Detecting *ALK* Gene Rearrangements in Lung Cancer Cytology Specimens

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Abstract

Molecular testing for *EGFR* mutations and *ALK* gene rearrangements has become a routine part of lung cancer pathological diagnosis, and is critical to determine the most effective therapies for patients with this poor prognosis cancer. *ALK* gene rearrangements are seen particularly in adenocarcinoma of the lung and are associated with an excellent response rate to targeted inhibition with crizotinib in clinical trials for many patients whose tumours harbour this change. *ALK* gene rearrangement is generally observed at low incidence posing challenges for routine detection. Since a high proportion of lung cancers are inoperable at presentation, cytology plays a central role in diagnosis and provision of material for ALK testing. The advantages offered by cytology specimens for ALK and other molecular testing are becoming increasingly recognised - cytology specimens tend to have a lower proportion of contaminating stromal and other non-neoplastic cells and often have higher quality DNA than routine histology specimens. One challenge is the often limited amount of cytological material obtaining in many lung cancer cytology specimens. FISH testing for *ALK* gene rearrangement using a break-apart probe is the gold standard for testing although there is a strong role for immunohistochemistry in ALK testing. This review highlights key aspects of ALK testing in cytology specimens.

Keywords: Lung adenocarcinoma; *ALK,* Anaplastic lymphoma kinase; FISH; Immunohistochemistry

Abbreviations: ALK: Anaplastic Lymphoma Kinase; EML4: Echinoderm Microtubule Associated Protein-like 4; FISH: Fluorescent *in situ* Hybridization; IHC: Immunohistochemistry; NSCLC: Non-Small Cell Lung Carcinoma; NOS: Not Otherwise Specified; RT-PCR: Reverse-Transcriptase Polymerase Chain Reaction; TKI: Tyrosine Kinase Inhibitor

Introduction

Accurate subtyping of non-small cell lung carcinoma (NSCLC) is important due to implications for treatment selection and due to the unique mutational profile of different lung cancer subtypes [1]. Adenocarcinomas, tumours with an adenocarcinoma component or NSCLC- not otherwise specified (NOS) (in cytology or small biopsy samples) may harbour driver mutations amenable to targeted therapy [2]. It is estimated that about 70% of patients with NSCLC present with advanced stage disease not amenable to surgical resection, so only diagnostic cytology or small biopsy tumour samples are available for molecular analysis. There is evidence that cytology and small biopsy specimens have equivalent capacity to accurately subtype NSCLC [3]. Use of immunohistochemistry has become routine for distinction of NSCLC subtypes in cytology (and small biopsy) samples where morphology alone is insufficient [2] and in one study immunohistochemistry was less frequently required in cytology compared to small biopsy samples [3].

ALK Rearrangements in Lung Cancer

Identification of driver mutations has become essential in lung cancer for selection of patients likely to respond to targeted therapies. A small proportion of lung adenocarcinomas harbour activating *ALK* (*anaplastic lymphoma kinase)* gene rearrangements and these tumours are highly responsive to targeted tyrosine kinase inhibitors (TKIs) such as crizotinib [4].

The *ALK* gene is located on the short arm of chromosome 2 and encodes a receptor tyrosine kinase that belongs to the insulin receptor family [5]. Activation of *ALK* most commonly results from a small chromosomal inversion (with or without a small deletion) resulting in fusion of the intracellular kinase domain of *ALK* with the amino terminal end of *echinoderm microtubule associated protein-like 4* (*EML4*) [6-8]. Different variants of *EML4-ALK* result from differing lengths of the *EML4* gene being incorporated into the fusion gene [6,9,10]. More rarely, different partner genes fuse with *ALK* including *KIF5B* (kinesin family member 5b), *TFG* (TRK-fused gene) and *KLC-1*(kinesin light chain1) [11,12].The various *ALK* fusion genes encode a constitutively activated tyrosine kinase [7,11] that stimulates cell proliferation, survival and migration mediated through RAS/RAF/MAPK1, PI3K/AKT and JAK3-STAT3 signalling pathways [10,13]. Mouse models expressing EML4-ALK develop multiple lung adenocarcinomas that are susceptible to pharmacologic ALK inhibition [14].

Studies from unselected Western populations mostly report *ALK* rearrangements in about 4% of NSCLC [5]. We found *ALK* rearrangements in 1% of lung adenocarcinomas in an Australian multicenter study [15]. The clinicopathological features associated with *ALK* rearrangements are similar to the profile associated with *EGFR* mutations. *ALK* rearrangements are associated with younger patient age and never-smokers or light smokers [6,15-19], however, racial and gender associations are less pronounced for *ALK*.

