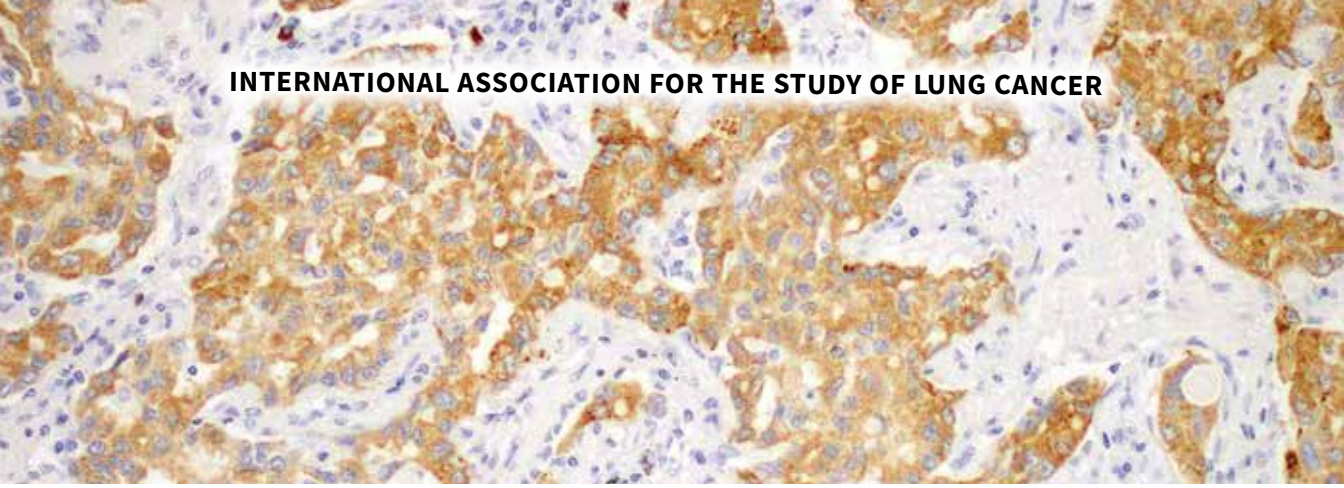

INTERNATIONAL ASSOCIATION FOR THE STUDY OF LUNG CANCER

SECOND EDITION

IASLC ATLAS OF ALK AND ROS1 TESTING IN LUNG CANCER

IASLC

Conquering Thoracic Cancers Worldwide

EDITED BY
MING SOUND TSAO, MD, FRCPC
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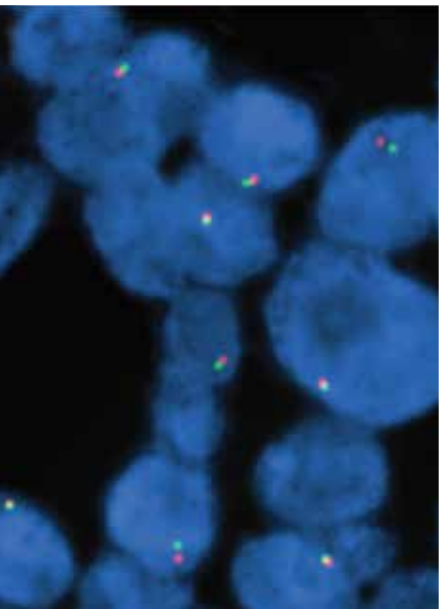

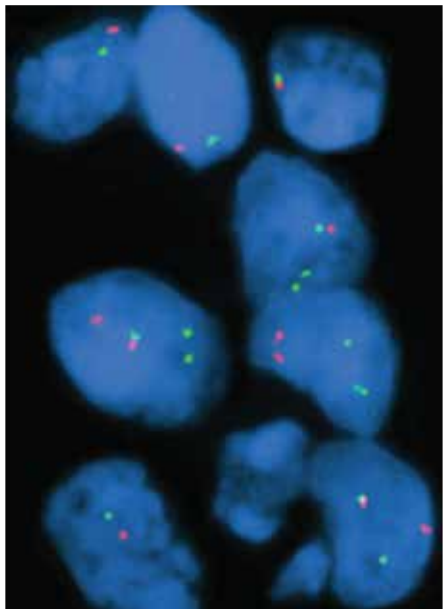

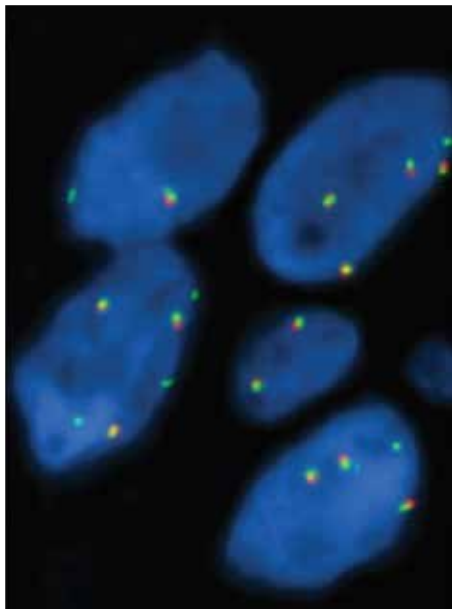

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International Association for the Study of Lung Cancer, Aurora, CO, USA

Editors:
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IASLC ALK and ROS1 Testing in Lung Cancer Workshop attendees, IASLC Headquarters, CO, USA, 2016. Left to right–Lynette M. Sholl, Andrew G. Nicholson, Fred R. Hirsch, Keith M Kerr, Yasushi Yatabe, Ming Sound Tsao, Marileila Varella-Garcia, Akihiko Yoshida, and Ignacio I. Wistuba. Not present: Jin-Haeng Chung, Sylvie Lantuéjoul, Neal I. Lindeman, Lukas Bubendorf, and Erik Thunnissen.

Abbreviations

The following abbreviations are used in the text.

ALK:	anaplastic lymphoma kinase (gene)
AMP:	Association for Molecular Pathology
CAP:	College of American Pathologists
CISH:	chromogenic in situ hybridization
DAB:	3, 3' diaminobenzidine
EDTA:	ethylenediaminetetraacetic acid
EGFR:	epidermal growth factor receptor (gene)
EML4:	echinoderm microtubule-associated protein-like 4 (gene)
FDA:	Food and Drug Administration
FFPE:	formalin-fixed paraffin-embedded
FISH:	fluorescence in situ hybridization
FNA:	fine-needle aspiration
H&E:	hematoxylin & eosin
HER2:	human epidermal growth factor receptor-2 (gene)
IASLC:	International Association for the Study of Lung Cancer
IHC:	immunohistochemistry
ISH:	in situ hybridization
KIF5B:	kinesin family member 5B (gene)
NSCLC:	non-small cell lung cancer
NGS:	next-generation sequencing
RET:	ret proto-oncogene (gene)
ROS1:	c-ros oncogene 1 (gene)
RT-PCR:	reverse-transcriptase polymerase chain reaction
SCLC:	small cell lung cancer
v:	variant

Introduction

By Ming Sound Tsao, Fred R. Hirsch, and Yasushi Yatabe

Since its original publication in 2013, the *IASLC Atlas of ALK Testing in Lung Cancer* has been widely requested all over the world and printed in four different languages.

At the time of that publication, ALK testing by FISH using the FDA-approved Vysis LSI ALK Break Apart FISH Probe Kit (Abbott Molecular) was the reference standard to detect *ALK*-rearranged lung cancers. In many countries, the higher cost of FISH and its need for technical expertise led to the evaluation of ALK IHC as a screening assay to detect *ALK* fusions in lung cancer. In November 2013, crizotinib (Xalkori, Pfizer Oncology) became the first ALK inhibitor to receive FDA approval for the treatment of patients with ALK-positive metastatic NSCLC. Since then, there has been much progress in the diagnosis and treatment of ALK-positive lung cancers. Several mechanisms of resistance were identified in ALK-positive lung cancers that progress during treatment with crizotinib, and the second-generation ALK inhibitors ceritinib (Zykadia, Novartis) and alectinib (Alecensa, Hoffmann La Roche) have received approval to treat patients who have disease that progressed with crizotinib. The ALK (D5F3) IHC CDx Assay (Ventana) has received approval by the FDA in the United States and in many other countries to select appropriate patients for treatment with an ALK inhibitor. ALK IHC has become a standard to screen for the expression of *ALK* fusion in lung tumors and, in many countries, it is also used to determine treatment eligibility. In 2012, *ROS1* rearrangement was identified in 1.7% of 1,073 advanced NSCLC tumors that were screened, and *in vitro* evidence emerged that these tumors were sensitive to crizotinib (Bergethon 2012). In 2014, the published results of a study on crizotinib to treat lung cancer with *ROS1* fusion led to the approval in the United States and Europe for expanded use of crizotinib in *ROS1*-rearranged lung cancer.

These developments have led to a call to update the *IASLC Atlas of ALK Testing in Lung Cancer* and include information on ROS1 testing methodologies. To address this issue, the IASLC Pathology Committee convened a panel of experts to publish this second edition, *IASLC Atlas of ALK and ROS1 Testing in Lung Cancer.* The Atlas is meant to serve as a resource to help pathologists, laboratory scientists, and practicing physicians better understand the background, protocol, and interpretation of results of ALK and ROS1 testing for patients with advanced NSCLC.

ALK and *ROS1* Gene Rearrangement

1

By Ming Sound Tsao, Yasushi Yatabe, and Fred R. Hirsch

It has been 9 years since the first report of the expression of oncogenic EML4-ALK and SLC34A2/CD74-ROS fusion proteins in lung cancer cell lines and primary tumor (Soda 2007, Rikova 2007). Although oncogenic *ALK* and *ROS1* gene rearrangements are found in approximately 4% and 2%, respectively, of NSCLC, primarily with adenocarcinoma histology (Table 1, Appendix 1 and 2), they epitomize the rapid advances being achieved in the basic science, diagnosis, and treatment of lung cancer. Three drugs (crizotinib, ceritinib, and alectinib) have become available for the treatment of *ALK*-rearranged lung tumors, and one drug (crizotinib) is now available for *ROS1*-rearranged tumors. The speed with which scientific discoveries led to effective clinical treatment and standard of care for patients with these lung tumors is unprecedented (Shaw 2013, Gainor 2013a, Facchinetti 2016). Furthermore, the rapid discoveries of resistance mutations for first-generation ALK and ROS1 inhibitors revealed the complexity of lung cancer genetics and biology (Lovly 2012, van der Wekken 2016). Fortunately, second-generation inhibitors were developed within a short time to overcome these resistance mechanisms (Dagogo-Jack 2016).

Table 1. Reported Prevalence of *ALK*- and *ROS1*-Rearranged NSCLCs[a]

	***ALK* fusion**		***ROS1* fusion**	
	No. of Patients Screened	**No. of ALK-Positive Tests (%)**	**No. of Patients Screened**	**No. of ROS1-Positive Tests (%)**
Asia	9,496	596 (6.2%)	5,375	121 (2.3%)
Europe	10,689	504 (4.7%)	1,828	37 (2.0%)
North America	4,473	239 (5.3%)	1,240	20 (1.6%)

[a] *Data presented as means; NSCLCs consist primarily of adenocarcinomas.*

ALK fusion genes were first identified in anaplastic large cell lymphoma, and subsequently in NSCLC and rare tumors such as inflammatory myofibroblastic tumor (Morris 1994, Hallberg 2013). Overall, more than 20 *ALK* fusion partners have been identified. In lung cancer, aside from the major partner *EML4*, fusions with *KIF*5B, *TFG*, *KLC1*, and *HIPI* have been reported (Figure 1) (Hallberg 2003, Takeuchi 2009, Wong 2011, Togashi 2012, Fong 2014, Hong 2014, Ou 2014). The breakpoints on the *ALK* gene almost always occur in intron 19 and, rarely, in exon 20, resulting in a constant inclusion of the ALK kinase domain in the fusion gene/protein. A common feature of the fused partner genes is the presence of basic coil-coil domain, which allows the spontaneous dimerization of the fusion proteins. *EML4-ALK*, the most common *ALK* fusion found in NSCLC, is formed by an inversion occurring on the short arm of chromosome 2 and involves the genes encoding for *ALK* (2p23) and *EML4* (2p21), with variants 1, 2, and 3a/3b being the most common fusion patterns among more than 13 variants (Yoshida 2016). Because the gene rearrangement involves large chromosomal inversion and translocation, FISH was the first method used for detecting all forms of *ALK* rearrangement, and until recently, FISH with ALK break-apart rearrangement probes was the reference criterion for the diagnosis of lung cancers with *ALK* rearrangement. More recently, the detection of ALK fusion protein by an ALK D5F3 IHC assay has received FDA approval for the selection of patients to be treated with an ALK inhibitor in the United States.

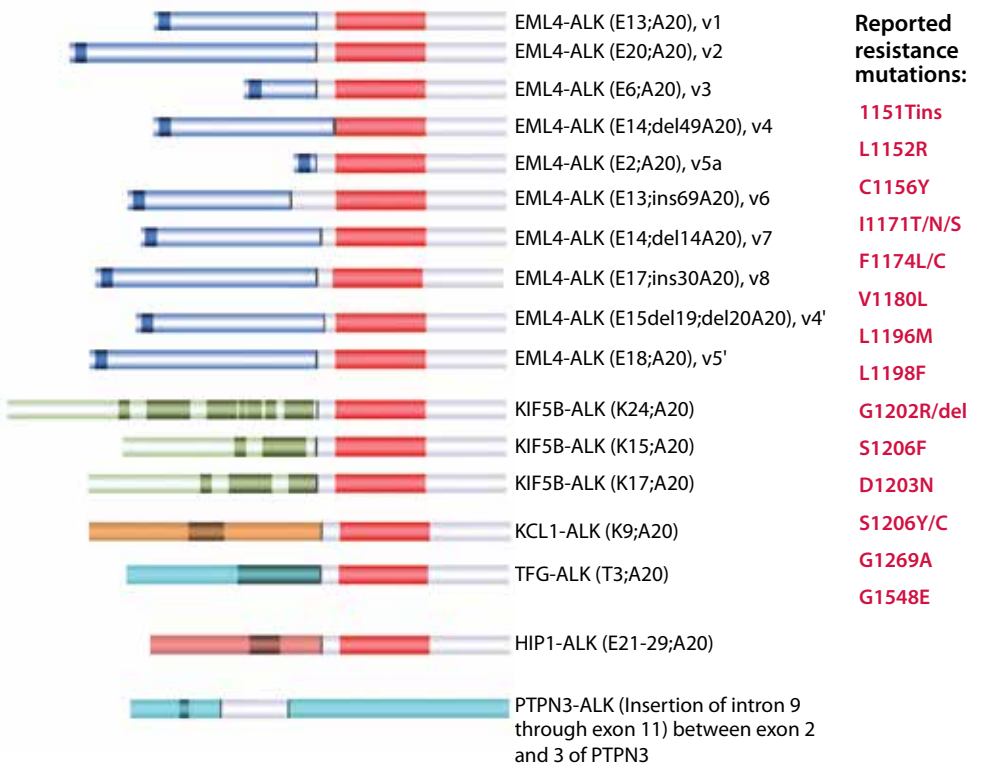

Figure 1. Schematic diagram of *ALK* rearrangement. The genes and domains are highlighted in different colors. Darker regions represent coil-coil domains in the fusion partner genes (*EML4, KIF5B, KLC1, TFG*), and the kinase domain in *ALK* (red).

Similar to the case with *EGFR*-mutated lung cancer, almost all *ALK*-rearranged tumors develop resistance to crizotinib treatment. Sequencing of the resistant tumor DNA has led to the identification of resistant point mutations on the *ALK* gene in 20% to 40% of patients (Figure 1) (Lovly 2012, van der Wekken 2016, Dagogo-Jack 2016). These mutations result in decreased binding of the inhibitor or increased ATP binding affinity (Lovly 2012). Other resistance mechanisms have also been identified, including the activation of EGFR and KRAS pathways by their respective mutations, and *ALK* and *KIT* gene amplification (Lovly 2012, van der Wekken 2016). Second-generation ALK inhibitors (ceritinib and alectinib) that may overcome some of these resistances have recently received approval in various parts of the world, including the United States, Europe, and Japan (Facchinetti 2016).

