A Guide to ALK Testing

This guide is intended to provide healthcare professionals with information on testing for anaplastic lymphoma kinase (ALK) fusions in non-small cell lung cancer (NSCLC).
ALK in NSCLC

The ALK protein, encoded by the \textit{ALK} gene, is a transmembrane receptor tyrosine kinase\(^1\) which, via ligand-dependent dimerisation and signalling,\(^2\) mediates cell proliferation and survival.\(^3\)

In 3–5\% of patients with NSCLC, \textit{ALK} gene rearrangement occurs\(^4\) and provides an oncogenic driver in these tumours.\(^6\) The most common ALK rearrangements involve a fusion between the echinoderm microtubule-associated protein-like 4 (\textit{EML4}) and \textit{ALK} genes, and were first discovered in NSCLC specimens in 2007.\(^6\) The fusion gene is created by an inversion in chromosome 2p resulting in fusion of the N-terminal portion of the \textit{EML4} gene with the sequence coding for the kinase domain of ALK (Figure 1).\(^6\) This results in replacement of the extracellular and transmembrane portions of the ALK protein with a coiled-coil portion of the EML4 protein.\(^7\)

\textit{EML4-ALK} is formed from the N-terminal portion of the \textit{EML4} gene and the kinase portion of \textit{ALK}.

While the physiological ALK protein is located at the plasma membrane, the EML4-ALK fusion protein is localised to the cytoplasm\(^6\) and forms constitutive dimers via the coiled-coil portion of EML4.\(^7\) This leads to activation and persistent mitogenic signalling (Figure 2).\(^7\) This unregulated signalling induces cancer progression through its impact on cell-cycle progression, survival, proliferation and metastasis.\(^3\)

\textbf{Figure 1.} Formation of the \textit{EML4-ALK} gene rearrangement

\textbf{Figure 2.} Activation of physiological ALK and EML4-ALK fusion protein
Several EML4-ALK fusion variants have been identified, all of which contain the sequence coding for the cytoplasmic portion of ALK, including its entire kinase domain. Additionally, other fusion partners for ALK have been identified, such as TFG and KIF5B.

Patients with tumours expressing ALK fusion proteins comprise a distinct molecular subset of patients with NSCLC for whom personalised treatment with ALK inhibitors is indicated. Therefore, tumour specimens from patients with NSCLC should be tested for ALK rearrangements in order to inform treatment decisions.

Testing for ALK

Rapid diagnostic procedures and treatment decisions are essential for patients with advanced NSCLC; therefore, effective collaboration between oncologists and pathologists is key to ensuring all patients are tested at initial diagnosis for all relevant molecular markers.

Due to an increasing number of molecular tests that should be performed on NSCLC specimens, tissue sample size should be maximised whenever possible. Tissue handling, processing and sectioning should be standardised to minimise wastage and optimised for the staining procedures and molecular tests required for NSCLC. Histological and cytological specimens are both potentially suitable for ALK testing. If the initial tissue sample is small, three to four spare sections should be cut upfront to avoid tissue loss from recutting.

According to current guidelines from the European Society for Medical Oncology and from the College of American Pathologists, the International Association for the Study of Lung Cancer and the Association for Molecular Pathology:

- ALK testing should be carried out systematically in advanced non-squamous NSCLC.
- All advanced NSCLC patients should be tested for ALK at initial diagnosis, allowing patients to receive the most appropriate treatment as early as possible.
- It is preferable to test for different molecular markers in parallel as sequential testing may delay treatment and is a less efficient use of limited tissue samples.
- Molecular test results should be available within 10 working days of receiving the specimen in the testing laboratory.

ALK-positive NSCLC can be identified using fluorescence in-situ hybridisation (FISH), immunohistochemistry (IHC), reverse transcription-polymerase chain reaction (RT-PCR) and next-generation sequencing (NGS). RT-PCR and NGS for ALK are emerging platforms and new data for these techniques will become available as they are further developed. Therefore, this guide will focus on testing for ALK using FISH and IHC.

