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Anaplastic lymphoma kinase (*ALK*) mutations in patients with adenocarcinoma of the lung

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ABSTRACT

Background: Non-small cell lung cancer (NSCLC) is a major cause of cancer-related death. Approximately 2–16% of NSCLC patients with wild-type epidermal growth factor receptor (*EGFR*) harbour anaplastic lymphoma kinase (*ALK*) mutations. Both *EGFR* and *ALK* mutations occur most commonly in Asian patients with NSCLC. As targeted therapy is available for NSCLC patients with these mutations, it is important to establish reliable assays and testing strategies to identify those most likely to benefit from this therapy.

Materials and methods: Patients diagnosed with adenocarcinoma of the lung between 2010 and 2014 were tested for *EGFR* mutations. Of these, 92 cases were identified as *EGFR* wild type and suitable candidates for *ALK* testing utilising immunohistochemistry and the rabbit monoclonal antibody D5F3. The reliability of the IHC was confirmed by validating the results against those achieved by fluorescence *in situ* hybridisation (FISH) to detect *ALK* gene rearrangements.

Results: Twelve (13%) cases were positive for *ALK* expression using immunohistochemistry. Of the 18 evaluable cases tested by FISH, there was 100% agreement with respect to *ALK* rearrangement/*ALK* expression between the assays, with 11 cases *ALK* negative and 7 cases *ALK* positive by both assays. *ALK* tumour expression was significantly more common in female compared to male patients (29.6% vs. 6.2%, $P < 0.001$), detected exclusively in patients that had never smoked ($P < 0.001$) and more frequently in metastases (22.7%) than in primary tumours (10%) ($P = 0.047$).

Conclusions: Detection of *ALK* expression by IHC is reliable and the most practical way of identifying NSCLC patients likely to benefit from crizotinib treatment.

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Introduction

Throughout the world, to include both Western and Asian countries, lung cancer continues to be the major cause of cancer-related death [1]. The largest proportion (approximately 85%), of these lung cancers are classified as non-small cell lung cancer (NSCLC), with a 5-year survival rate of 16% [2,3]. A history of tobacco smoking has been attributed to most of these cancers, though some patients have never smoked [4]. It has been shown that 10–30% of patients with NSCLC carry specific epidermal growth factor receptor (*EGFR*) mutations, and that patients with tumours that harbour these mutations benefit from treatment with the tyrosine kinase inhibitor Tarceva (erlotinib) [5–9]. More recently, anaplastic lymphoma kinase (*ALK*) gene rearrangements caused by translocations or inversions, resulting in high levels of *ALK* protein expression, have been found to be present in approximately 2–16% of NSCLC [10–13]. Patients with these *ALK* mutations benefit from treatment with the drug crizotinib [14,15].

To date, the vast majority of patients with *ALK* rearrangements have NSCLC, and in particular adenocarcinomas, which contain the 'wild type' *EGFR* gene i.e. their tumours are negative for *EGFR* mutations [12]. For practical purposes therefore the expression of the mutated *EGFR* and *ALK* are mutually exclusive. This is an important consideration when identifying patients likely to benefit from therapies that target either *EGFR* mutations or expression of the *ALK* protein, as the laboratory testing for *EGFR* and *ALK* can be relatively expensive. Consequently, current guidelines consider it acceptable to restrict *ALK* testing to those patients shown to be *EGFR* negative, i.e. possess wild type *EGFR* in their tumour samples [16].

The alterations in the *ALK* can be detected by fluorescence *in situ* hybridisation (FISH) using a multicolour break-apart DNA probe to *ALK*. Until recently, this was considered to be the most reliable way of identifying *ALK* rearrangements and predicting which patients are likely to respond to crizotinib therapy [15,17]. However,

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evidence from recent studies employing highly specific and sensitive rabbit monoclonal antibodies to demonstrate *ALK* protein over-expression suggest that this may be a more reliable test to predict response to crizotinib [17,18]. In this paper we investigate the expression of *ALK* protein in an Asian cohort of patients with lung adenocarcinomas, utilising an immunohistochemical assay and a highly sensitive and validated rabbit monoclonal antibody.

Materials and methods

A total of 120 patients diagnosed with NSCLC adenocarcinomas between 1 January 2010 and 31 December 2014, were tested for *EGFR* mutation analysis using the cobas® *EGFR* Mutation test kit (v1.0 CE-IVD) (Roche Molecular Diagnostics, California, U.S.A.). Of these, 92 cases were identified as *EGFR* wild type with no mutation detected in exons 18–21 of the *EGFR* and were therefore considered as suitable candidates for *ALK* testing. Seventy cases were primary NSCLC (lung biopsies and lung resections) and 22 cases were metastatic NSCLC (pleural, lymph node, brain, bone and liver biopsies). The study was approved by the Medical Ethics Committee of the University Malaya Medical Centre (Ref. no: 1150.25). All specimens were fixed in 10% formalin and paraffin wax embedded (FFPE).