The human *ROS1* gene is located on chromosome 6p22 and encodes a tyrosine kinase receptor that is evolutionally related to the ALK receptor. It is a homologue of the chicken c-*ros*, proto-oncogene of *v-ros* from UR2 avian sarcoma virus (Matsushime 1986, Birchmeier 1986). The first *ROS1* fusion gene discovered was *FIG(GOPC)-ROS1* in human glioblastoma cell line U-118 MG; it resulted from a 6p deletion between the *FIG* and *ROS1* genes (Birchmeier 1990, Charest 2003). The *FIG-ROS1* fusion subsequently was found in cholangiocarcinoma and lung adenocarcinoma (Peraldo Neia 2014). Other *ROS1* fusion partners that have been identified in lung cancer include *SLC34A2, CD74, TPM3, SDC4, EZR, LRIG3, KDEL R2, LIMA1, MSN, CLTC, CCDC6, TMEM106*, and *TPD52L1* (Figure 2) (Bergethon 2012, Gainor 2013a, Davies 2013, Zhu 2016). With more widespread profiling of tumors with NGS, the number of *ROS1* fusion partners likely will continue to grow.

The mechanism by which ROS1 fusion proteins become oncogenic remains unclear. Unlike *ALK*-fusion oncogenes, a majority of *ROS1* fusions lack coil-coil domain that promotes spontaneous dimerization and kinase activation (Takeuchi 2012). Nevertheless, some ALK

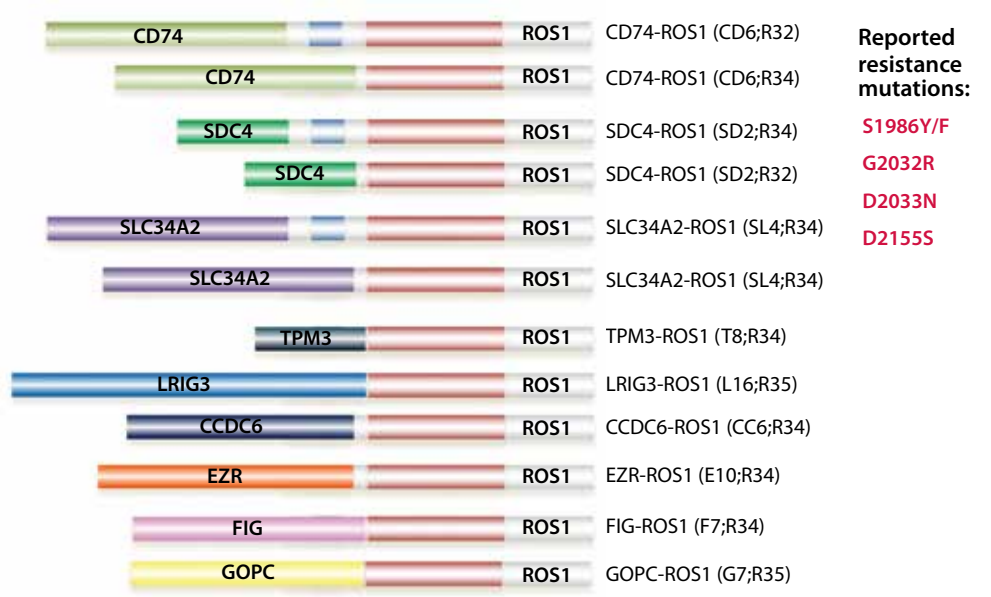

Figure 2. Schematic diagram of *ROS1* rearrangement. *ROS1* kinase and transmembrane domains are highlighted in brown and blue, respectively. Partner genes are shown as different colors.

inhibitors inhibit proliferation of the cell line HCC-78, which harbors *ROS1* rearrangement (Rikova 2007, McDermott 2008), probably as a result of evolutional correlation of both molecules. Furthermore, in the expansion cohort of the PROFILE 1001 trial (NCT00585195), the response rate to crizotinib was 72% among patients with lung cancer in which *ROS1* rearrangement was identified by break-apart FISH assay (Shaw 2015). This result formed the basis for approval of crizotinib for the treatment of *ROS1*-rearranged lung cancer in the United States and Europe. However, several resistance mutations in *ROS1* fusion genes acquired during the treatment of *ROS1*-rearranged lung cancer with crizotinib have already been identified (Figure 2). Considering the rapid evolution of ALK inhibitor therapies, one can expect that a strategy for overcoming the resistance mechanism in *ROS1*-rearranged lung cancer will soon be forthcoming.

Conclusion

ALK and *ROS1* gene rearrangements occur in approximately 4% and 2% of lung adenocarcinoma, respectively. Although the frequency of these genomic aberrations is low, their diagnosis offers patients with lung cancer the opportunity to receive highly effective targeted therapies. The story of *ALK* and *ROS1* reflects the current exciting state in lung cancer research.

Candidates for ALK and ROS1 Testing

By Fred R. Hirsch, Murry W. Wynes, and Ming Sound Tsao

2

The detection of *ALK* and *ROS1* gene rearrangements or aberrant expression is widely recognized as being highly important for selecting efficacious therapy for patients with advanced NSCLC. The prevalence of patients with NSCLC harboring *ALK* rearrangements is about 4% and the rate is slightly lower for patients with *ROS1* rearrangements, with a prevalence of 2% (Appendix 1 and 2). Numerous fusion partners have been described for both *ALK* and *ROS1* but the clinical significance of these fusions partners requires further investigation (see Chapter 1). The rearrangement and aberrant expression of the genes are mostly found in lung tumors from never- or light smokers, women, and younger patients, and in tumors classified as adenocarcinomas. However, it is unclear whether clinicopathologic features may help in determining which patients should have ALK or ROS1 testing. Is it plausible to exclude older patients and smokers with squamous histology from ALK and ROS1 testing?

In published studies, approximately 70% to 80% of patients with ALK-positive NSCLC are never-smokers, which means that 20% to 30% are previous or current smokers. Individuals with ALK-positive NSCLC tend to be 40 to 50 years old, which is considerably younger than both patients with NSCLC (median age, 70 years) and patients with tumors harboring *EGFR* mutations (60 to 65 years). (Rodig 2009, Shaw 2009, Bang 2010, Kwak 2010, Shaw 2011). However, in all studies, *ALK*-rearranged tumors have also been found in patients older than 70 years and younger than 40 years. The clinical profile is similar for people with *ROS1*-rearranged tumors; the median age is significantly younger, but the alteration is detected in tumors from patients older than 70 years (Shaw 2014, Sholl 2013a, Mazieres 2015, Cai 2013, Bergethon 2012). As with *ALK* rearrangements, *ROS1* rearrangements tend to be found in tumors in never-smokers, with 30% found in current or former smokers. Thus, according to these data, it is not clear that smoking history and age should preclude ALK and ROS1 testing in any patient.

Histology appears to be a more important selection criterion; among more than 12,000 lung cancer specimens that have been reported on in the literature, *ALK* and *ROS1* rearrangements were found predominately in nonsquamous and non-neuroendocrine lung cancers (Figure 1). Testing for ALK and ROS1 is not routinely performed for patients with advanced

NSCLC of squamous histology, and this has been the recommendation of guidelines, including the CAP/IASLC/AMP Molecular Testing Guideline for Selection of Lung Cancer Patients for EGFR and ALK Tyrosine Kinase Inhibitors, published in 2013 (Lindeman 2013) (see Chapter 13). However, *ALK* rearrangements have been detected in about 1.3% of more than 1,400 squamous cell lung cancers (Figure 1) and in several case reports in which IHC was used to detect ALK protein expression (Alrifai 2013, An 2012, Ochi 2013). Studies on *ROS1* alterations have been limited, with no ROS1-positive tumors identified among 861 squamous cell cancers, and one ROS1-positive tumor among 47 (2.1%) of adenosquamous carcinomas (Bergethon 2012, Wu 2016, Jin 2015, Cha 2014, Go 2013, Rimkumas 2012).

The discordance among studies of squamous cell lung cancers may be related to the difficulties that still exist in diagnosing subtypes of NSCLC. A lung cancer diagnosis is often made according to the examination of a small biopsy specimen or cytology samples, but histopathologic diagnoses made on these small specimens are not always representative of the whole tumor. Reassessments of a squamous cell cancer diagnosis in such specimens with no evidence of *EGFR* and *KRAS* mutations demonstrated components of adenocarcinoma in 15 of 16 tumors (Rekhtman 2012). Therefore, the updated CAP/IASLC/AMP guideline suggests that ALK and ROS1 testing be done for patients with adenocarcinoma and lung cancers of mixed histology with an adenocarcinoma component in the setting of a fully excised lung cancer specimen (see Chapter 13). ALK and ROS1 testing is also recommended for limited specimens, such as biopsy and cytology specimens, where an adenocarcinoma component cannot be completely excluded. Furthermore, the updated CAP/IASLC/AMP guideline recommends ALK and ROS1 testing for never-smokers who are younger than 50 years and have a tumor of squamous histology.

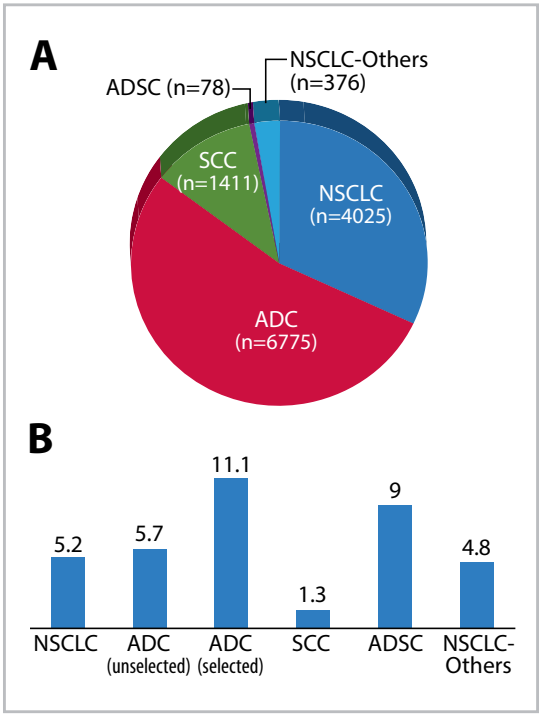

Figure 1. Summary of studies on *ALK* gene rearrangements in NSCLC. **A.** Number of NSCLC cases that have been studied for *ALK* aberration and reported in the literature up to May 2013. "NSCLC" refers to cases in studies that did not specify tumor types, and "NSCLC-others" include adenosquamous carcinoma (ADSC) and large cell/sarcomatoid carcinoma. **B.** Estimated rate of ALK-positive cases according to tumor histology. For adenocarcinoma (ADC), prevalence in studies with or without clinical selection criteria (eg, smoking history, negative for *EGFR* or *KRAS* mutation) are provided. The numbers are based on data presented in Appendix 1 of the first edition of this Atlas (Tsao 2013). SCC = squamous cell carcinoma.

Screening for ALK and ROS1 expression with IHC may represent an ideal solution to the concern of ALK and ROS1 testing in squamous cancers, given its low cost and high reproducibility, sensitivity, and specificity.

For patients with localized or local-regional NSCLC, testing for *ALK* and *ROS1* rearrangements are not associated with any immediate targeted therapies outside a clinical trial.

However, testing for *ALK* and *ROS1* in the setting of earlier stage NSCLC may be beneficial, as disease will recur in many patients with earlier disease, and previously determined test results may save time, effort, and patient distress when subsequent systemic therapy is required.

Conclusion

Current recommendation for ALK and ROS1 testing is mainly for adenocarcinoma, lung cancers with adenocarcinoma component, or NSCLC samples in which adenocarcinoma component cannot be reliably ruled out. However, ALK and ROS1 analyses in squamous cell carcinoma has been limited, and testing is warranted in non-smokers with this tumor histology.

Sample Acquisition, Processing, and General Diagnostic Procedures

3

By Andrew G. Nicholson, Keith Kerr, Ignacio Wistuba, and Yasushi Yatabe

Testing for *ALK* and *ROS1* gene rearrangements is one of several diagnostic procedures that may be required on a tissue sample containing lung cancer. In most patients, a single sampling procedure generates a relatively small amount of tissue that must then be used as efficiently as possible to allow for the most fully informed diagnosis possible. Two important points about tissue samples should be remembered: a sample may contain limited amounts of tumor tissue, and only one opportunity is available to fix and process the tissue. Thus, acquisition and processing are crucial steps in quality control in order to facilitate all the diagnostic procedures that may need to be done on a tissue sample.

Obtaining Tissue for Diagnosis

ALK and ROS1 testing is usually performed on a small biopsy specimen or a cytology sample taken from a patient who has advanced disease. Less often, the whole tumor is available from a patient who has had surgical resection of early-stage disease and subsequent recurrence, or who has unexpected advanced disease at resection. Tissue sampling for diagnosis should be aimed at obtaining the largest yield of tumor in the safest and least invasive way possible (Thunnissen 2012b). Sampling may involve the primary tumor, intrathoracic metastatic disease, or extrathoracic metastases. Although discrepancies in ALK status between primary and metastatic disease have been reported (Kim 2013), these discrepancies are rare. The primary tumor may be sampled at endoscopy (by endobronchial or transbronchial forceps biopsy, cryobiopsy, or FNA, or with a percutaneous, transthoracic approach (by core-needle biopsy or FNA). Intrathoracic metastatic disease is now routinely sampled with use of endobronchial ultrasound (EBUS) or transesophageal ultrasound (EUS) guidance; pleural disease (either pleural biopsy or fluid cytology) is often a good source of diagnostic material. Distant extrathoracic metastatic disease can be sampled as appropriate to the site; in all cases, several imaging techniques are helpful in targeting the sampling to improve tumor yield (Rivera 2007). In most centers, surgical procedures may be used to obtain tissue if sufficient material has not been obtained with image-guided procedures or when such procedures are thought unlikely to be successful. Samples from all these sites when

obtained with the noted methods, provide sufficient material for ALK and ROS1 testing in most cases (Neat 2014).

Tissue Processing

The key issues for ALK and ROS1 testing on either tissue biopsy or cytology samples are that the material must be processed and handled appropriately and the sample must contain a sufficient number of tumor cells (Thunnissen 2012a). The number of tumor cells required for IHC assessment of ALK and ROS1 proteins remains undefined, but a minimum of 50 assessable tumor cells are required for FISH testing. Alternative approaches for cytology smears are available, but the most appropriate approach with cytology samples is usually the preparation of a cell block that allows sections to be prepared and treated in the same way as sections of tissue biopsy samples. In general, all of the tissue or cellular material received in the pathology laboratory should be processed. Surgical resection specimens are an exception, although, as a general rule, tumors with a diameter of 3 cm or less should be processed in their entirety. Large pleural effusions should also be processed in part, as their preservation as a cell block or blocks can be a good source of tumor cells.

Fixation by immersion, or where appropriate, by inflation, with 10% neutral buffered formalin is recommended. Pre-fixation in some alcohol-based fixatives may alter tissue antigenicity or DNA integrity. Acidic decalcifying solutions used on bone biopsy samples may interfere with IHC, frequently compromise FISH testing, and often degrade DNA, making mutation testing less reliable. Fixatives that are acidic (such as Bouin's fluid) or based on hard-metal salts should also be avoided. In general, a period of fixation of more than 6 hours and less than 72 hours is recommended, especially when biomarker testing is to be done (for which DNA integrity is important) (Wolff 2013, Hunt 2008). Underfixation or overfixation may have deleterious effects on DNA and protein antigen epitopes (Werner 2000, Atkins 2004, Oyama 2007, Eberhard 2008, Bussolati 2008). One of the crucial parts of this phase in tissue handling is the period of time beginning immediately after the sample is removed from the patient and placed in preservative. Most laboratories have neither control of nor data on how much time elapses between tissue removal and immersion in a fixative and its arrival in the laboratory. In addition, most tissue processing machines include a fixation step, which increases the fixation time. In practice, most laboratories will adjust their staining processes relevant to IHC and in situ hybridization (ISH) to allow for their own average fixation time. Determining the nature and duration of fixation is a greater challenge in laboratories that receive samples from many outside sources with widely differing fixation procedures.

