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

N Mohamad, P Jayalakshmi, A Rhodes, C-K Liam, J-L Tan, S Yousoof and P Rajadurai

Department of Pathology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia; Department of Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia; Subang Jaya Medical Centre, Selangor, Malaysia

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.

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,
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 analysis kit (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 (v1.0 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 Kit (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).
requires specialist equipment; all of which are impor-
tance, its interpretation is time consuming and it is
relatively expensive, requires considerable technical
rangements on tissues samples was considered the
use with immunohistochemistry, with some reports
However, very recently two rabbit monoclonal antibod-
ies to determine in order to identify patients likely to ben-
ef 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 adenocarcin-
omas 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 inter-
national 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 rear-
rangements 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 impor-
tant and limiting factors when providing this service in a
diagnostic laboratory. The use of an immunohistochem-
ical 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 antibod-
ies 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 cri-
zotinib 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 rear-
rangement, as determined by FISH, and recorded com-
plete 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 anti-
bodies of similar specificity and sensitivity, immunohis-
tochemistry 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 recep-
tors 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 adenocarcin-
oma, 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 compris-
ing 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 test-
ing for ALK tumour expression by immunohistochemistry.

Table 1. Case details and ALK expression.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No of patients (n = 92)</th>
<th>Positive (n = 12)</th>
<th>Negative (n = 80)</th>
<th>Significance P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>Age (±SD)</td>
<td>ALK tumour expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (±SD)</td>
<td>61.2 ± 11.7</td>
<td>60.5 ± 7.8</td>
<td>61.3 ± 12.2</td>
<td>0.823</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>Positive (n = 12)</td>
<td>Negative (n = 80)</td>
<td>Significance P-value</td>
</tr>
<tr>
<td>Male</td>
<td>65</td>
<td>4 (6.2%)</td>
<td>61 (93.8%)</td>
<td>&lt;.0000</td>
</tr>
<tr>
<td>Female</td>
<td>27</td>
<td>8 (29.6%)</td>
<td>19 (70.4%)</td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Ever smokers</td>
<td>51</td>
<td>0</td>
<td>51 (100%)</td>
<td></td>
</tr>
<tr>
<td>Never-smokers</td>
<td>36</td>
<td>12 (33.3%)</td>
<td>24 (66.7%)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>5</td>
<td>0</td>
<td>5 (100%)</td>
<td></td>
</tr>
<tr>
<td>Type of specimen</td>
<td></td>
<td></td>
<td></td>
<td>0.047</td>
</tr>
<tr>
<td>Primary tumour</td>
<td>70</td>
<td>7 (10%)</td>
<td>63 (90%)</td>
<td></td>
</tr>
<tr>
<td>Metastatic lesion</td>
<td>22</td>
<td>5 (22.7%)</td>
<td>17 (77.3%)</td>
<td></td>
</tr>
</tbody>
</table>
No potential conflict of interest was reported by the authors.

**ORCID**

A Rhodes [http://orcid.org/0000-0002-1187-0939](http://orcid.org/0000-0002-1187-0939)

S Yousoof [http://orcid.org/0000-0002-6266-0878](http://orcid.org/0000-0002-6266-0878)

P Rajadurai [http://orcid.org/0000-0002-9199-1940](http://orcid.org/0000-0002-9199-1940)

**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 DSF3 is a reliable alternative to FISH for determining ALK mutations in NSCLC.

**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 separated 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).
References