For each case, multiple slides corresponding to whole tissue sections were reviewed by a pathologist and classified according to the 2015 World Health Organization (WHO) classification [19]. Other data collected for analysis included; the patients' age, gender, ethnicity and smoking status.

EGFR mutation analysis was as follows. DNA was isolated from the FFPE tissue sections containing at least 70% of tumour cells using the cobas® DNA preparation kit. The cobas® *EGFR* Mutation Test kit (v1 CE-IVD) utilising a cobas®4800 real-time PCR system was then used to identify mutations in exons 18, 19, 20 and 21 of the *EGFR* (Roche Molecular Diagnostics, California, U.S.A.).

Immunohistochemistry (IHC) for *ALK* protein was performed on all 92 cases using the rabbit monoclonal antibody clone D5F3 (Ventana Medical Systems, Tucson, USA). The antibody was visualised using the OptiView DAB IHC Detection (Ventana Medical Systems) and the Ventana OptiView amplification Kit. The entire staining procedure was performed on a fully automated Ventana Bench-Mark XT instrument. A case identified as having *ALK* rearrangement by FISH was used as a positive control for all runs. A binary scoring system (positive or negative for *ALK* status) was used to evaluate the staining; with the presence of strong, granular cytoplasmic staining in tumour cells (any percentage of positive tumour cells) considered *ALK* positive, and the absence of granular cytoplasmic staining in tumour cells considered *ALK* negative.

FISH analysis was performed on 23 FFPE cases, comprising 12 *ALK* immunohistochemically positive cases and 11 *ALK* IHC negative cases, in order to determine the level of agreement between the two assays and validate the immunohistochemical assay. The FDA-approved Vysis LSI *ALK* Dual Color Break Apart FISH Probe Kit (Abbott Molecular, Des Plaines, IL, U.S.A.) was used according to the manufacturer's instructions. The dual-colour probe includes the 5′*ALK* Spectrum Green and 3′*ALK* Spectrum Orange fluorophores, which appears as a yellow fusion signal (or adjacent red and green signals) without an *ALK* gene rearrangement. A result was considered positive for *ALK* rearrangement when >15% of the cells scored showed split green and red signals by a length equivalent to at least two signal diameters and/or an isolated red signal.

Statistical analysis was as follows. Continuous data of the patients' age is presented as the mean ± the standard deviation and was analysed using the Student's *t*-test. Categorical data are presented as frequency and percentage and were analysed using the chi-square test or Fisher Exact test. This statistical analysis utilised the Statistical Package for the Social Sciences (SPSS) software, Version 20 (SPSS Inc. Chicago, IL, U.S.A.).

Results

Twelve (13%) of the 92 cases tested were positive for *ALK* protein expression using immunohistochemistry. Of the 23 cases tested by FISH, a total of 5 cases were non-interpretable due to either insufficient numbers (<50) of tumour cells ($n = 3$), or comprising mainly fibrous tissue ($n = 2$). All 5 of these cases were *ALK* positive by immunohistochemistry. For the remaining 18 cases there was 100% agreement with respect to *ALK* rearrangement/*ALK* expression, between the two assays, with 11 cases *ALK* negative by both FISH and immunohistochemistry, and 7 cases *ALK* positive by both assays. The type or rearrangements observed in the FISH positive cases comprised; 5′*ALK* deletions ($n = 2$) and *ALK* translocation/inversions ($n = 5$).

The clinical characteristics of the cases with respect regards to *ALK* expression is summarised in Table 1. The mean age of patients with *ALK* positive tumours and that of those without *ALK* mutations was not significantly different ($P = 0.823$). *ALK* expression showed a significant association with gender, with a higher incidence of *ALK* positive tumours in female compared to male patients (29.6% vs. 6.2% $P < 0.001$). *ALK* tumour expression was detected exclusively in patients that had never smoked (never smokers), as opposed to patients who had smoked at some time during their life (ever smoked)($P < 0.001$). *ALK* expression was only detected in lung adenocarcinoma showing solid (4/24, 16.7%) or acinar (4/46 (8.7%) histological patterns (See Figure 1(A) and (B)) and was more frequently detected in metastatic lesions (22.7%) than in the primary tumours (10%) ($P = 0.047$).

Table 1. Case details and *ALK* expression.