Tissue Handling

Most biomarker investigations (IHC, ISH, or RNA/DNA studies) are performed during the initial diagnostic workup. In these circumstances, freshly cut sections should be used for biomarker testing. Tissue stored on glass sections will start deteriorating in a matter of days or weeks and certainly over months. Degradation depends on the storage conditions and most likely also on the specific biomarker (Atkins 2004). The stability of ALK and ROS1 protein on unstained cut sections has not yet been studied systematically. Therefore, similar to the case of HER2 testing in breast cancer, slides with tissue sections stored for longer than 6

weeks should not be used for IHC testing for ALK. If storage is necessary, the sections should be coated in wax or a similar medium to prevent air oxidation and the sections should be kept in cool, dry, and dark conditions. Tissue in FFPE blocks is less prone to deterioration, and recutting the tissue block as needed at a later time works well in most circumstances.

Various strategies can help limit the number of times the block needs to be cut to provide material for initial morphologic assessment, IHC staining, and subsequent molecular analysis. Extra sections may be cut at the first cutting session, and many laboratories already have protocols set up according to local requirements. Any protocol, however, requires close supervision by pathologists to ensure that material is not wasted, not only by cutting too many sections and undertaking unnecessary IHC but also by not cutting enough sections, which requires further trimming of the block for a second round of sectioning (Figure 1).

The pathologist must be in close communication with oncologists and those undertaking interventional procedures (surgeons, respiratory physicians, and radiologists) to ensure that any known diagnosis is apparent before sectioning is done. This communication is especially important for samples from patients in whom resistance to ALK and ROS1-related targeted therapies has developed. In such cases, only a routinely stained slide is needed to confirm the presence of malignancy and to assess tumor load.

Tissue may be preserved in other simple practical ways, such as placing material in multiple blocks (multiple cores or bisected nodules) to minimize waste.

Evaluation of Tissue Samples

Once sections are prepared, the first step in the evaluation of a sample is to identify the presence or absence of malignancy. Depending on patient selection, choice of sampling technique, and operator skill, the rate of positive tumor findings is generally high and may range from approximately 60% to more than 90% (Schreiber 2003). It is well recognized that, even when tumor is present in the sample, it may not be present in all tissue fragments and it generally comprises a small proportion of the tissue submitted (Coghlin 2010). Once malignancy is confirmed, the next step is to exclude the possibility of nonepithelial malignancy (such as lymphoma or sarcoma) and/or the possibility that a cancer, especially adenocarcinoma, is not lung metastasis from another organ. Most often, this step can be done easily, based on the evaluation of adequate clinical and radiographic information accompanying the sample and the basic H & E-based morphologic assessment. In particular, a lack of clinical information may lead to unnecessary ancillary IHC testing on the sample in an attempt to exclude possible extrathoracic sources for an adenocarcinoma, which may leave insufficient material for molecular testing.

Assuming the tumor is primary lung cancer, the next step is to distinguish SCLC from other types, as advanced SCLC is treated differently from NSCLC. This discrimination can usually be made with high accuracy on the basis of morphologic characteristics (Burnett 1994), but IHC may be required. Most cases that are not SCLC can be accurately and consistently classified morphologically as squamous cell carcinoma, adenocarcinoma, or, rarely, another NSCLC type. In 25% to 40% of cases, however, depending on the sample type and case mix, morphologic features are not adequate for accurate and consistent NSCLC subtype classification (Chuang 1984). Diagnostic IHC can then be used to predict the likely NSCLC subtype (Nicholson 2010, Loo 2010, Travis 2011, Travis 2015). This approach, using a limited

IHC panel, can reduce the proportion of NSCLC-not otherwise specified (NOS) cases to less than 20% and predict the NSCLC subtype in most cases with an accuracy of more than 90% (Nicholson 2010, Loo 2010). Cases reported in conjunction with this use of IHC should be described by the recommended terminology (eg, NSCLC, favor adenocarcinoma on IHC) in a morphologically undifferentiated case in which IHC predicts adenocarcinoma (Travis 2015). Only those samples lacking evidence of both adenocarcinomatous and squamous differentiation after IHC should be classified as NSCLC-NOS.

When FISH testing is requested, the pathologist should review any additionally cut slides to ensure that tumor cells are still present in the specimen. Slides can also be marked to show

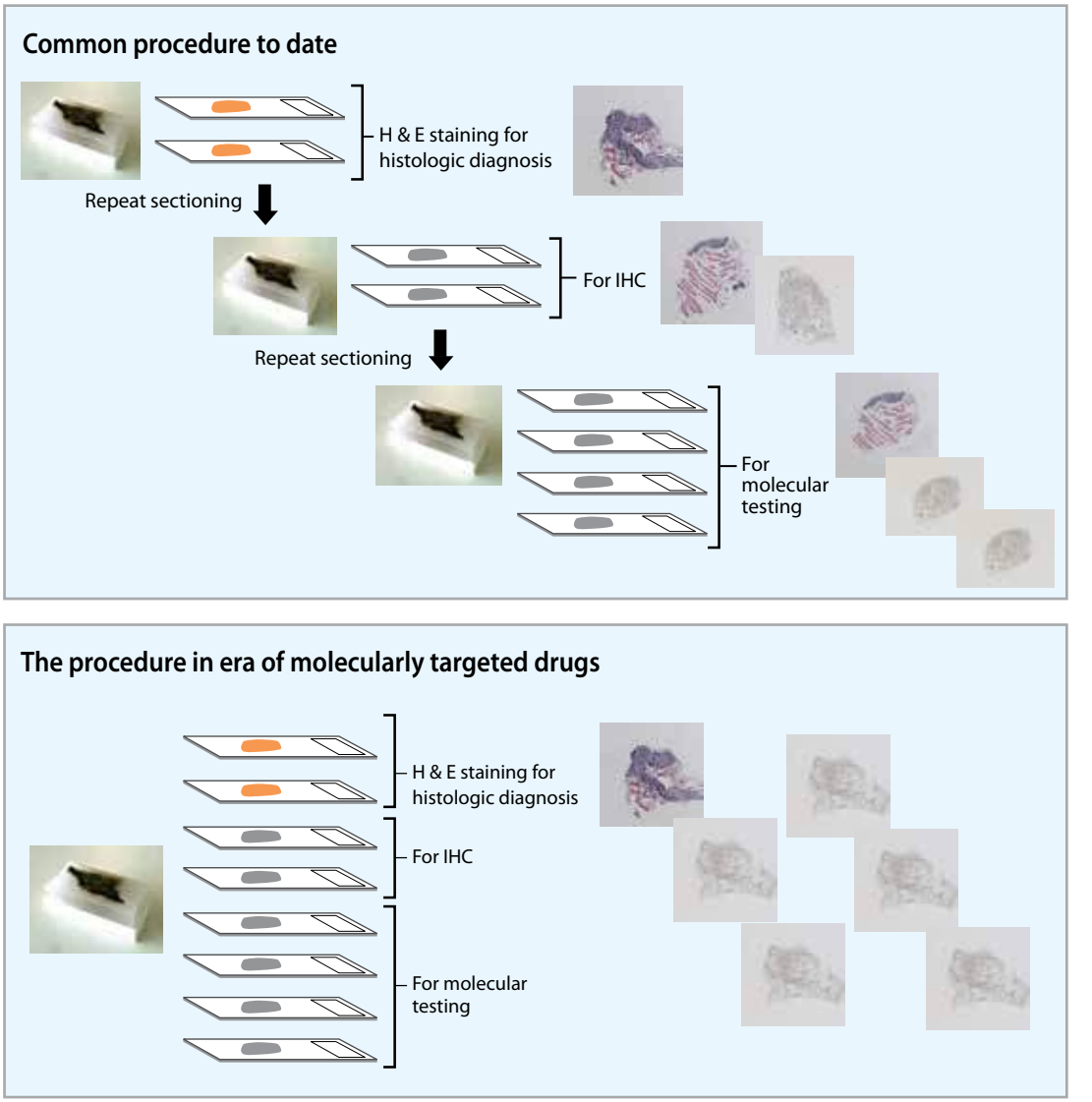

Figure 1. Preparation of tissue sections during diagnostic workup. Current routine practice involves making additional sections for IHC assay and/or molecular testing after the initial sectioning and hematoxylin and eosin (H & E) staining for histologic diagnosis. Multiple sequential sectioning may deplete the tumor volume each time block trimming is necessary. In the era of molecularly targeted therapies, the preparation of additional unstained sections for possible IHC analysis and/or molecular testing may substantially reduce the amount of tissue sample lost and improve turnaround time.

areas of highest density for potential microdissection. For samples to be sent for sequencing, documentation should be sent regarding the tumor percentage (number of tumor cells as a percentage of total number of cells on the slides), as well as the presence or absence of necrosis.

New Techniques and ALK and ROS1 Testing

ALK rearrangements have been successfully identified with both FISH and IHC in isolated circulating tumor cells (CTCs), which potentially opens the door for noninvasive screening (Ilie 2012, Pailler 2013, Ross 2015). One study has also shown that *ROS1* rearrangements can be identified in CTCs from patients who have the rearrangement in the primary tumor (Pailler 2015). In addition, rearranged *EML4-ALK* fusion transcripts have been found to sequester in circulating blood platelets, which has helped to predict and monitor outcomes after treatment with crizotinib (Nilsson 2016). Although this technique is not in routine use, capture of CTCs, or even cell-free tumor DNA (cfDNA), is likely to increase in clinical practice over the next few years (Paweletz 2016). Pathologists will need to adapt and be part of the management of the tissue sample pathway in relation to all molecular testing of CTCs and cfDNA, including testing for *ALK* and *ROS1* rearrangements.

Conclusion

The identification of patients with therapeutically targetable molecular drivers in their tumors is now a standard of care. The potential need for molecular testing beyond that required for initial morphologic diagnosis and refinement of tumor classification by IHC makes the acquisition, handling, processing, and judicious use of diagnostic tumor tissue of crucial importance.

ALK Testing with IHC

By Erik Thunnissen, Sylvie Lantuéjoul, Jin-Haeng Chung, Keith M. Kerr, Fred. R. Hirsch, Ming Sound Tsao, and Yasushi Yatabe

4

Molecularly targeted therapy is critically dependent on a validated test to detect the corresponding molecular alteration, especially when the molecular alteration is present in a small subgroup of patients. ALK IHC holds promise as a rapid and relatively inexpensive screening method that involves the use of bright-field examination, which is preferred by most pathologists primarily because it allows the evaluation of tissue architecture and tumor cell histology. Potentially, IHC can be interpreted with fewer malignant cells than needed for FISH. IHC can be performed successfully on a variety of different tumor specimens; FFPE tissue blocks, fluid, and FNA cytology cell blocks or smears can be tested, as long as at least a few clusters of viable tumor cells are present in the specimen. In addition, a disease of low prevalence such as *ALK*-rearranged NSCLC calls for an economic screening method (Soda 2007, Koivunen 2008, Perner 2008, Takeuchi 2008, Palmer 2009). A validated and reliable ALK IHC assay can provide a cost-effective platform for routine ALK screening of NSCLC in clinical practice at reduced costs. The ALK (D5F3) CDx Assay (Ventana) has been approved by the US FDA and by several other regulatory authorities worldwide as a stand-alone ALK diagnostic test, suggesting an algorithm for patient selection that is based on a definitive IHC result (positive or negative), regardless of the antibody used.

The Challenge of ALK IHC

It is important to standardize the ALK IHC assay as a screening method and to establish the evaluation criteria. The tumor cells in ALK-positive lung cancer usually express the protein product of the various *ALK* chimeric genes (Figures 1 and 2). The fusion protein of the intracellular tyrosine kinase domain of *ALK* with various (N-terminal) truncated portions of the partner gene is responsible for constitutively increased *ALK* kinase activity (Morris 1994, Allouche 2007). However, the ALK fusion protein in NSCLC may be more difficult to detect with the ALK1 antibody, which is used to diagnose anaplastic large-cell lymphoma, as the protein expression is generally lower in NSCLC (Mino-Kenudson 2010). To overcome this issue, several technical steps have been introduced, including antigen retrieval, use of a primary antibody with higher affinity and at a sufficiently high concentration, strong

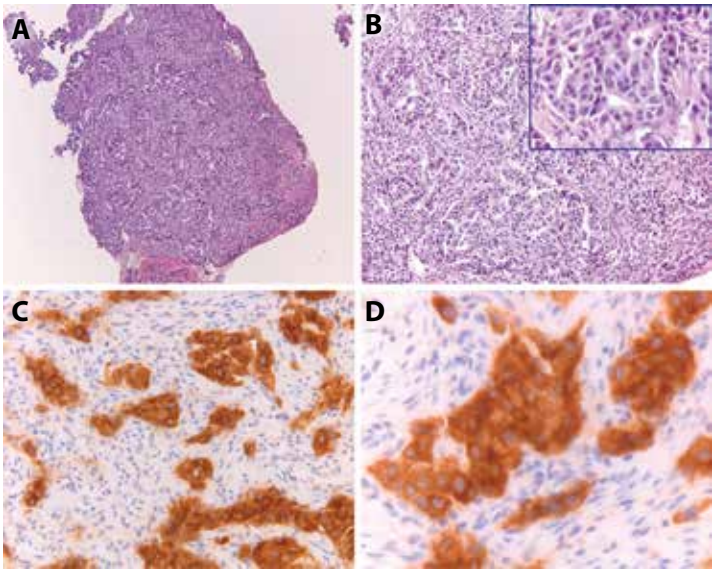

Figure 1. An adenocarcinoma that is strongly ALK positive. A and B: H & E-stained slides, with an overview (A) and at a magnification of x10 (B). The inset in B is at a magnification of x40. No signet ring cells are seen. C and D: ALK IHC with the 5A4 antibody shows intense cytoplasmic staining of *ALK* gene product (magnification, C: x20, D: x40).

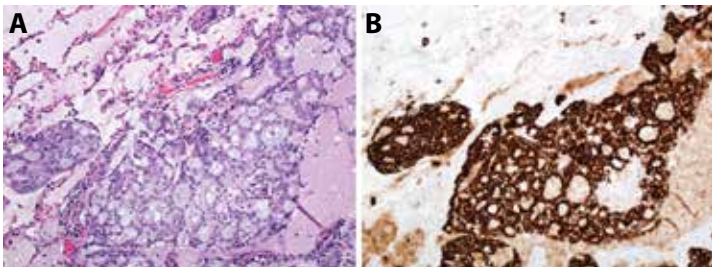

Figure 2. An adenocarcinoma that is strongly ALK positive. A: H & E-stained slide. B: ALK IHC with the D5F3 antibody and tyramide amplification shows intense cytoplasmic staining of *ALK* gene product (magnification, x40).

signal amplification steps (eg, with a tyramide cascade and intercalation of an antibody-enhanced polymer), and the development of novel antibodies (Table 1).

Because ALK protein is not expressed in lung tissue, an internal positive control for immunostaining is lacking, which makes it difficult to judge whether a negative IHC result is truly negative for expression of the ALK fusion protein. Nevertheless, diffuse expression of ALK protein in lung cancer cells is almost always associated with expression of the aberrant ALK fusion protein (Takeuchi 2013). FFPE cell blocks with *ALK*-rearranged cell lines (H3122-variant 1 and H2228-variant 3) may be used as external positive controls to set up the staining conditions, but epitope concentration in these cell lines is high (3+). The optimal staining control has weak epitope concentration, allowing easy detection of any variations in the staining protocol. Recently, a cross section of the appendix has been used for this purpose with ALK IHC, where ganglion cells should stain at least weakly positive (1+/3+) [http://www.nordiqc.org/downloads/assessments/45_14.pdf] (Ibrahim 2016).

Table 1. Commercially Available Antibodies for IHC to Detect ALK

Clone	Clone Type	Isotype	Immunogen
ALK1	Mouse monoclonal	IgG3, kappa	Amino acids 1359–1460 of the full length human ALK protein, corresponding to amino acids 419–520 of the chimeric NPM-ALK protein
5A4	Mouse monoclonal	IgG1	C-terminus of the NPM-ALK transcript (419-520 amino acids)
D5F3	Rabbit monoclonal	IgG	Carboxyl terminus of human ALK
Anti-ALK	Rabbit monoclonal	IgG	Recombinant protein representing amino acids 426-528 of human ALK

Fixation and Sectioning

The preanalytic steps for ALK IHC are the same as those for other IHC procedures. Regardless of origin, diagnostic biopsy or surgical specimens should immediately be fixed in an adequate amount (ratio of 10 times more than the volume of the specimen) of neutral buffered 4% formalin and embedded in paraffin. Fixation must be done as soon as possible to avoid cold ischemia effects. Fixation times of less than 6 hours are not recommended because conventional staining as well as IHC can be adversely affected. Antigen preservation for IHC is epitope dependent, and some epitopes may not be hampered by fixation times of as long as 120 hours. For practical purposes, a fixation interval of 6 to 48 hours is recommended for all specimens. A study at different centers with different fixatives (formalin-fixed or alcohol–formol–acetic-acid–fixed) demonstrated substantial discordances, with different fixatives as possible confounders, emphasizing the need for fixation in a standardized manner (Cabillic 2014).