Fluorescence in-situ hybridisation

FISH involves the hybridisation of a fluorophore-labelled single-stranded DNA probe which is complementary in sequence to the genetic region of interest. The fluorophore signal can be visualised using a fluorescence microscope.

In NSCLC specimens, ALK gene rearrangements can be detected using a break-apart FISH probe assay. The assay uses two fluorophore-labelled probes that flank the break point of the ALK gene, one on the 3' segment (orange) and one on the 5' segment (green).

**Positive for ALK**
- If rearrangement has occurred, nuclei will contain ‘broken apart’ orange and green signals, which appear separated by at least two signal diameters.
- If deletion has occurred, nuclei will contain single orange signals.

**Negative for ALK**
- If no activating rearrangement or deletion in the ALK gene locus has occurred, nuclei will contain fused orange and green signals (either overlapping, adjacent, or less than two signal diameters apart) or nuclei will contain single green signals, in addition to fused signals.
A specimen is positive for ALK if >25/50 cells are judged positive. A specimen is negative for ALK if <5/50 cells are judged positive. If there is uncertainty, a second count should be conducted and an average calculated. If the average percentage of positive cells is ≥15% (of 100 cells) the sample is considered positive.

Immunohistochemistry

IHC detects the expression of ALK protein and is a valuable screening tool for testing NSCLC samples for ALK rearrangement. One of the challenges with IHC is that even in ALK-rearranged NSCLC, ALK protein expression is relatively low. Standard detection methodology, as used in the identification of ALK-rearranged anaplastic lymphomas, is inadequate in NSCLC. Thus in NSCLC, primary anti-ALK antibodies are often used in conjunction with enhanced detection systems for signal amplification.

The VENTANA ALK (D5F3) CDx assay is an FDA-approved companion diagnostic and IVD-labelled ALK IHC assay.

According to the package insert:

- A specimen is positive for ALK if there is strong, granular, cytoplasmic, brown staining in the tumour cells (any percentage of positive tumour cells); staining is usually homogenous, with a uniform level of intensity throughout the neoplastic portions of the tumour.
- A specimen is negative for ALK in the absence of strong, granular, cytoplasmic staining in the tumour cells.
A positive control slide should be included with every test staining run to confirm reagents are functioning properly and guard against false-negative results. A negative control slide should also be included, to check for background staining and confirm the absence of target antigen labelling.

Pathologists should be aware of various artefacts that may lead to false-positive staining, including:

- light cytoplasmic stippling in alveolar macrophages
- cells of neuronal origin
- glandular epithelial staining
- cells with lymphocytic infiltrate
- normal mucosa in NSCLC (including mucin)
- necrotic tumour areas.

**ALK testing centres**

Laboratories conducting molecular testing on NSCLC specimens should consider participating in external quality assurance (EQA) programmes, which can help to ensure and enhance proficiency in molecular testing. Below is a list of websites of medical and pathology societies which conduct EQA programmes. Here you can also find a list of testing centres which have successfully participated in EQA programmes for ALK testing.

**European Society of Pathology**
http://lung.eqascheme.org/info/public/alk/previous_participants.xhtml

**Institut National du Cancer**
http://www.e-cancer.fr/Professionnels-de-sante/Les-therapies-ciblees/Les-plateformes-de-genetique-moleculaire-des-cancers/Missions-et-localisation-des-plateformes

**Associazione Italiana di Oncologia Medica**
http://www.aiom.it/area+pubblica/area+medica/prodotti+scientifici/tavoli+di+lavoro/Tavolo+di+lavoro+AIOM+--+SIA-PEC/1%2C604%2C1%2C

**Deutsche Gesellschaft für Pathologie**
http://www.quip-ringversuche.de/

**UK NEQAS**
http://www.ukneqas.org.uk

**Useful resources and publications**

www.ALKTesting.org


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Vysis ALK Break Apart FISH assay

ALK Break Apart FISH protocol for cytological specimens (courtesy of Professor Lukas Bubendorf, Institute of Pathology, University Hospital Basel, Switzerland)

VENTANA ALK (DSF3) CDx assay interpretation guide
http://www.ventana.com/product/1816?type=2326

References