Characteristics	No of patients (n = 92)	*ALK* tumour expression		Significance *P*-value
		Positive (n = 12)	Negative (n = 80)	
Age (year)				0.823
Mean (±SD)	61.2 ± 11.7	60.5 ± 7.8	61.3 ± 12.2	
Gender				<.0000
Male	65	4 (6.2%)	61 (93.8%)	
Female	27	8 (29.6%)	19 (70.4%)	
Smoking status				<0.001
Ever smokers	51	0	51 (100%)	
Never-smokers	36	12 (33.3%)	24 (66.7%)	
Unknown	5	0	5 (100%)	
Type of specimen				0.047
Primary tumour	70	7 (10%)	63 (90%)	
Metastatic lesion	22	5 (22.7%)	17 (77.3%)	

Discussion

The treatment of patients with advanced NSCLC with *ALK* rearrangement with the tyrosine kinase inhibitor, crizotinib, has been found to significantly increase progression free survival compared to existing chemotherapeutic regimes [15]. Whilst the incidence of *ALK* rearrangements in NSCLC in Western populations can be as low as 2–6% [11,13], the incidence is frequently higher in Asian studies and ranges from 3 to 16% [10,12]. The increased incidence emphasises the importance of establishing reliable assays and testing strategies for *ALK* determination in order to identify patients likely to benefit from crizotinib therapy.

This is the first documented study of *ALK* tumour expression in Malaysia, a multi-racial country comprising the three most populous ethnic groups in Asia; Malay, Chinese and Indian. The incidence of *ALK* tumour expression was found to be 13% of all the lung adenocarcinomas tested and with wild type *EGFR* expression and of a similar incidence to that found in Chinese studies (10, 12). This strategy for predictive *ALK* testing follows international guidelines recommending a rational approach in which *ALK* testing can be restricted to *EGFR* wild type NSCLC, due to the reciprocal expression of these mutations in NSCLC i.e. the vast majority of *ALK* positive tumours do not harbour *EGFR* mutations [16].

Until recently, FISH and the detection of *ALK* rearrangements on tissues samples was considered the most reliable way to determine patient's eligibility for crizotinib therapy [15,17]. However, FISH methodology is relatively expensive, requires considerable technical expertise, its interpretation is time consuming and it requires specialist equipment; all of which are important and limiting factors when providing this service in a diagnostic laboratory. The use of an immunohistochemical assay for *ALK* is far more practical and economically viable in this setting. The initial antibodies available to *ALK* though, showed variable levels of sensitivity, with the risk of producing false negative results with some cases known to have *ALK* rearrangements by FISH [10,11]. However, very recently two rabbit monoclonal antibodies to *ALK* have become commercially available and for use with immunohistochemistry, with some reports showing that these highly sensitive antibodies to *ALK* are more accurate at predicting patient response to crizotinib than the FISH assay [20,21]. In the current study, we validated the reliability of one of these antibodies, clone D5F3, against cases with and without gene rearrangement, as determined by FISH, and recorded complete concordance on all evaluable cases.

Participation in an external quality assessment programme, such as the U.K. National External Quality Assessment Scheme (UK NEQAS) for Immunocytochemistry and *In Situ* Hybridisation [22], is an essential quality assurance component of all clinically accredited laboratories and is particularly important for assays such as those to *ALK*, which effectively predict those lung cancer patients most likely to benefit from life extending targeted therapy. Recent results from the UK NEQAS programme for *ALK* immunohistochemistry show that in the U.K. over 80% of laboratories testing for *ALK* are using the D5F3 clone, as used in the current study, with 85% of participating laboratories achieving acceptable results [22]. As more studies are published validating the usefulness of the D5F3 clone and antibodies of similar specificity and sensitivity, immunohistochemistry may well become the primary screening assay for *ALK* testing, in a similar way as it has for other predictive markers, such as those to oestrogen receptors and the human epidermal growth factor receptor-2 (HER2) in breast cancer [23,24]. As well as reporting on the reliability and practicality of this approach, we show interestingly that *ALK* tumour expression is significantly associated with female patients with lung adenocarcinoma, and patients that have never smoked. Whilst the numbers are small and require further investigation, this is an important finding as the incidence of lung cancer in both these categories is reported to be rising [25,26]. The other notable finding is that *ALK* tumour expression was significantly more common in the cases of metastatic NSCLC than it was in cases of primary tumour comprising mainly lung biopsies, though again the numbers are small.

This work represents an advance in biomedical science because it confirms the usefulness and reliability of testing for *ALK* tumour expression by immunohistochemistry.