After tumor tissue has been embedded in paraffin, the tissue is stable and preserved against oxidative influences. However, once 3- to 4-µm thick slides are cut from the FFPE block, the storage time of these sections mounted on glass microscope slides at room temperature is limited to a maximum of 3 months (Blind 2008). The slides remain adequate for a longer period of time when stored at a colder temperature (4°C). However, slides of tissue sections that were prepared more than 6 weeks earlier should be interpreted very carefully, as they may present false-negative results.

Immunostaining: Antibody, Detection Method, and Interpretation

For the analytic procedure (ie, actual ALK IHC testing), several issues need to be controlled and optimized: epitope retrieval, type and concentration of the antibody, incubation time, incubation temperature, and amplification.

A single uniform technique, or comparator, has not been evaluated in studies on ALK IHC in NSCLC. Instead, the type or source of antibodies, the process of antigen retrieval and antibody detection, and the amplification techniques have varied substantially (Table 2). Head-to-head comparison of different antibodies shows that D5F3 (Cell Signaling Technology) and 5A4 (Novocastra) with the ADVANCE system (Dako) appear to be equally sensitive (Conklin 2013). Other studies also demonstrated comparability of the two antibodies. (*See Chapter 10.*) The D5F3 is part of a commercial companion diagnostic ALK IHC assay kit, whereas 5A4 is commonly used as a laboratory-developed test.

The ALK1 antibody (Dako) is less accurate and should not be used. A novel monoclonal anti-ALK antibody 1A4 (Origene) was compared with D5F3 and described as a promising candidate for screening lung tumors for the presence of *ALK* rearrangements (Gruber 2015). In contrast to the ALK (D5F3) CDx Assay (Ventana), 1A4 IHC was performed using a conventional staining procedure, without signal enhancement. The 1A4 antibody was subsequently examined in an independent cohort, and the sensitivity was comparable to D5F3, but specificity was much lower (70%) (Wang 2016). Therefore, with this approach, tumors that are positive for ALK on testing with 1A4 IHC will require an additional predictive technique before treatment advice can be given.

The sensitivity for detecting the ALK fusion protein has been enhanced by using several signal amplification steps (Figure 3) (Rodig 2009, Sakairi 2010, McLeer-Florin 2012).

Table 2. Immunostaining Conditions Using Commercially Available ALK Antibodies in Selected Published Studies in which Commercially Available Kits Were Not Used

Study	Antibody	Antigen Retrieval	Dilution	Incubation	Detection System
Yi et al., 2011	ALK1	EDTA, pH 8.0, 30 min in PT Link	1:100	30 min at room temperature	ADVANCE
Mino-Kenudson et al., 2010	ALK1 D5F3	EDTA, pH 8.0, in pressure cooker	1:2 1:100	Overnight	EnVision+
Minca et al., 2013	D5F3	Heat mediated with BenchMark XT	1:100	Not specified	OptiView
Martinez et al., 2013	D5F3	Standard on BenchMark XT	1:50	16 min at 37°C	ultraView
Paik et al., 2011	5A4	CC1 solution, 100°C, 20 min	1:30	2 hr at 42°C	iVIEW
Hofman et al., 2011	5A4	Target Retrieval Solution, pH 9.0, 97°C, 40 min	1:50	30 min at room temperature	EnVision FLEX
McLeer-Florin et al., 2012	5A4	CC1 solution with EDTA, pH 8.4, 1 hr	1:50	2 hr at 37°C	Amplification Kit
Kim et al., 2011	5A4	CC1 solution, 100°C, 20 min	1:30	2 hr at 42°C	iVIEW
Sholl et al., 2013b	5A4	Citrate buffer, pH 6.0, in pressure cooker, 122°C, 30-45 min	1:50	40 min at room temperature	EnVision FLEX+
Wong et al., 2009	Anti-ALK	Citrate buffer, pH 6.0, in microwave, 95°C, 30 min	1:1000	Overnight at 4°C	Streptavidin-biotinylated horseradish peroxidase complex
Chen et al., 2012	Anti-ALK	CC1 solution, 95°C, 30 min	1:500	Overnight at room temperature	ultraView

Antibodies: ALK1 is a product of Dako; D5F3 is a product of Cell Signaling Technology; 5A4 is a product of Novocastra in the studies by Paik et al., Kim et al., and Sholl et al. and is a product of Abcam) in the studies by Hofman et al. and McLeer-Florin et al; and anti-ALK is a product of Invitrogen, Life Technologies Corporation. Antigen Retrieval: PT Link and Target Retrieval Solution are products of Dako; BenchMark XT is a product of Ventana Medical Systems, Inc. Detection systems: ADVANCE, EnVision+, and EnVision FLEX+ are products of Dako; OptiView (DAB Kit), ultraView (DAB Kit), iVIEW (DAB Kit), and Amplification Kit are products of Ventana Medical Systems, Inc.

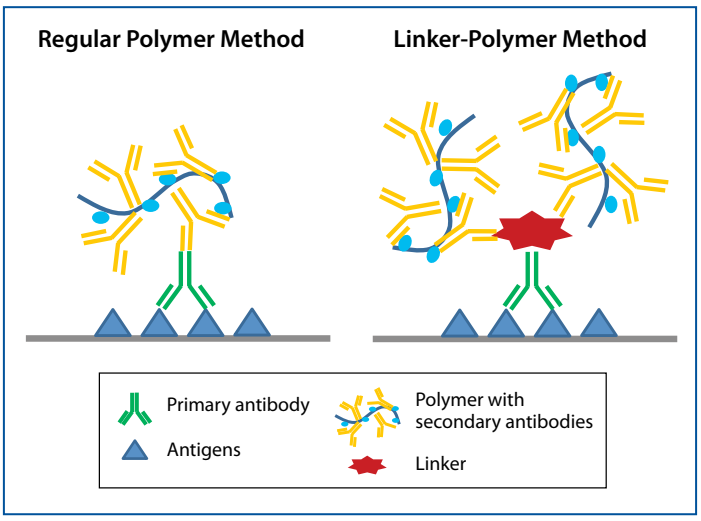

Figure 3. Schemes of regular polymer and linker-polymer methods.

The D5F3-based immunoassay (ALK [D5F3] CDx Assay; Ventana) was developed and standardized on the automated immunostaining platform BenchMark XT combined with the OptiView Amplification Kit. This kit includes an amplification step that can reduce or eliminate equivocal results by increasing the signal difference between the specific immunoreaction and the background signal. Thus, the D5F3 assay produced more intense cytoplasmic signals but with higher background and focal staining, which raises the possibility of false-positive interpretation (Ibrahim 2016).

The postanalytic phase starts with microscopic evaluation of the stained slide. In NSCLC, ALK staining is cytoplasmic; it may have a granular character and, in some cases, there may be membrane accentuation. As mentioned earlier, the intensity of the staining is dependent on the enhancement system used (Figure 4). The assessment of staining intensity is subjective, but the use of successive microscope objective lenses with inherent related spatial resolution is a physical aid in establishing the intensity level, as first applied to HER2 testing (Ruschoff 2012). The use of this approach may lead to more uniformity in intensity scoring. Strong staining (3+) is clearly visible with use of a x2 or x4 microscope objective lens; moderate staining (2+) requires a x10 or x20 objective lens to be clearly seen; and weak staining (1+) can be seen only with a x40 objective lens. The classic histo-score (H-score) is derived by multiplying the percentage of tumors that stain positively by the intensity (0, 1, 2, or 3), giving a range of 0 to 300. This approach takes greater account of the heterogeneity of the staining. Interestingly, with tyramide enhancement the difference in epitope concentration between a negative and a strong positive staining intensity is reduced to the extent that scoring is either negative or positive (Figure 4). This tyramide enhancement works similarly for both of the currently used antibodies (D5F3 and 5A4) (Savic 2015, Ibrahim 2016).

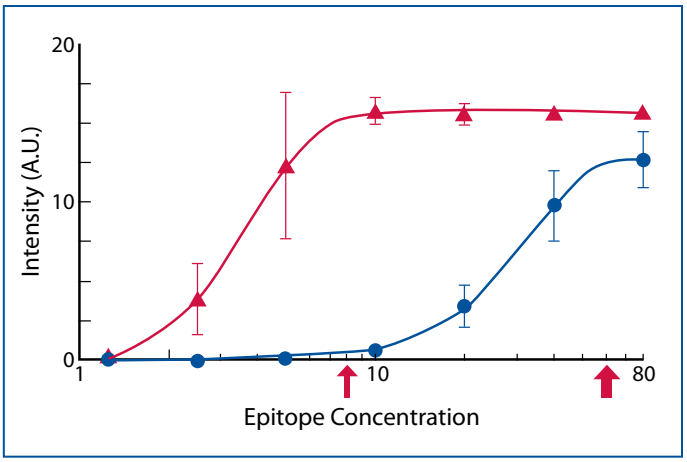

Figure 4. For immunohistochemical visualization the effect of a low (blue circles) and a high (red, triangles) signal enhancement system is shown. Note that with a high signal enhancement system a low epitope concentration may become positive (small arrow), while negative with a low enhancement system. In addition, a higher intensity plateau is reached: once positive a higher epitope concentration will not lead to darker staining. Applied to ALK immunohistochemistry: in lymphomas the epitope concentration is higher (thick arrow) than in NSCLC (thin arrow) and a low enhancement system may suffice. In NSCLC high affinity antibody with high concentration and high enhancement is necessary. Epitope concentration has logarithmic scale and intensity linear scale. Modified from Prinsen et al. 2003.

Different criteria for ALK-positive and ALK-negative results on IHC have been applied in different studies. Some authors have scored the intensity from 1+ to 3+ (Figure 5), with an ambiguous threshold around 1+ or 2+ ; this scoring approach seems to be mainly related to the amplification system used. In a recent analysis of pooled data, the diagnostic operating characteristics in 12 studies (3,754 NSCLC specimens) were analyzed, taking the different scoring systems into account (Jiang 2016). The IHC 3+ and the binary ALK-positive category matched for both antibody procedures with ALK FISH-positive cases and the IHC-negative cases

matched FISH-negative cases. The nearly 100% concordance in these IHC categories favors the use of IHC as a screening method to identify ALK-positive NSCLC. However, for the lower intensity staining in the four-tiered IHC approach, tumors with 1+ and 2+ intensity need additional validation with ALK FISH testing.

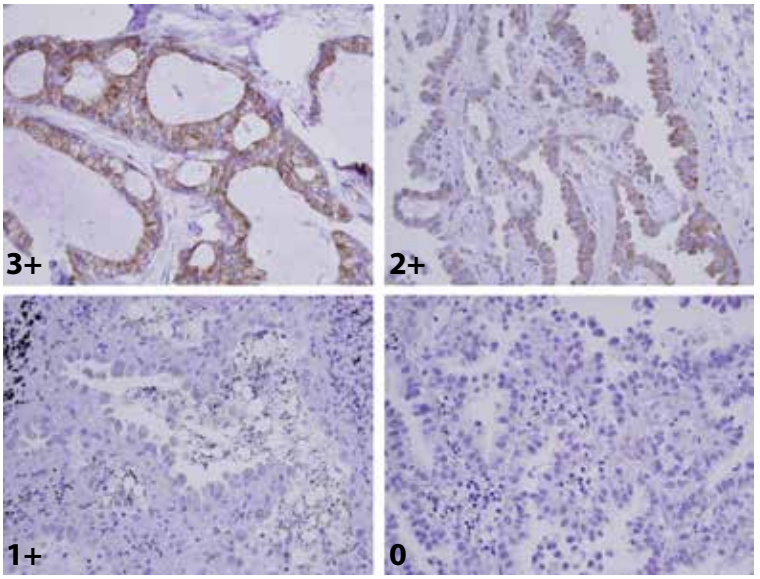

Figure 5. An example of ALK IHC scores ranging from 0 to 3+.

The reproducibility of ALK IHC results among different laboratories and pathologists is high for validated protocols (Wynes 2014, Blackhall 2014, Cutz 2014, Ibrahim 2016, Thunnissen 2012a).

Practical Implementation of ALK IHC

Generally, because ALK protein is not expressed in lung tissue, strong IHC amplification systems can be used. However, pathologists should be familiar with various artifacts that may lead to false-positive staining: light cytoplasmic stippling in alveolar macrophages (Figure 6), cells of neural origin (nerve and ganglion cells), glandular epithelial staining, extracellular mucin, and necrotic tumor areas. Background staining is rarely observed within normal lung parenchyma, but several staining pitfalls have been noted (Table 3). False-positive cytoplasmic staining in NSCLC has been noted with the tyramide amplification system using D5F3. This staining may be weaker than usual positivity in lung cancers that are ALK-positive on IHC.

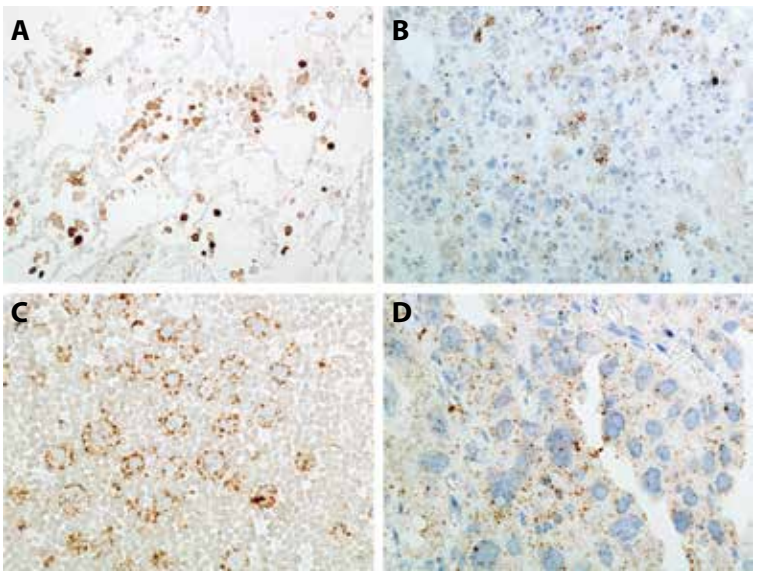

Figure 6. Nonspecific staining with IHC with the D5F3 antibody. A: Alveolar macrophages at the margin of an ALK-negative tumor. B: Cytology cell block of a needle aspirate from a lung nodule, showing NSCLC. Light cytoplasmic stippling in alveolar macrophages is an artifact that may lead to a false-positive interpretation. Cell block (C) and biopsy specimen (D) of adenocarcinoma with stippling, which was negative for *ALK* rearrangement on FISH.

Histologically, mucin-containing cells such as signet ring cells require careful interpretation of

ALK immunoreactivity. A thin membranous positive pattern on ALK IHC may be masked by an intracellular mucin vacuole (Figure 7), and the positive pattern may then be difficult to detect in the signet ring cells (Rodig 2009, Yoshida 2011a, Popat 2012).

Some researchers have noted membranous staining, particularly in the apical portion, in FISH-negative cancer (Murakami 2012, Mino-Kenudson [personal communication]). This finding was not specific to cancer cells and was also seen in some non-tumor cells, such as reactive type II pneumocytes. Thus, it is important to see background staining in the staining specimens. In addition, some neuroendocrine carcinomas have also been associated with positive reactions (Murakami 2012, Nakamura 2013). Merkel cell tumors of the skin may also be ALK-positive on IHC, but have no *ALK* rearrangement detected by FISH or NGS.