In NSCLC, *ALK* rearrangements occur almost exclusively in

adenocarcinomas [19] particularly with solid, acinar, cribriform with extracellular mucin or signet ring cell morphology in resection specimens [16,20-22]. In cytology (and small biopsy) specimens signet ring cells have been the only distinguishing morphological feature in *ALK* rearranged lung adenocarcinomas [23].

While almost all *ALK* rearrangements occur in adenocarcinoma, they may also occur in adenosquamous carcinomas [24] or combined small cell carcinoma and adenocarcinoma [25]. For this reason, it is recommended that cytology or small biopsy samples showing squamous or small cell carcinoma features can be considered for *ALK* (and *EGFR*) testing if the clinical profile is suspicious (i.e., young never smoking patient) [26] as there is increasing recognition that the adenocarcinoma component of a mixed tumour may be unapparent in limited sample specimens.

As expected for a driver mutation, *ALK* rearrangements almost always occur in tumours lacking other significant mutations such as *EGFR* or *KRAS* alterations [15,19,20,27], however, rare cases of tumours harbouring activating alterations of both *ALK* and *EGFR* have been reported [6,28,29].

Identification of *ALK* Rearrangements

Recent consensus guidelines for molecular testing of lung cancer from the College of American Pathologists in conjunction with the International Association for the Study of Lung Cancer and the Association for Molecular Pathology [26] recommend testing all patients for *EGFR* mutations and *ALK* rearrangements at the time of diagnosis of advanced stage lung adenocarcinoma. They recommend that *EGFR* testing is prioritised over other tests, followed by *ALK* testing.

Fluorescence *in situ* hybridisation (FISH) using a dual colour probe that targets the breakpoint of the *ALK* gene is the standard method for identifying *ALK* rearrangements in current clinical practice and this technique has been validated in clinical trials [4]. FISH is a relatively costly and labour intensive assay that can be technically challenging to interpret in *ALK* assays due to the small inversion resulting in a subtle split in signals. More recently studies have shown that ALK overexpression resulting from *ALK* gene rearrangement can be identified using immunohistochemistry (IHC) using both the 5A4 clone [28,30,31] and the newly available D5F3 clone [15,32]. IHC screening with confirmation by FISH provides a more cost-effective means of identifying *ALK* rearrangements in low prevalence populations and can be used if carefully validated [26]. Furthermore, occasional tumours show *ALK* expression by IHC and are sensitive to ALK-TKI treatment despite being technically negative by FISH [33,34].
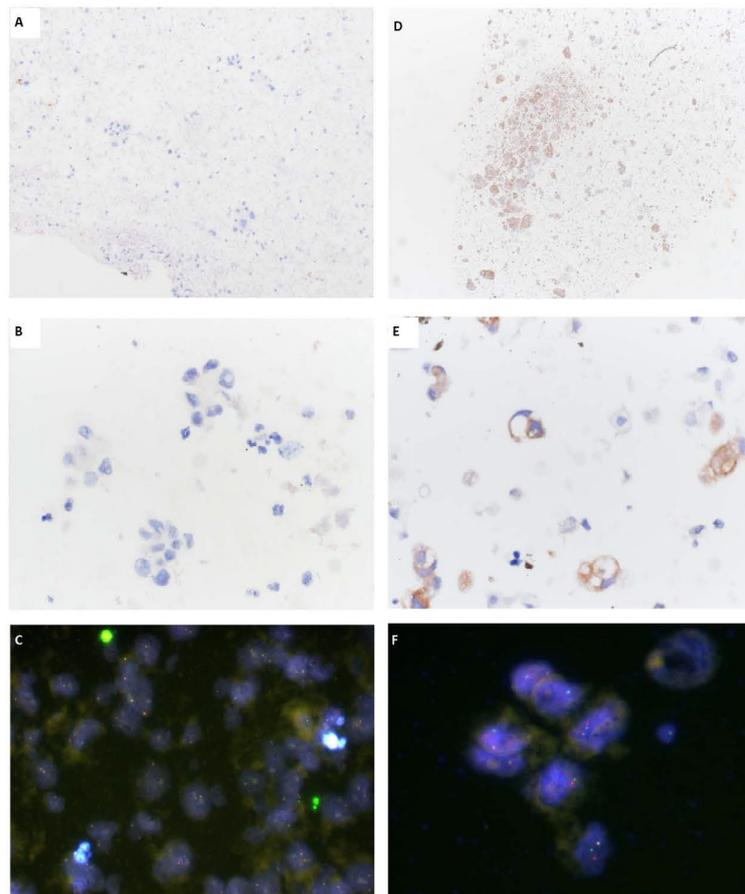
Figure 1: IHC and FISH for ALK in cytological specimens of lung adenocarcinoma. Cell block of pleural fluid with metastatic lung adenocarcinoma from an elderly woman. The tumour cells were negative for ALK IHC (A) low power and (B) high power. (C) FISH using a break apart probe was negative for ALK gene rearrangement with two fused red/green signals. Cell block from a fine needle biopsy of metastatic lung adenocarcinoma in a cervical lymph node from a 34 year old woman. The tumour cells were positive for ALK IHC (D) low power, (E) high power. (F) FISH showed ALK gene rearrangement with split red and green signals.