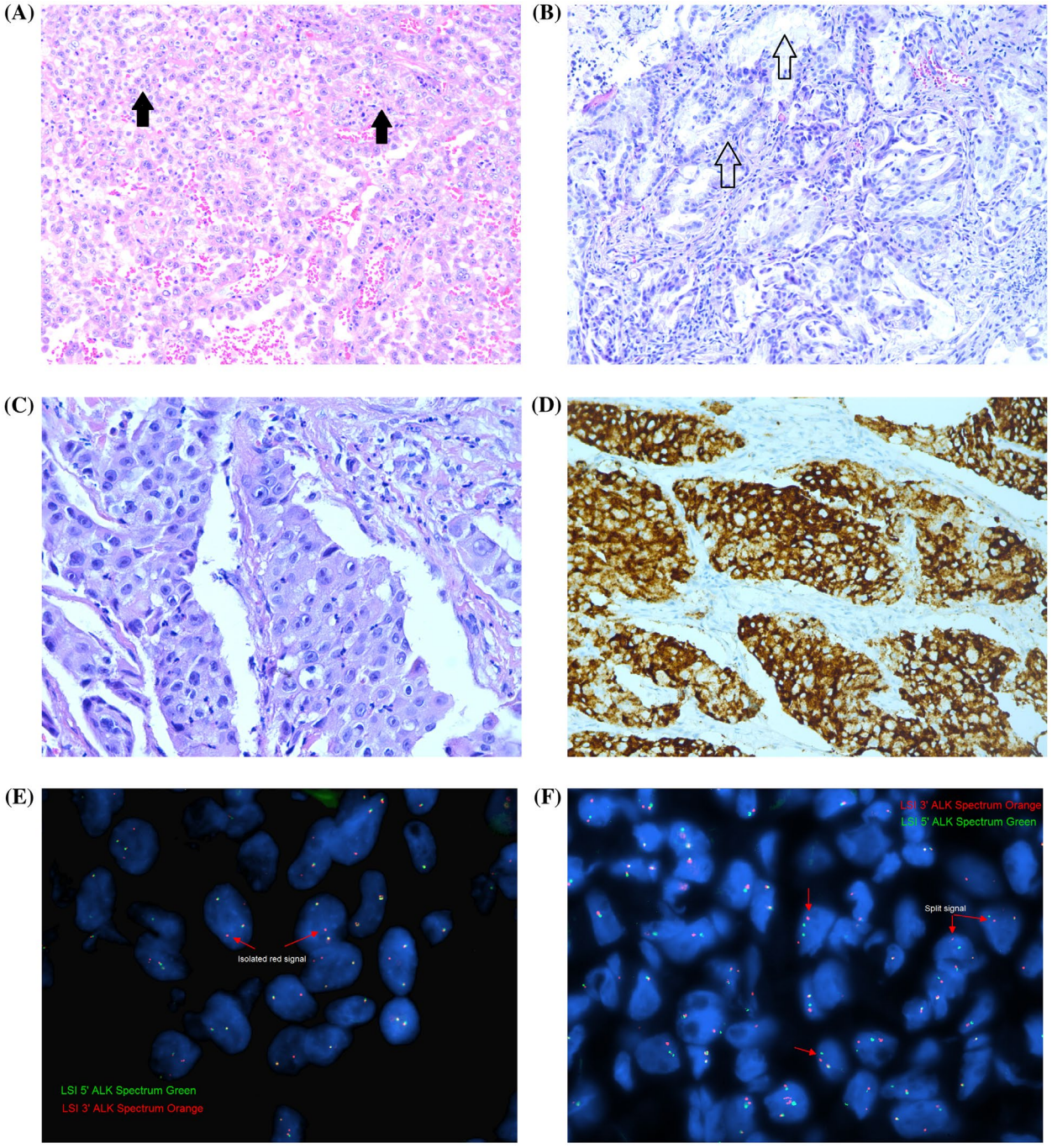

Figure 1. Staining characteristics of the *ALK* mutated lung adenocarcinomas; (A and B) Haematoxylin and Eosin (H&E) of tumours showing solid (solid arrows) and acinar type (open arrows) histological patterns, respectively, (C, D and E) H&E, *ALK* expression (immunohistochemistry) and FISH, respectively of a case showing 5′*ALK* deletion (depicted by isolated red signals), (F) FISH of a case with *ALK* translocation/inversion (depicted by seperated red and green signals). NB: Cells without *ALK* rearrangement are shown to have either yellow signals or closely adjacent red and green signals. Magnifications ×20 (A, B), ×40 (C, D), ×100 (E, F).

Summary table

What is known about this subject:
- Non-small cell lung cancer (NSCLC) is a major cause of cancer-related death.
- Its incidence in the female Asian population is increasing.
- Approximately 2–16% of NSCLC patients carry anaplastic lymphoma kinase (*ALK*) mutations and are eligible for crizotinib therapy.

What this paper adds:
- *ALK* expression shows a higher incidence in females compared to male patients and in patients that have never smoked.
- Immunohistochemistry using the rabbit monoclonal antibody D5F3 is a reliable alternative to FISH for determining *ALK* mutations in NSCLC.

Disclosure statement

No potential conflict of interest was reported by the authors.

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