Table 3. Potential Pitfalls in Interpreting the Results of Immunohistochemistry (IHC)

Mucin-producing cells	Cytoplasm is masked by intracellular mucin, with absence of ALK protein, leading to negative staining or marginally membranous-like staining, and a false-negative interpretation.
Membranous staining	Nonspecific membranous staining, particularly prominent in the apical portion, is seen occasionally. This finding is not specific to tumor cells and is also seen in normal pneumocytes.
Neuroendocrine cells	Some squamous cell carcinomas, large cell neuroendocrine carcinomas, and normal ganglion cells show positive reactions.
Nonspecific mucin staining	Depending on the amplification system used, some background can be found on extracellular mucin and within the cytoplasm of alveolar macrophages and bronchial cells.

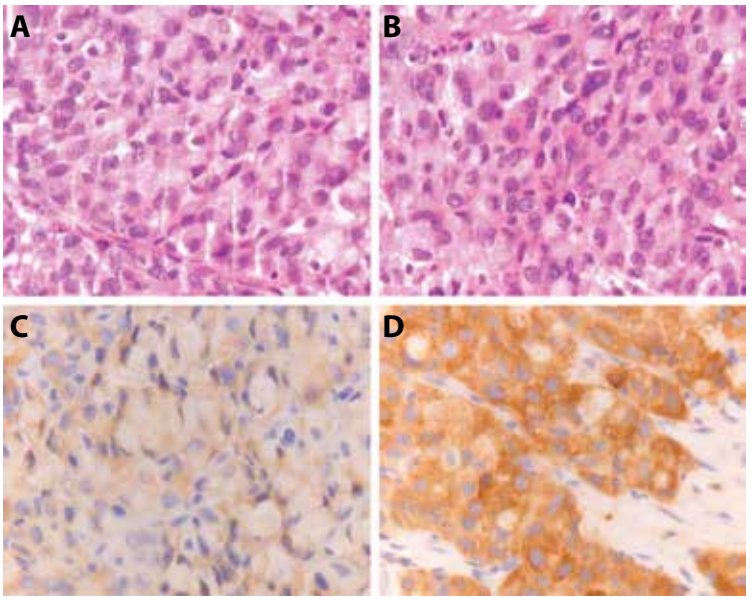

Figure 7. A tumor with variation in morphology and staining. H & E staining shows (A) an area with many signet ring cells, and (B) area with solid pattern with few signet ring cells. ALK 5A4 IHC shows (C) small cytoplasmic rim low (+1) and (D) high (+2/+3) staining intensities (magnification, x40).

The staining may appear heterogeneous in some tumors, particularly in surgical specimens (Figure 8); however, if preanalytic conditions are controlled for, the vast majority of tumor cells are stained, paralleling the homogeneous distribution of the *ALK* gene rearrangement in FISH analysis. The heterogeneity is likely related to heterogeneity of the fixation and does not seem to be related to the presence of a different histologic pattern. The sensitivity of ALK protein to delay of fixation is not an issue for biopsy specimens, but may be an issue when using tissue microarrays for ALK screening of archived specimens. The para-nuclear dot-like pattern reported as typical of the *KIF5B-ALK* rearrangement may require further confirmation (Figure 9) (Takeuchi 2009).

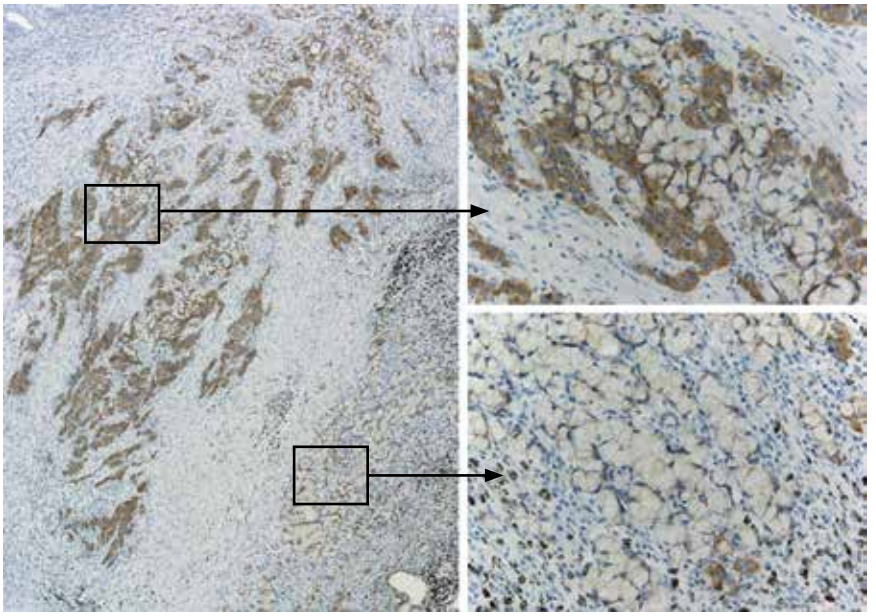

Figure 8. An ALK-positive tumor with heterogeneous positive staining. The boxes in the left panel correspond to the images on the right. Signet ring cell carcinoma component is a potential pitfall for negative staining.

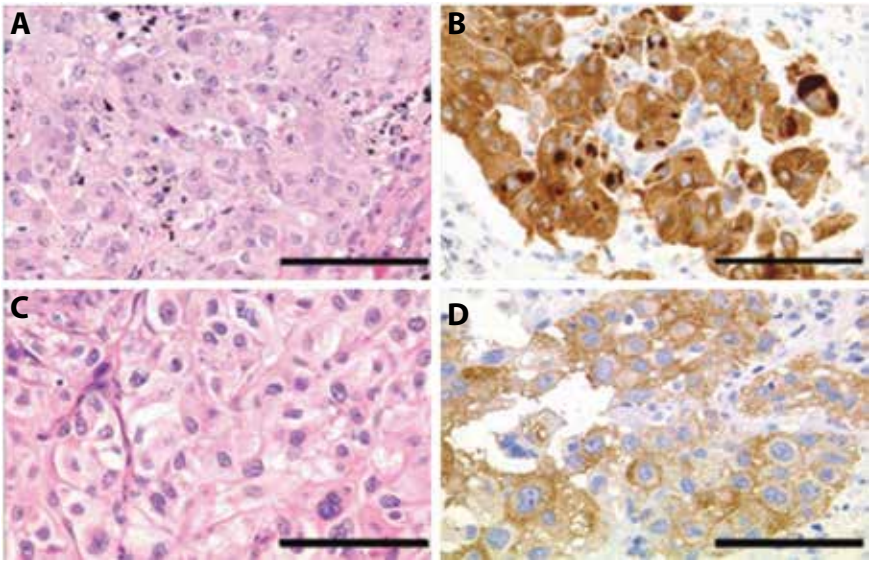

Figure 9. Unusual positive reactions on IHC of a KIF5B-ALK-positive adenocarcinoma, showing a strong Golgi area-highlighted staining pattern (A: H & E staining, B: ALK staining) and a perinuclear halo pattern (C: H & E staining, D: ALK staining). The bars = 100 µm. Reprinted from Takeuchi K, et al. KIF5B-ALK, a novel fusion oncokinase identified by an IHC-based diagnostic system for ALK-positive lung cancer. *Clin Cancer Res.* 2009;15:3143-3149.

Discordant NSCLC cases, with ALK protein expression but no detectable *ALK* rearrangement on FISH, have been reported. The explanations for this discordance include the following: (1) false-negative interpretation of FISH results, especially for results that are close to the threshold of 15% (von Laffert 2015); (2) amplification of the *ALK* gene (which

has been associated with ALK protein expression in some but not all cases), possibly leading to 1+ or 2+ staining (Kim 2013, Salido 2011); (3) false-positive interpretation of ALK IHC results; (4) double rearrangement involving *ALK*, reducing the visible distance of the two FISH probes; and (5) an indeterminate mechanism.

In an animal model, expression of non-rearranged *ALK* transcripts was relevant, because ALK-inhibitor treatment of NSCLC cells and xenograft tumors expressing wild-type *ALK* transcripts resulted in tumor regression and suppression of metastasis (Wang 2011). The clinical relevance remains the question, as it is unclear whether lung cancers that are ALK IHC-positive and FISH-negative respond to treatment with an ALK inhibitor. (*See Chapters 13 and 14.*)

Conclusion

ALK IHC assays are validated and standardized and are clinical tools for cost-effective screening for the presence of *ALK* rearrangement in NSCLC and is already recommended by organizations in Europe, Japan, and Asia. In the United States, the IHC CDx Assay has been approved by the US FDA, and patients with positive results on ALK IHC are eligible for treatment with an ALK inhibitor. To improve the reliability of assays for detecting ALK positivity, as well as optimal information regarding patient selection for ALK inhibitors, further studies should be performed to compare and validate these different diagnostic assays to correspond with the response to ALK inhibitors with appropriate external quality assessment programs.

ROS1 Testing with IHC

By Lynette Sholl, Akihiko Yoshida, Andrew Nicholson, Sylvie Lantuéjoul, and Fred R. Hirsch

5

Gene rearrangements leading to ROS1 activation and overexpression are detected in 1% to 2% of people with lung adenocarcinoma. ROS1 is a receptor tyrosine kinase that is phylogenetically similar to ALK. As with *ALK*-rearranged tumors, *ROS1*-rearranged lung tumors arise predominantly in younger, nonsmoking individuals and may have a distinct morphology, including solid or cribriform growth with mucin production and/or frequent signet ring cells (Bergethon 2012, Yoshida 2013, Lee 2015). Patients with *ROS1*-rearranged tumors have had substantial and durable responses to the multitargeted tyrosine kinase inhibitor crizotinib (Shaw 2014), which is approved by the FDA in the United States and the European Medicines Agency (EMA) in Europe for *ROS1*-rearranged NSCLC. It is therefore important to test appropriate NSCLC tumors for this genetic alteration.

ROS1 gene fusions typically result from interchromosomal rearrangements, although rare intrachromosomal rearrangements have been reported in glioblastoma and NSCLC (Rimkunas 2012). Most of these fusions are readily detected by FISH. However, given the high cost and technical challenges of FISH, coupled with the rarity of *ROS1* rearrangements in lung cancer, alternative screening tests may be appropriate in some settings. The first report of ROS1 IHC used with a sensitive rabbit monoclonal antibody was published in 2012 (Rimkunas 2012); since that time, many studies in which IHC was used to screen for *ROS1* fusions in lung cancer have been published. In this chapter, we describe the published methods, performance characteristics, and potential pitfalls of ROS1 IHC in clinical practice; in most contexts, ROS1 IHC will be used as a screening tool, with results confirmed by an orthogonal method such as FISH or molecular tests (eg, RT-PCR and NGS).

Preanalytic Considerations

In studies published to date, ROS1 IHC has been done only on FFPE tissues. Specimens should be placed in fixative as quickly as possible after they are obtained to minimize antigen degradation as a result of cold ischemia. Fixation should be carried out in 10% neutral buffered formalin for at least 6 hours and for as long as 48 to 72 hours. Once tissue is embedded in paraffin, it can be stored in a climate-controlled environment (approximately 25°C in dry

conditions) for 10 years or more with relative retention of protein signals (Nuovo 2013). However, once a 4- to 6-micron tissue section is cut onto glass slides, exposure to the air may lead to oxidative damage and signal degradation, as demonstrated by studies of ALK IHC antibodies, for which antigenicity of cut tissue sections appears to decline after 3 months. In the absence of clear published data on preanalytic factors affecting ROS1 antigenicity, it may be prudent to apply guidelines similar to those established for ALK IHC (*see Chapter 3*).

Immunostaining

At this time, only one ROS1 antibody is commercially available: clone D4D6 (Cell Signaling Technology). However, detection systems and staining conditions vary considerably across studies (Table 1). In the absence of a uniform technique, variability in study results may reflect substantial differences in approaches to antigen retrieval or signal amplification (linker, tyramide, etc.) Cross-platform studies have not yet been performed.

Evaluation of Staining

ROS1 overexpression in *ROS1*-rearranged lung adenocarcinomas is typically cytoplasmic, but the actual pattern varies considerably among individual tumors. *ROS1* fuses with a variety of different partners to promote lung tumorigenesis, and evidence suggests that

Table 1. Staining Conditions for ROS1 IHC with Use of Clone D4D6

Study	Antigen Retrieval	Dilution	Incubation	Detection System	Staining Platform
Rimkunas et al., 2012	EDTA, pH 8.0	0.19 ug/mL	Overnight	EnVision+	Dako
Sholl et al., 2014	EDTA, pH 8.0, 125°C, 30 sec	1:1000[a]	1 hr	SignalStain Boost	Leica Novolink
Mescam-Mancini et al., 2014	CC1 solution, 1 hr	1:50	2 hr at 20°C	UltraView	Ventana BenchMark
Cha et al., 2014	CC1 solution, 100°C, 64 min	1:50	32 min at 37°C	OptiView	Ventana BenchMark
Warth et al., 2014	pH 8.0	1:100		Dako Autostainer	Dako
Yoshida et al., 2014	Target Retrieval Solution, pH 9.0	1:100	Overnight at 4°C	EnVision Flex+	Dako
Lee et al., 2015	Not described	1:50	1 hr at room temperature	EnVision+	Dako
Shan et al., 2015b	EDTA, pH 9.0, in steam cooker, 1.5 min	1:40	1 hr	Not described	Not described
Rogers et al., 2015	CC1 solution, 64 min	1:50	32 min at 37°C	OptiView	Ventana BenchMark
Boyle et al., 2015	CC1 solution, 60 min	1:100, 1:250	1 hr at 37°C	UltraView	Ventana BenchMark

[a]*Using concentrated form of the antibody before release of commercial product.*

some fusion partners are associated with unique patterns of ROS1 protein overexpression. *CD74-ROS1* is a commonly reported fusion in lung cancer and has been associated with ROS1 expression that has a granular cytoplasmic pattern with focal or diffuse intensely stained globular aggregates of protein (Figure 1A) (Yoshida 2014). *EZR-ROS1* fusions are also common and appear to correlate with weak cytoplasmic expression with membranous accentuation (Figure 1B) (Yoshida 2014, Boyle 2015). Other fusions, *SLC34A2-ROS1* and *SDC4-ROS1,* have been reported to show solid cytoplasmic ROS1 staining (Figure 1C). These correlations; however, are based on a small number of observations, as most studies correlate the results of ROS1 IHC with those of break-apart FISH alone, an approach that does not identify the fusion partner. On the basis of currently available, albeit limited, data, the precise fusion does not appear to influence the response to crizotinib (Shaw 2014); however, the spectrum of protein overexpression that signals the presence of an underlying oncogenic *ROS1* rearrangement should be recognized.

Similar to the situation with ALK IHC, *ROS1*-rearranged tumors are almost always diffusely positive (Figure 1D), typically in a homogeneous manner, but with staining intensity that ranges from weak to strong. Some degree of heterogeneity may be seen within a tumor, possibly as a result of variable fixation in larger resection specimens. In contrast, false-positive ROS1 expression is most often focal or patchy (discussed in detail in the next section).

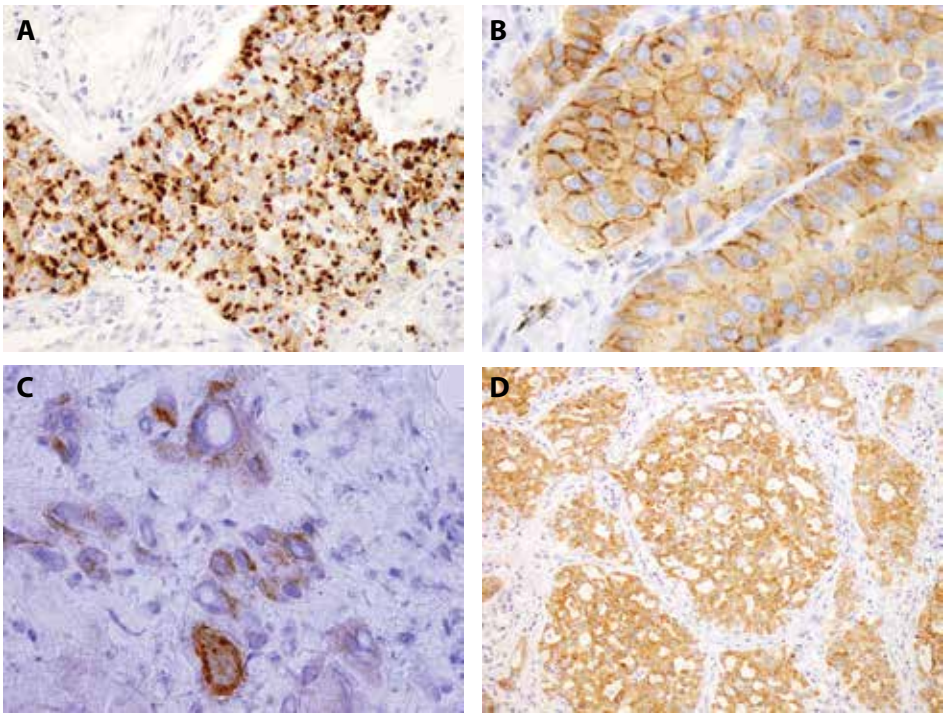

Figure 1. Patterns of expression of ROS1 fusion proteins in lung cancer are diverse and appear to correlate with the identity of the fusion partner. *CD74-ROS1* fusion correlates with prominent cytoplasmic globules (A), whereas *EZR1-ROS1* fusion may show diffuse cytoplasmic expression with membranous accentuation (B). *SLC34A2-ROS1* (C) and *SDC4-ROS1* fusions are relatively rare but appear to correlate with diffuse, finely granular cytoplasmic expression of ROS1. In nearly all cases containing confirmed ROS1 fusions, protein expression is homogenously expressed across the tumor cells (D), albeit to varying degrees of intensity in different tumors.

