases of squamous cell carcinoma, 3 cases of large cell undifferentiated carcinoma, 2 mixed adenosquamous carcinoma, 1 case each of mixed squamous and large cell neuroendocrine carcinoma, carcinoid tumour, pleomorphic carcinoma, sarcoma, sarcomatoid carcinoma and mixed malignant peripheral nerve sheath tumour and large cell neuroendocrine carcinoma). Common to these 21 other tumours was young age and/or a history of non-smoking which was deemed by the oncologist as a reason for molecular testing. Of these 37 samples were insufficient due to low volume of tumour or tissue. In January 2013, KRAS mutation testing was introduced and was carried out on 242 cases. ALK rearrangement testing was introduced in 2012 and carried out on 304 cases. Most of these samples were biopsy specimens, 428 followed by 160 cell blocks (prepared from cytological specimens such as EBUS aspirates or pleural fluids), and 94 surgically resected samples either from the primary tumour or site of metastasis. Clinical data and demographics of all patients were collected retrospectively from electronic clinical records. This study was conducted as part of an audit on the clinical testing services for lung adenocarcinomas in molecular pathology. Lung cancer staging was done in accordance with the 7th Edition "TNM classification of malignant tumours" (10). Smoking status was classified as never smokers (<100 cigarettes in lifetime), former smokers (stopped smoking for at least one year before diagnosis) and current smokers (including those who stopped smoking less than one year prior to diagnosis) (9). Smoking exposure was measured in pack years whereby one pack year was defined as smoking 20 cigarettes per day in one year.

ALK, EGFR and KRAS mutation testing

ALK IHC was carried out using the D5F3 clone (Cell Signaling Technology) to identify expression of the abnormal ALK fusion protein. The scoring criteria used was based on a

binary scoring system positive of negative ALK status. Positive staining constitutes any cytoplasmic and/or nuclear staining. An equivocal pattern of staining was used for cases showing apical or focal membranous staining and for these cases FISH analysis was requested to determine the ALK rearrangement status. The tumours showing ALK positivity were also further tested by fluorescence in-situ hybridization (FISH). FISH analysis was performed using the Vysis LSI ALK Break Apart Rearrangement Probe from Abbott Molecular (UK) and the evaluation was carried out as recommended by the manufacturer.

The EGFR and KRAS mutation status was determined using DNA extracted from formalin-fixed, paraffin embedded sections. Macrodissection was carried out on a large number of cases to enrich the tumour DNA. The amount of tissue used varied from 3 to 6 10μm thick sections depending on the amount of tumour tissue present in each case. DNA was analysed for 29 EGFR mutations using the Qiagen's therascreen® EGFR RGQ PCR method. KRAS testing was carried out using an in-house pyrosequencing method to detect mutations in codons 12, 13 and 61 of the KRAS oncogene using the primer sequences described (please see Supporting Information).

Statistical Analysis

Data were analysed using χ^2 test for association and trend (where categories were ordered, eg smoking). Where expected observations were less than 5, Fisher's Exact test was employed. Multivariable explanatory models were analysed using logistic regression.

RESULTS

Of 682 patients with lung cancer included 680 were tested for EGFR mutations, 304 for ALK and 242 for KRAS mutations concomitantly.

Of 304 cases tested for the presence of and ALK abnormality by IHC, 9 were positive (2.9%), 3 were equivocal (1%) and 292 (96.1%) were negative. Of these, 125 cases were also analysed by FISH. Out of the 9 IHC positive cases, 7 cases were confirmed by FISH as showing an ALK gene rearrangement, 1 case showed a definite ALK IHC positive staining but FISH analysis showed no evidence of an ALK rearrangement and 1 case

failed to hybridise. The 3 cases with an equivocal ALK IHC pattern showed no ALK gene rearrangement by FISH. Taken as a whole, ALK IHC+/FISH+ was found in 2.3% of the cases in our study. We report 100% sensitivity (7 IHC+/7 FISH+) and 96.6% specificity (113 IHC-/117 FISH-) when comparing results of ALK protein IHC expression with ALK FISH analysis. In our cohort, ALK rearrangements were found to be associated with never smokers (p<0.001) and younger patients (≤50 years old) (p<0.001).

Table 1 summarises the results for EGFR, KRAS mutations and ALK rearrangement with demographical and clinical characteristics. Cases with insufficient material for analysis were excluded from the statistical analysis. None of the cases in our cohort had concomitant mutations of EGFR, KRAS or ALK translocation, supporting the adenocarcinoma oncogenome pattern of molecular exclusivity (11).