The optimal tumour specimen for *ALK* FISH testing contains plentiful non-overlapping tumour cells that can easily be distinguished from interspersed inflammatory and stromal cells based on nuclear or architectural features using a high magnification fluorescent microscope [35]. It is imperative that a pathologist mark the appropriate area for FISH evaluation on an H&E stained slide. Alcohol fixed specimens (or those that have undergone acid decalcification) should be avoided [35]. Pathological assessment for specimen suitability, cellularity and marking up of an appropriate area for expert FISH evaluation is essential to achieve accurate results [26]. Using a break-apart dual labelled FISH probe, cases are considered positive for *ALK* gene rearrangement if they show split red and green signals in at least 15% of tumour nuclei with a minimum number of 50 cells analysed. Alternatively, an unbalanced *ALK* rearrangement displays single red signals from the 3' probe with loss of the 5' green probe signal [35]. It is increasingly recognised that atypical patterns may also be observed on FISH and data on these unusual changes is currently being accumulated. Testing with second method such as immunohistochemistry may be helpful to resolve some of these atypical FISH patterns.

Reverse-transcriptase polymerase chain reaction (RT-PCR) can also be used to identify *ALK* rearrangements so long as comprehensive primer sets are used to detect all possible known *ALK-EML4* variants as well as other *ALK* fusion partners [36]. This technique is not currently recommended by the CAP/IASLC/AMP guidelines for routine practice due to the difficulty obtained high quality RNA from formalin fixed paraffin embedded tissues [26]. However, RT-PCR is highly sensitive on fresh tumour samples and may be particularly suitable for cytology specimens [36].

Cytology and *ALK*

Testing for *ALK* rearrangements may be undertaken on histology or cytology specimens from primary or metastatic sites. While FISH for *ALK* rearrangements can be undertaken on a range of cytology specimens including conventional smears, cytospins or liquid-based preparations [35]we use paraffin-embedded cell block preparations from our cytology specimens in routine clinical practice. Smears have the advantage of avoiding nuclear truncation, tend to have high tumour cellularity and DNA quality and may be superior to formalin fixed cell block preparations [35], however, we find it more convenient to perform ALK IHC and FISH on consecutive sections cut from a cell block (Figure 1) where morphology can be compared to the H&E stained slide and this is the current recommendation [26]. *ALK* gene rearrangements can be identified by FISH, RT-PCR and IHC using endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) specimens [17,37,38] and some have reported almost 100% of lung cancer cytological specimens are suitable for *ALK* FISH assessment [35]. ALK IHC can be performed with high sensitivity on smears from a range of lung adenocarcinoma specimens including pleural effusions, fine needle aspirates of lymph nodes and bronchial brushings [39], although in our practice we usually perform ALK IHC on cell block preparations.

Summary

The need to identify molecular markers that predict patient response to targeted therapies has revolutionised pathological assessment of lung cancer specimens. Testing for *ALK* rearrangements should be considered in non-squamous NSCLC (i.e. adenocarcinoma and NSCLC-NOS in cytology samples) that lack *EGFR* mutations. A range of molecular techniques including IHC, FISH and sequencing based molecular genetic assays are expected to play an increasing role in the diagnostic workup of lung cancer and as many cases only have cytology specimens available, it is essential that cytologists are ready to meet the challenge.

Conflict of interest statement

WC and SO report having received honoraria for lectures and advisory boards from Pfizer, manufacturers of crizotinib.

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