Challenges of ROS1 IHC

ROS1 IHC differs from ALK IHC in that ALK expression is virtually specific to tumors with *ALK* gene rearrangements, whereas the levels of ROS1 mRNA and protein may be expressed at typically low and very occasionally prominent levels in tumors lacking *ROS1* fusions. ROS1 mRNA expression has been reported in *ALK*-rearranged and *EGFR*-mutated tumors, and ROS1 protein overexpression has been reported in *ERBB2*-mutated lung tumors (Li 2011, Acquaviva 2009, Mescam-Mancini 2014). As a result, apparently nonspecific ROS1 protein expression may be seen in *ROS1*-negative tumors. Although nonspecific expression typically presents in a patchy, weak pattern (Figure 2A), in some instances it may appear widespread or strong, or both. In one study, ROS1 expression was present in 80% of invasive mucinous adenocarcinomas that tested negatively for *ROS1* rearrangement (Figure 2B) (Yoshida 2014). ROS1 immunoreactivity in this tumor subtype should therefore be interpreted with caution. ROS1 expression may also be seen in reactive pneumocytes, macrophages, and giant cells in the lung (Figures 3A, 3B) (Sholl 2013a). In some cases, the ROS1 expression level in benign pneumocyte proliferation can be strong, and care must be taken not to misinterpret this level as representing ROS1 positivity in tumor cells.

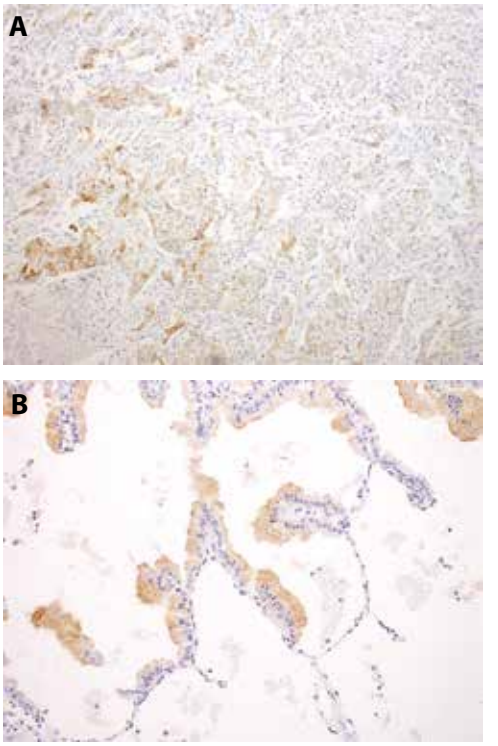

Figure 2. Nonspecific ROS1 protein expression is typically weak and patchy; when more intense nonspecific expression is seen, it is usually heterogeneous (A). Some authors have reported diffuse staining in ROS1 fusion-negative invasive mucinous adenocarcinomas (B). The intensity of this nonspecific staining will be driven in part by the sensitivity of the detection system used.

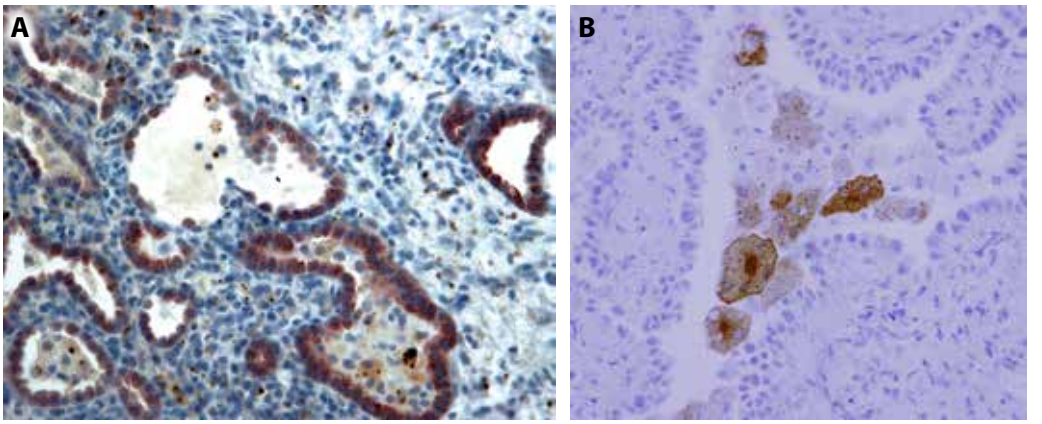

Figure 3. ROS1 protein expression may be seen in reactive type II pneumocytes (A) and giant cells (B).

Practical Implementation

Laboratories should run a paired positive control to ensure that the assay is performing as expected. Although internal positive-control staining can be found in background non-neoplastic lung tissue, small biopsy specimens or cytology specimens may lack the cellular components that typically have ROS1 staining (eg, reactive pneumocytes, giant cells). Endogenous ROS1 expression is reported in a variety of normal tissues, including that of the adrenal gland, kidney, stomach, small bowel, colon, peripheral nerve, skeletal muscle, and cerebellum. A paraffin-embedded cell block of the *ROS1*-rearranged cell line HCC-78 can be used in daily practice as an external positive control. However, when a screening assay is implemented, use of a positive control that expresses the target at a low level will help to confirm sensitivity. The commercially available glioblastoma cell line U-118 MG contains a *FIG (GOPC)-ROS1* fusion and expresses ROS1 at a low level by Western blot and IHC (Rimkunas 2012).

The findings of most published studies demonstrate that ROS1 IHC is nearly 100% sensitive for *ROS1* rearrangement as detected by other methods, with a specificity ranging from 70% to 98% (Yoshida 2014, Sholl 2013a, Mescam-Mancini 2014, Cha 2014). The rates of ROS1 IHC positive results range from 5% to 35% across studies (Table 2) (Cha 2014, Warth 2014, Mescam-Mancini 2014, Yoshida 2014). This variability is likely multifactorial, driven in part by detection methodology, selection bias in the tested cohort, and different thresholds for a weak or focal positive result. The selected detection system, in particular, a platform that involves a robust signal amplification system, may substantially increase the rate of false-positive results. At the same time, a *ROS1*-rearranged cancer may have modest, albeit diffuse, protein expression, which may be missed if a sufficiently sensitive method is not used (Yoshida 2014); however, such cases are rare. In one study, the rate of ROS1 positivity on IHC ranged from 10% in a retrospective cohort to 31% in a prospective cohort (Cha 2014). The authors of that study noted that ROS1 expression was more common in tumors from smokers, a population more highly represented in the prospective cohort. Although demographic factors may influence the false-positive rate, more liberal criteria may be used in prospective clinical testing to maximize the sensitivity of the assay. In contrast, the percentage of true-positive cases, based on comparison with FISH, has

Table 2. Rates of ROS1 IHC Positivity[a]

Study	No. of Specimens	IHC+, FISH-	IHC+, FISH+	% Positive IHC Overall	% True-Positive IHC
Mescam-Mancini et al., 2014	121	3	9	10	7
Cha et al., 2014 (retrospective cohort)	219	14	8	10	4
Cha et al., 2014 (prospective cohort)	111	29	5	31	5
Warth et al., 2014	1,478	59	9	5	1
Yoshida et al., 2014	270	78	17	35	6
Total	2,199	183	48	11	2

[a]*Studies are restricted to those in which confirmatory fluorescent in situ hybridization (FISH) was performed on all specimens that tested positively on immunohistochemistry (IHC). Percentages are rounded to the nearest whole number.*

ranged from 0.6 to 7.4. Studies in which the rate of true-positive results was high included cohorts that had been enriched for tumors containing *ROS1* fusions.

Overall, the data published to date suggest that, on average, five ROS1 IHC-positive cases will require orthogonal testing, such as FISH, RT-PCR, or NGS, to identify one true-positive finding of *ROS1* rearrangement. Experienced pathologists; however, will recognize that the patterns of ROS1 expression in tumors harboring a *ROS1* rearrangement are usually distinctive and readily distinguished from false-positive staining. Although individual laboratories may choose to perform confirmatory assays on only those samples with diffuse and homogeneous protein expression, initial confirmatory analysis on all IHC samples is recommended to gain experience on the rate of false-positive results. In France, a national pathology expert panel recommends that ROS1 IHC be followed by confirmatory FISH (Mescam-Mancini 2014, Mazières 2015). The results of some studies have suggested that application of H-score cutoffs of 100 or 150 (Boyle 2015, Yoshida 2014) can maximize the sensitivity and specificity of ROS1 IHC. Reported discrepancies between FISH and IHC may also reflect false-negative or false-positive results by FISH (Yoshida 2014), although the contribution of such a factor should be minor, as the level of interpretative challenge for ROS1 FISH differs from that for ALK FISH assays. In particular, the *FIG-ROS1* rearrangement may be missed by some of the commercially available probes (*see Chapter 7 for details*). Use of additional molecular techniques, such as NGS, should be considered for cases with unexpectedly discordant IHC and FISH results.

Conclusion

ROS1 IHC using the D4D6 antibody is a robust screening tool for detection of *ROS1*-rearranged lung tumors because of its excellent sensitivity, rapid turnaround time, and low cost relative to FISH-based methods. ROS1 IHC screening is becoming a routine component of testing on lung adenocarcinomas across institutions. ROS1 protein expression may be seen in tumors without *ROS1* rearrangement, mostly in a focal heterogeneous pattern. Because of this potential discrepancy, establishing optimal staining conditions and interpretative cutoffs are crucial for clinical application, and orthogonal techniques are necessary to confirm the presence of a rearrangement.

ALK Testing with FISH

By Akihiko Yoshida, Lukas Bubendorf, and Marileila Varella-Garcia

6

FISH with a break-apart probe set was originally developed for detecting gene fusions created by interchromosomal translocations. Break-apart FISH is a reliable diagnostic method in surgical pathology because it is easily applicable to FFPE specimens even when the exact fusion partners are not known. FISH with break-apart probes for *ALK* has been successfully incorporated into diagnostic practice for lymphomas and mesenchymal tumors, and the discovery of *ALK* rearrangement in a rare subset of NSCLCs broadened the application (Soda 2007). However, in the latter setting, FISH has been associated with higher level of interpretational challenges, primarily because the fusion typically occurs between *ALK* (2p23.2) and the closely situated gene *EML4* (2p21) through intrachromosomal inversions; only rarely is *ALK* fused with other genes through interchromosomal translocations. Thus, break-apart FISH for the diagnosis of lung cancers with *ALK* rearrangement must be performed with close attention to technical details and interpretational guidelines.

FISH Probe Design

The ALK break-apart probe is typically designed by labeling the 3' (telomeric) part of the fusion breakpoint with one fluorochrome and the 5' (centromeric) part with another fluorochrome. Some variation exists among different commercial and custom-made reagents as to the specific genomic areas covered by the probes and the distinct fluorochromes used for labeling. In the kit developed by Abbott Molecular (Figure 1; Vysis LSI ALK Break Apart FISH Probe Kit), the 3' part (approximately 300 kb) is represented by an orange signal (SpectrumOrange, often referred to as red), and the 5' part (approximately 442 kb) is represented by a green signal (SpectrumGreen). This probe set was approved as a companion diagnostic assay by the US FDA for an ALK inhibitor and is commonly used worldwide.

Preanalytic Requirements

As a DNA-based assay, FISH has an important advantage in its robustness. However, the assay is affected by many factors, especially time to fixation, time of fixation, and type of fixative,

all of which can lead to DNA degradation (Table 1) (Hunt 2007, Babic 2010). For example, long cold ischemia, or more than 1 hour from the time the tissue is excised to when it is placed in fixation, may result in DNA degradation and failure of FISH testing (Khoury 2012). The ideal fixation time is considered to be between 6 and 48 hours (Hunt 2007, Babic 2010); both shorter and longer fixation times may influence test performance significantly.

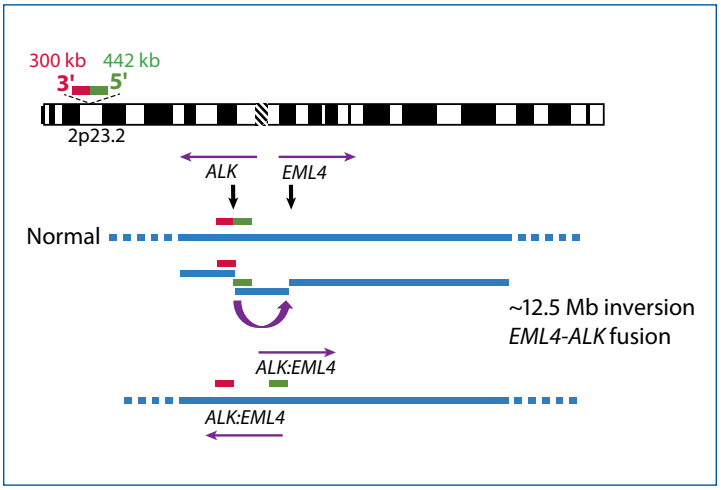

Figure 1. The Vysis LSI ALK Break Apart FISH Probe Kit (Abbott Molecular) used to test for the presence of *EML4-ALK* fusion gene (*ALK* rearrangement) in lung cancers.

The optimal fixative is 10% neutral buffered formalin; Prefer and Bouin's fixative will prevent hybridization. Tissues decalcified by strong acid solutions almost certainly fail to hybridize; mild decalcification with EDTA or formic acid generally does not substantially impair the test performance. Decalcification is particularly relevant when a metastastic bone lesion is the only sample available for molecular analysis; in such cases, the degree of bone decalcification can be inferred from the appearance on tissue stained with hematoxylin and eosin (H & E). This appearance may guide the decision of whether to submit the specimens for FISH testing (Rumery 2016).

Another relevant factor in the success of the assay is the age and storage conditions of the slide. Sectioned tissue on slides that are archived for a prolonged period at room temperature tend not to be successful in standard FISH assays, and customized protocols are required. Therefore, tissue in an embedded block is the ideal storage format. The acceptable storage

Table 1. Preanalytic Recommendations for Successful FISH

Parameter	Recommendation
Time to fixation	As short as possible, not exceeding 1 hr
Fixative	10% neutral buffered formalin
Time of fixation	6-48 hr
Preparation	Paraffin-embedded sections, cut at a thickness of 5 ±1 μm
Specimen storage	Tissue blocks (ideal)
Storage time for blocks	Not relevant if in proper conditions
Storage conditions for blocks	Protected from light, heat, and humidity
Storage time for cut sections	4-6 weeks (ideal); older slides require customized protocol
Decalcification	EDTA, if necessary

time for paraffin blocks is a function of the storage condition (eg, temperature, exposure to light, heat, humidity), and materials subjected to DNA degrading conditions may fail to hybridize.

Numerous variables before and after hybridization can also affect testing. The prehybridization procedure includes a series of steps to facilitate probe penetration into the nucleus of the tumor cells. Tissue permeabilization is achieved by digestion of large protein structures, but not all specimens respond identically to a given protocol. Tumors with poor differentiation are more sensitive to prehybridization procedures, whereas fibrotic and mucinous tumors are more resistant. Post-hybridization washes must allow adequate elimination of unbound probe without decreasing signal intensity. Many alternative protocols may generate excellent results; thus, laboratories may choose any of these protocols, as long as conditions are properly adjusted to the characteristics of the specimen.