EGFR mutations show a slight female predominance with 345 females (53.7%) and 298 males (46.3%). 14.5% of females and 5.7% of males had EGFR mutation in their tumours, with a prevalence of 10.4% (n=67) EGFR mutations in our cohort. Deletions in exon 19 (24/67) and L858R exon 21 mutation (32/67) are the two most prevalent mutations as shown in Table 2. In 3 cases a double mutation of EGFR gene was present, including two cases of L858R exon 21 and T790M exon 20 mutations, and one case of L861Q exon 21 and exon 18 mutations. Of note, in our cohort, two cases of a rare deletion and insertion in exon 19 were also reported.

EGFR mutations were found in 35.1% (27/77) of never smokers, 9.3% (23/247) of former smokers and 5.0% (14/280) of current smokers. There was a significant association between EGFR-mutant tumours and never smoking status (p<0.001). There is evidence to suggest a linear association with smoking history as increasing duration of smoking is associated with a decreasing proportion of EGFR mutations (p<0.001). Females had a significant increased likelihood of EGFR mutation compared to males (OR 2.80, 95% CI 1.59-4.97, p<0.001). However, this effect was lost when smoking pack-years were taken into account. An increase in one pack years of smoking resulted in a decrease in the odds ratio of EGFR mutations (OR 0.94, 95% CI 0.92 - 0.96, p<0.001).

KRAS mutations were found in 35.8% (86/240) of cases successfully tested. The most frequent mutation found was in codon 12 with 74 cases (86%) followed by 7 cases with codon 61 mutations (8.1%) and 5 cases with mutations in codon 13 (5.8%), as shown in Figure 1. KRAS-mutation status was associated with a history of smoking, in both former (OR 6.26, 95% CI 2.00-19.56, p=0.002) and current smokers (OR 6.82, 95% CI 2.18-21.35, p=0.001) significantly higher than in non-smokers. Neither gender (p=0.09) nor the number of smoking pack years (p=0.13) had an influence on the rates of KRAS mutations. The frequency of EGFR and KRAS mutations by smoking status is illustrated in Figure 2.

DISCUSSION

The approval of EGFR and ALK TKIs for use in subsets of patients with activating mutations means there is a requirement to provide a molecular pathology service capable of multiplex testing in a cost-effective manner. As these mutations tend to be mutually exclusive (12), we have introduced KRAS testing as a tool to help select patients for ALK testing and at the same time enable a wise use of the budget. KRAS testing offers a more cost-effective and clinically useful alternative compared to ALK FISH testing (13). Moreover, despite not being linked to predicting a direct response to therapy, there is strong evidence that KRAS mutations are involved in the mechanism of resistance to crizotinib (14) and in addition, KRAS mutations act as a stronger predictor of response to EGFR TKI therapy than the EGFR mutation status alone (15). Therefore, based on the reasons above, our testing algorithm for NSCLC testing includes EGFR, ALK IHC and/or FISH and KRAS mutations testing (13).

Our study reports the prevalence of EGFR, ALK and KRAS mutations in South East of Scotland as 10.4%, 2.3% and 35.8% respectively.

There is large variation in the prevalence of ALK abnormalities as highlighted in a large systematic review (7). This variation in reporting is due to the selection criteria, with some studies including squamous cell carcinomas in their testing algorithms. There is not much data published from UK based population studies but data presented at local or national meetings describes the actual prevalence of ALK rearrangement in lung NSCLC

in the UK to be lower than initially reported at approximately 1 to 2% (unpublished data). Although ALK rearrangements are mutually exclusive with EGFR and KRAS mutations in our cohort, small numbers of tumours with concomitant ALK and EGFR or ALK and KRAS mutations have been reported in the literature (16). In our cohort six out of the seven ALK rearranged tumours were non-smokers, only one patient was identified to be a smoker.

We have not identified any false negative IHC negative / FISH positive cases and this could be due to the relative small numbers in out cohort. Other studies report a discrepancy rate of up to 24% possibly due to hierarchical screening (17). While the method commonly used to detect ALK abnormalities is FISH, most labs are now adopting immunohistochemistry as a screen test followed by confirmatory FISH (18). This is cost-effective and offers a quicker turn-around time. More importantly, a recent study argues that the detection of the ALK fusion protein by immunohistochemistry is superior to an ALK FISH test in predicting tumour response and survival to crizotinib (19). In view of this new data and our own experience, molecular testing of lung NSCLC should include ALK immunohistochemistry either as a screening tool or perhaps as a primary diagnostic test to detect ALK rearrangements.

Variable EGFR mutation rates have been reported across the world. One study in a single centre in the US reported an EGFR mutation rate of 20%, while a European study reported EGFR mutation rate of as low as 4.9% in an unselected cohort of patients whereby all newly diagnosed NSCLC cases were screened for EGFR mutations (20). Data from Asian populations generally report higher mutation rates, in some studies as high as 66.3% (21). One likely explanation for the higher prevalence reported in most studies, including our study, was the possible selection bias when referring cases for mutation testing. At the early stages of EGFR mutation testing services, undoubtedly most of the cases referred seemed likely to be selected on the basis of never smoking status and younger age.

Our study highlights the association of female gender and never smoking status with the presence of EGFR mutations. Among the non-smokers tested, 74.6% were female. Despite the association found between the non-smoking status and the presence of EGFR

mutations, we do not recommend using smoking status as a selection criterion for excluding smokers from EGFR testing. As demonstrated, EGFR mutations are also present in smokers and former smokers, although to a much lesser degree.

We demonstrate that in smokers with any smoking history, there is a significant association with an increased likelihood of KRAS mutations regardless of smoking pack years.

In conclusion, ALK protein expression in tumour cells is 100% sensitive and 96.6% specific for ALK rearrangements by FISH. Our findings support the use of ALK IHC as an effective screening tool for this rare but clinically important molecular subgroup of lung adenocarcinomas.

DISCOSURES

Yuan Chen Kheng, Kathy Walsh and Anca Oniscu drafted the manuscript, collected and analysed the data.

Linda Williams carried out the statistical analysis.

William Wallace, David J Harrison and Anca Oniscu designed the study and drafted the manuscript.

The authors have no conflicts of interest to declare.

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	EGFR mutations			KRAS mutations			ALK mutations		
	All patients	Patients with EGFR mutations	All 680 patients	All patients	Patients with KRAS mutations	All 242 patients	All patients	Patients with ALK mutations	All 304 patients
	(N = 680)	(N = 67)	% (95% CI)	(N = 242)	(N = 86)	% (95% CI)	(N = 304)	(N = 7)	% (95% CI)
Sex									
Male	316	17	46.5 (42.4-50.1)	116	35	47.9 (41.8-54.3)	148	4	48.7 (42.3-54.7)
Female	364	50	53.5 (49.9-57.6)	126	51	52.1 (45.7-58.2)	156	3	51.3 (45.3-57.7)
Age Median (Mean)	67 (66)	67 (67)		68 (67)	68 (67)		68 (66)	50 (58)	
IQR	15	13		16	17		16	25	
Smoking history	(N = 637)	(N = 64)		(N = 230)	(N = 82)		(N = 289)	(N = 7)	
Never smokers	79	27	12.4 (10.0-15.0)	35	4	15.2 (10.7-20.7)	36	6	12.5 (9.0-16.8)
Former smokers	263	23	41.3 (27.4-45.5)	93	37	40.4 (33.4-47.4)	121	0	41.9 (36.2-47.2)
Current smokers	295	14	46.3 (42.0-50.1)	102	41	44.3 (36.8-51.6)	132	1	45.7 (40.7-51.5)

Table 1. Frequency of patients tested for EGFR, KRAS and ALK and their demographical and clinical characteristics.

EGFR mutation	Frequency (%)
L858R exon 21	32 (47.8%)
Deletion in exon 19	24 (35.8%)
Mutation in exon 18	3 (4.5%)
Insertion in exon 20	2 (3.0%)
L861Q exon 21	1 (1.5%)
L858R exon 21 & T790M exon 20	2 (3.0%)
Deletion and insertion in exon 19	2 (3.0%)
L861Q exon 21 & mutation in exon 18	1 (1.5%)
Total	67

Table 2. EGFR mutations detected in our cohort.

Figure Legends

Figure 1: KRAS mutations detected in our cohort.

Figure 2: Frequency of EGFR and KRAS mutations according to smoking history.

Supporting Information

KRAS primers sequences: forward 5'-GGCCTGCTGAAAATGACTG -3' and reverse 5'-Biotin-GCTGTATCGTCAAGGCACTCT-3' for KRAS codons 12 and 13, forward 5'-Biotin-TGGAGAAACCTGTCTCTTGGATAT-3' and reverse 5'-CTGGTCCCTCATTGCACTGTACTC-3' for KRAS codon 61.