Quality Assessment of the Hybridized Specimen and Selection of Scorable Cells

It is essential that the quality of tissue morphology and signal intensity be rigorously assessed before a specimen is accepted for analysis. Specimens are optimal for analyses when they exhibit excellent morphology and signal intensity with very low background noise (Figures 2 and 3). Specimens with evidence of chromatin overdigestion or poor probe penetration are not acceptable and must be retested after troubleshooting technical conditions. For example, specimens are not acceptable when the pretreatment of tissue is insufficient or excessive (Figure 4) or when technical sectioning artifacts that generate overlapped nuclei with stringy signals make it impossible to measure the separation between red and green (Figure 5).

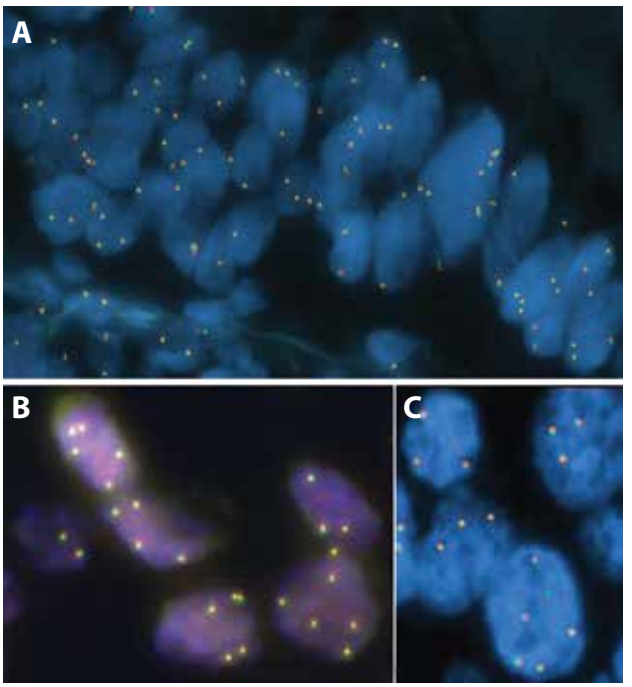

Figure 2. Microscopic fields of *ALK*-nonrearranged lung cancers, showing predominantly fused signal pattern.

In general, *ALK* rearrangement is evenly distributed within the tumor, reflecting its critical oncogenic role (Camidge 2010). Therefore, it is not necessary to select a specific tumor area based on morphology or immunoprofile. Scoring must be done on the well-preserved nonoverlapping tumor cells that have at least one copy each of the 5' and 3' signals. Because lung cancers tend to assume a wide range of growth patterns, and because tumor cells may closely intermingle with non-neoplastic tissue elements (eg, alveolar macrophages and lymphocytes), the accurate identification of tumor cells may be difficult in a dark field. It is advisable to always refer to a serially cut, H & E-stained tissue slide for appropriate morphologic adjustment.

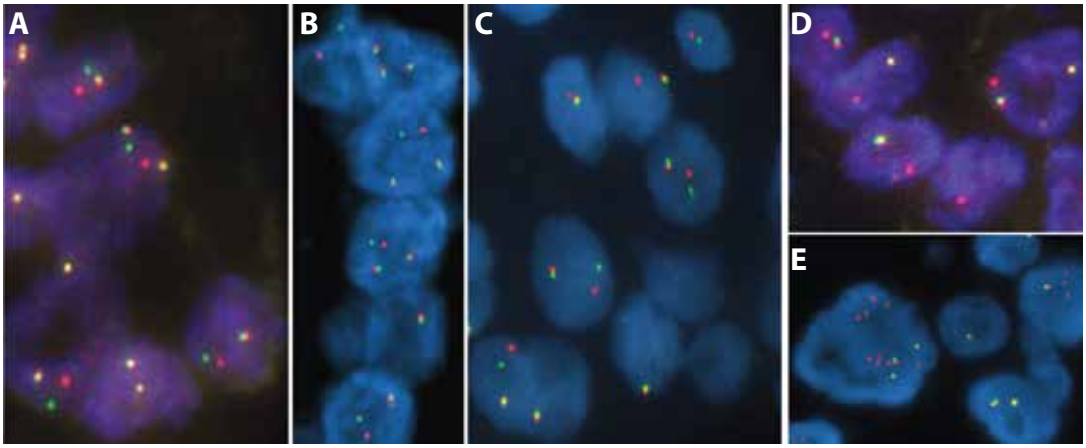

Figure 3. Microscopic fields of *ALK*-rearranged lung cancers, showing predominantly the split 3′-5′ pattern (3A-3C) and the isolated 3′ pattern (3D, 3E).

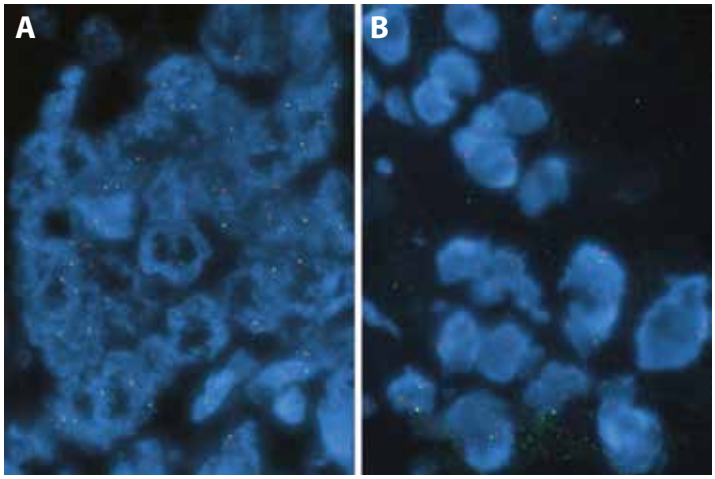

Figure 4. Specimens unacceptable for analyses because of tissue overdigestion (4A) or tissue underdigestion (4B).

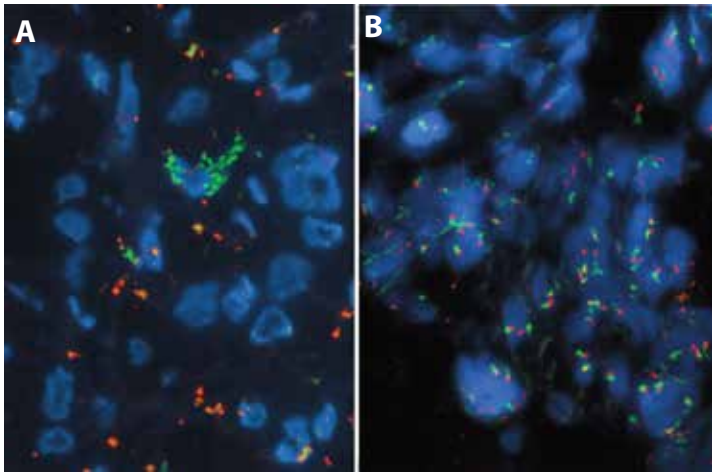

Figure 5. Specimens unacceptable for analyses because of high background noise (5A) or stringy signals (5B).

Cell Classification: Signal Patterns

In concept, the genomic areas homologous to the 5' and 3' probes are molecularly very close and these signals are seen as fused, touching, or adjacent in normal cells. In contrast, when the *EML4*-ALK fusion gene is present, the 5' *ALK* green signal becomes far removed from the 3' *ALK* red signal (by approximately 12.5 Mb), and the signals are seen as being split. In reality, however, the 3' and 5' signals may be seen as far apart from or as close to each other in normal host cells because of various degrees of condensation and three-dimensional arrangement of the chromatin. Similarly, because of the proximity of *EML4* and *ALK*, the split can be so narrow that the signals may seem fused in *ALK*-rearranged tumor cells. Furthermore, this genomic region seems to be highly unstable, and the homologous regions to one of the probes can be lost, with the corresponding signal being missing. As a result, each tumor cell may display a variety of combinations of co-localized 5'-3' *ALK* signals and isolated 5' or 3' *ALK* signals.

Despite this diversity in signal profile, cells can be classified into one of the following four patterns based on each signal number and location.

Fused pattern (Figure 6A). A cell is interpreted as having a fused pattern when the 5' and 3' signals are fused (Figure 2). Any separation of 5' and 3' signals by a distance of less than two signal diameters should be classified as fused. The number of fused 5'-3' signals per tumor nucleus is not relevant for pattern classification.

Split pattern (Figure 6B). A cell is interpreted as having a split pattern when the 5' and 3' signals are separated, regardless of the number of actual isolated signals (Figure 3). The separation between the 5' and 3' signals must be two or more times the diameter of the largest signal (Camidge 2010). The number of isolated 5' and 3' signals does not need to be equal; for example, a cell with two copies of isolated 5' signal and three copies of isolated 3' signals is classified as split. The number of accompanying fused 5'-3' signals in the cell is not relevant for pattern classification.

Isolated 3' pattern (Figure 6C). A cell is interpreted as having an isolated 3' pattern when isolated 3' signals are present with no isolated 5' signals. When a cell has both isolated 3' and 5' signals, with more 3' signals than 5' signals, the pattern

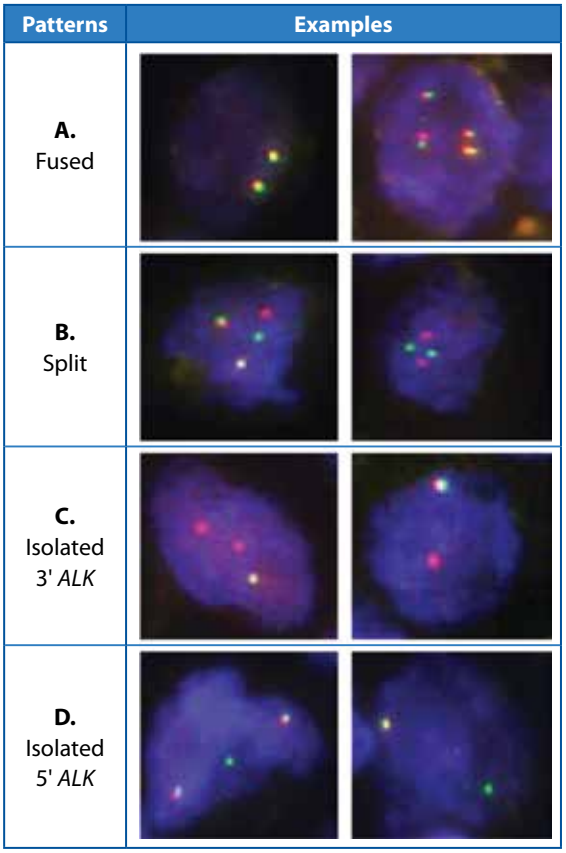

Figure 6. Tumor cell classification based on the *ALK* signal pattern on FISH.

is classified as the split pattern, not isolated 3'. The number of accompanying fused signals is not relevant for pattern classification.

Isolated 5' pattern (Figure 6D). A cell is interpreted as having an isolated 5' pattern when isolated 5' signals are present with no isolated 3' signals. When a cell has both isolated 3' and 5' signals, with more 5' signals than 3' signals, the pattern is classified as the split pattern, not isolated 5'. The number of accompanying fused signals is not relevant for pattern classification.

Note: The criteria for the split pattern are primarily based on testing of FFPE tumor sections with the Vysis LSI ALK Break Apart FISH Probe Kit (Ventana), and the criteria should be validated when a different analytic reagent or biologic specimen is used. The probe size may differ among probe designs, and a larger probe size results in both a larger signal size and a shorter distance required for the definition of a split.

Scoring

A minimum of 50 tumor cells is needed when there is one scorer and a minimum of 100 tumor cells is needed when there are two scorers. (*See more information in the "Specimen Classification" section.*) Specimens with fewer assessable cells are not suitable for FISH analysis (Camidge 2010). The signal pattern for each tumor cell should be recorded on a scoring worksheet (Figure 7). Scoring may be more accurate when it is done while viewing the tissue under a microscope with single (red and green) and dual interference filters. When using image-based scoring, the image must represent all section depths in order to avoid false interpretation of isolated signals (Figure 8).

Cell	Fused signal	5' signal	3' signal	Pattern
Cell 1	2	0	0	Fused
Cell 2	2	1	1	Split
Cell 3	2	0	1	Isolated 3'
Cell 4	1	1	0	Isolated 5'
Cell 5	0	1	2	Split
Cell 6	1	0	0	Fused
Cell 50	2	0	0	Fused

Summary of Scoring:
Total # of cells scored: 50
Total # of cells with fused pattern: 19
Total # of cells with split pattern: 22
Total # of cells with isolated 3' pattern: 5
Total # of cells with isolated 5' pattern: 4
Total # of cells with rearrangement-positive patterns: 22+5=27
Rearrangement-positive cell rate: 27÷50x100=54%

Figure 7. An example of a worksheet for scoring cells.

Copy number gain of native *ALK* is common in NSCLC (Figure 9) and there is no indication that it is associated with protein overexpression. At this time, we do not recommend that *ALK* copy number be routinely included as part of scoring.

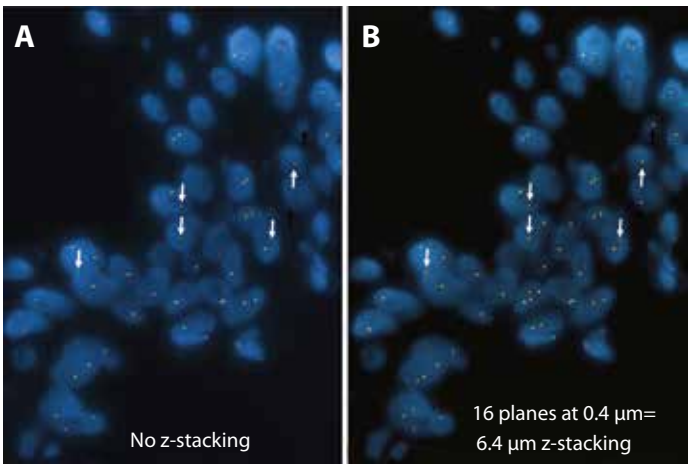

Figure 8. Image-based analysis requires attention to z-stacking (consolidation of multiple focus levels into one plane). Isolated signals (8A) become fused signals (8B) with z-stacking.

Note: The signal size in captured

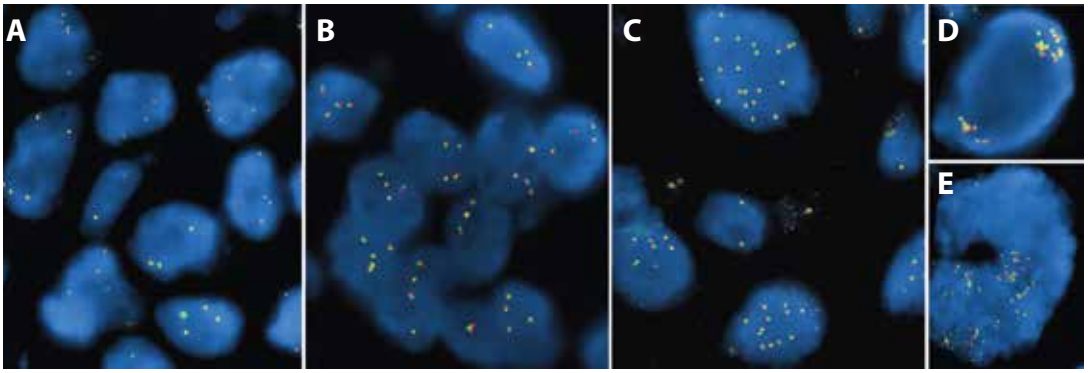

Figure 9. Copy number gain of native *ALK* signals is commonly observed in lung cancer specimens, with levels ranging from low (9A) to very high (9C). A cluster of numerous copies suggests gene amplification (9D, 9E). Copy number gain should not be interpreted as rearrangement.

images tends to be slightly larger than that seen on actual examination under fluorescent microscopy. When scoring is done on captured images, the distance between signals may be underestimated, which may compromise the results of analysis. Another pitfall of using images for scoring is that image capturing often consolidates multiple focus levels into one plane, and as a result, a vertically split signal along the z-axis of the tissue plane may be indistinguishable from a fused signal.

Calculation of Rearrangement-Positive Cell Rate

The rate of rearrangement-positive cells is defined as follows:

> Rearrangement-positive cell rate (%) = [(number of cells with split pattern + number of cells with isolated 3' pattern) /Total number of cells evaluated] × 100

Note: Because the kinase domain of *ALK* tyrosine kinase is encoded by the 3' part of the gene, it is the unpaired 3' signal that indicates the oncologically relevant fusion gene, whereas the unpaired 5' signal represents a likely nonfunctional reciprocal fusion product. Therefore, cells with an isolated 3' pattern are categorized as rearrangement-positive cells along with those with a split pattern, while, as a rule, cells with an isolated 5' pattern should not be interpreted as rearrangement-positive cells. (*See "Atypical Signal Profile" later for cautionary statement regarding the latter rule.*) Dismissing the isolated 3' pattern and limiting the definition of rearrangement to the split pattern reduces the sensitivity of the ALK FISH assay to 60% to 70% (Yoshida 2011a, Paik 2011).

Cutoff Value

Because the *EML4-ALK* fusion gene is typically created by a small intrachromosomal inversion involving two genes located in close proximity, the distance between the split signals representing an *ALK* rearrangement is typically narrow when the break-apart FISH assay is used. Because of the degree of chromatin condensation in the cells or its physical distribution, narrow splits are sometimes technically indistinguishable from fused signals, which can cause the rate of rearrangement-positive cells in *ALK*-rearranged NSCLCs to be low (40% to 70%) (Perner 2008, Camidge 2010, Camidge 2012b). In addition, NSCLCs without *ALK* rearrangement may have rearrangement-positive patterns (ie, split pattern or isolated

3' pattern) in a fraction of cells (Perner 2008, Camidge 2010, Yoshida 2011a), likely because of truncation artifact or perhaps a stochastic genomic alteration that does not indicate a specific fusion gene. As a result, the distribution of rearrangement-positive cell rates in NSCLC is continuous (Martin 2015) rather than separated into two discrete groups, and setting a cutoff value to discriminate between *ALK*-rearranged and *ALK*-wild-type NSCLCs is based on the accumulated experience correlating FISH results with other fusion detection methods (such as RT-PCR). A practical cutoff value of 15% has been established to allow for the best separation between *ALK*-rearranged (ALK-positive) and *ALK*-wild-type (ALK-negative) NSCLCs (Camidge 2010, Kwak 2010, Yoshida 2011a).

Note: The 15% cutoff is primarily based on testing with the Vysis LSI ALK Break Apart FISH Probe Kit and should be validated when a different reagent is used.

Specimen Classification

When the rearrangement-positive cell rate is 15% or more, the specimen is interpreted as positive for *ALK* gene rearrangement; when the rate is less than 15%, the specimen is interpreted as negative for *ALK* gene rearrangement. In order to minimize technical bias, the Vysis LSI ALK Break Apart FISH Probe Kit recommends a two-step assessment strategy with two independent scorers (Figure 10). The first scorer scores 50 tumor cells. A rate of rearrangement-positive cells less than 10% (ie, rearrangement in fewer than five of the 50 cells) is considered negative; a rate greater than 50% (ie, more than 25 of 50 cells) is considered positive; and a rate of 10% to 50% (ie, 5 to 25 of 50 cells) is considered equivocal. In the latter scenario, a second independent scorer scores an additional 50 tumor cells, and a final rate of rearrangement-positive cells is calculated on the sum of the first and second scores. The specimen is then classified based on the final rate in relation to the cutoff of 15%.

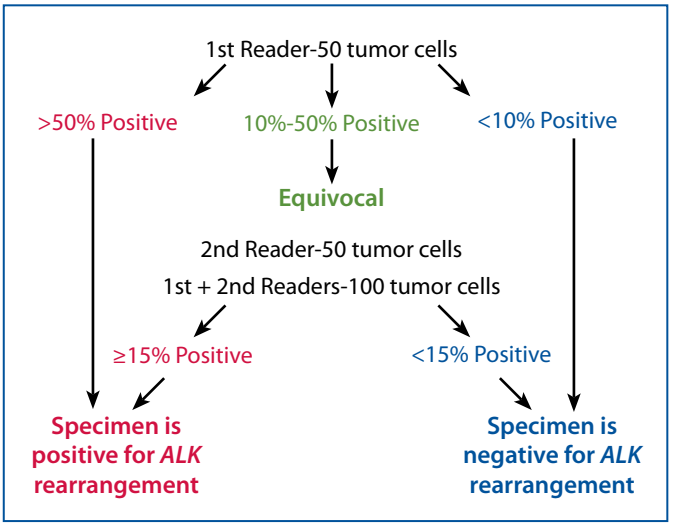

Figure 10. The scoring algorithm recommended by Vysis LSI ALK Break Apart FISH Probe Kit (Abbott Molecular).

Laboratory Validation

The ALK FISH assay should be properly validated in the laboratory before testing is offered in a clinical setting (Halling 2012, Saxe 2012). The accuracy of the results—that is, the degree to which the assay discriminates between negative and positive—should be compared with the accuracy at another laboratory where the validated assay is being performed properly and/or compared with the accuracy for a previously validated method in the same laboratory. The precision or reproducibility of results should be verified according to the degree of agreement between measurements conducted on the same specimen by different technologists

and/or at different times, and the entire analytic process should be verified. Verification of accuracy and precision should be repeated periodically. Moreover, the analytic sensitivity and specificity of the assay should be verified in specimens with known genotype. *ALK*-rearranged NSCLC cell lines (such as NCI-H3122 and NCI-H2228) can be used as positive controls (Koivunen 2008). Experience on a large number of *ALK*-wild-type NSCLCs and benign tissues will help gain an understanding of the appearance of nonsignificant split between signals (less than two signal diameters). These tasks are simpler when commercial probes are used and require a higher level of attention to details when laboratory-developed reagents are used.

Challenges

ALK break-apart FISH has been associated with four primary challenges that may increase the risk of test errors: interpretation, borderline rates of rearrangement-positive cells, erroneous results for biologic reasons, and atypical signal profile.

Interpretation

The most common source of ALK FISH error is inaccurate signal interpretation (Sholl 2013b, Minca 2013, Cutz 2014). The inaccuracy may result from dismissing narrow splits inherent to *EML4-ALK* as fused, overcalling noise split signals in *ALK*-wild-type cases, and/or mistaking nontumor cells as neoplastic elements in a dark field. Strict adherence to the enumeration rules and careful correlation with morphology on H & E-stained tissue should improve the performance. Participation in external quality assurance programs such as proficiency testing surveys is recommended to maintain the test quality (Cutz 2014, Marchetti 2014).

Borderline Rates of Rearrangement-Positive Cells

In approximately 5% to 10% of NSCLCs, the rate of rearrangement-positive cells falls within the range of 10% to 20% (Camidge 2013, Ilie 2015, Selinger 2015, Von Laffert 2015). Although the currently accepted cutoff of 15% could technically classify such cases as either positive or negative for *ALK* rearrangement, studies have shown that such equivocal counts represent one of the major sources of discrepancy between FISH and other modalities (Ilie 2015, Selinger 2015, Von Laffert 2015). For a borderline count, we recommend that cells be carefully counted again, with particular attention paid to the morphologic differentiation between tumor and nontumor cells. Including nontumor cells in the count dilutes the rate of rearrangement-positive cells. Similar attention should be paid to the vertically split signals along the z-axis of the tissue plane, which could be mistaken as a fused signal. This latter pitfall is particularly relevant in a laboratory in which the evaluation is performed on the captured digital images that consolidate multiple focus levels to produce one image (z-stacking). These borderline cases may also harbor atypical signal profiles, as described later (Yatabe 2015). Specifically, a red-doublet pattern may initially stand out as a borderline rate of rearrangement-positive cells. Analysis using a single-color filter may facilitate the identification of closely apposed signals that may be overlooked by a dual-color (red and green) filter. If the rate of rearrangement-positive cells is still borderline on careful reassessment, the report could be issued based on the 15% cutoff. However, an additional note may be necessary that recommends correlation with other diagnostic modalities, such as

IHC, RT-PCR, or NGS, or even ancillary FISH with different probe designs (Selinger 2015, Von Laffert 2015).

Erroneous Results for Biologic Reasons

Most discordance between FISH and other modalities (IHC or RT-PCR) that has been reported to date likely stemmed from imperfect performance of one of the latter modalities, or interpretational or borderline issues of FISH as described earlier. However, it has become clear that FISH may also generate true false-positive or false-negative results.

False-positive FISH results have been difficult to demonstrate, mainly because of the well-acknowledged limited sensitivity of RT-PCR and IHC. However, these errors will be increasingly detected by the emerging wide application of NGS, and to date, a few such cases have been reported (Jang 2016). One mechanism to explain false-positive FISH results is the *ALK* gene rearrangement that produces nonfunctional *ALK* fusion. The findings of one study suggested that tumors that predominantly harbor isolated 3' pattern may be more frequently associated with false-positivity determined by NGS (Gao 2015).

In contrast, a small but significant number of cases of false-negative FISH results has been well documented (Yoshida 2011a, Murakami 2012, Peled 2012, Ren 2014, Shan 2015a, Takeuchi 2016, Houang 2014, Roth 2014, To 2013 , Ying 2013, Selinger 2013). In such cases, atypical FISH signal patterns are observed in some examples (see later), whereas in others, FISH patterns are nonatypical. The genomic mechanisms underlying false-negative FISH results have not been fully clarified, but it is conceivable that complex gene rearrangements and cryptic insertions may be contributors (Takeuchi 2016, Ali 2016, Jang 2016).

Atypical Signal Profile

FISH may produce an atypical signal profile in rare instances (approximately 6% of cases) (Camidge 2013). At least some such patterns are known to be associated with false-negative results.

One example is when most of the tumor cells harbor an isolated 5' predominant pattern, with only a few cells having a split or an isolated 3' pattern (5' predominant pattern, Figure 11). By conventional enumeration rule, the isolated 5' pattern should be classified as ALK negative and these cases would be interpreted as negative for *ALK* rearrangement. Nevertheless, some of the cases with this signal pattern have been reported to carry an *EML4-ALK* fusion transcript when the results were confirmed by other modalities (Yoshida 2011a, Ren 2014, Takeuchi 2016),

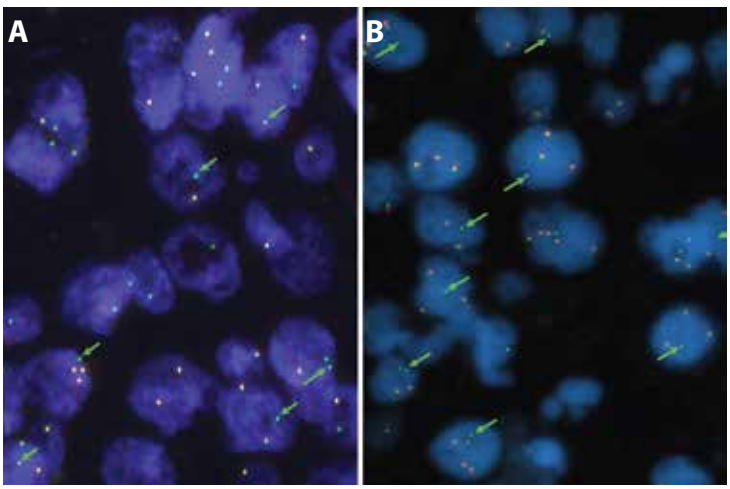

Figure 11. An atypical isolated 5′ predominant pattern observed in lung cancers tested by ALK break-apart FISH. Arrows indicate single green signals (5′ *ALK*).

and one such case harbored a rare *BIRC6-ALK* fusion (Shan 2015a). Some of these tumors responded well to crizotinib, highlighting the clinical value of recognizing this signal pattern (Ren 2014, Shan 2015a, Takeuchi 2016). However, the association between 5' predominant pattern and *ALK* fusion is not entirely consistent, because some NSCLCs with this FISH pattern lacked *ALK* fusions but harbored *KRAS* mutations (Gainor 2013b).

Another atypical FISH signal is a so-called red-doublet pattern, in which a pair of 3' signals fuses with a 5' signal (Figure 12A) (Peled 2012). ALK-positive NSCLCs with a red-doublet pattern may be misinterpreted as negative for *ALK* rearrangement because such a signal cluster may mimic a conventional fused signal. Sometimes, three or more copies of 3' signals may cluster and fuse with a 5' signal (red-triplet pattern, etc.; Figure 12B, 12C). In yet other rare instances, most of the tumor cells in ALK- positive NSCLCs may exhibit only isolated 3' signals without normal copies of *ALK* (Figure 12D). Cells with such a pattern are regarded as nonevaluable by conventional scoring rules because of the possibility of a hybridization failure of the 5' probe, and ALK-positive cancers with such a pattern may be overlooked.

Although it is not yet completely clear how consistently atypical signal patterns predict fusion status, these patterns should at least raise suspicion and prompt testing with other diagnostic modalities. Future studies may identify other atypical signal profiles that are associated with false-negative FISH results.

Emerging Issues

A few reports have suggested that the copy number of rearranged *ALK* may change, particularly after treatment with an ALK inhibitor (Doebele 2012; Kobayashi 2013). Although the exact incidence and mechanism of such change are not yet clear, repeat testing might be helpful when the tumor demonstrates acquired resistance following treatment with an ALK inhibitor, so that a new therapeutic regimen can be considered.

The findings of recent studies have suggested that *ALK* rearrangement may be heterogeneously distributed within tumor tissue (Abe 2015, Cai 2015, Zito Marino 2015). Although

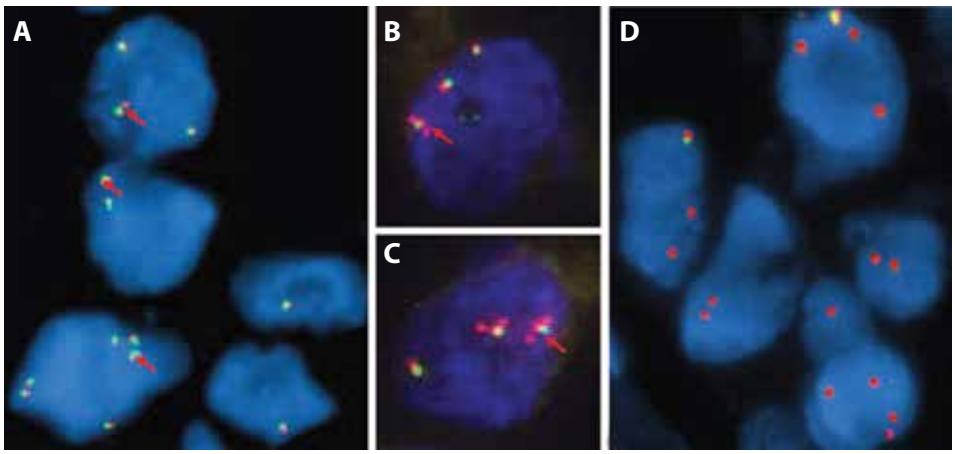

Figure 12. Atypical patterns seen in lung tumors with ALK break-apart FISH. 12A: 3′-5′-3′ (red- doublet pattern); 12B and 12C: 3′-3′-5′-3′ (red-triplet pattern); 12D: Isolated 3′ signals, mostly without normal *ALK* signals. Arrows indicate atypical signals.

these observations contrast with the conventional view that *ALK* fusion exists uniformly (Camidge 2010) and with the almost invariable findings of diffuse ALK IHC staining in *ALK*-rearranged NSCLCs, this issue is worth attention, as it may hold significant clinical implication.

The data are still immature in these areas and further studies are needed.

Conclusion

Break-apart FISH is a reliable technique for the diagnosis of *ALK*-rearranged NSCLCs and has been accepted as the criterion standard to select patients for treatment with an ALK inhibitor. However, FISH testing heavily depends on careful preparation and interpretation with strict adherence to guidelines. Furthermore, FISH may rarely produce equivocal or even erroneous results. As any other clinical test, ALK break-apart FISH has unique strengths and limitations and should be used within an appropriate diagnostic context. It is also strongly recommended that each laboratory perform internal validation studies using known controls before this method is introduced as a routine test. In addition, laboratories should participate in periodic slide exchange programs with other accredited clinical laboratories or in proficiency testing surveys provided by approved vendors.

ROS1 Testing with FISH

By Marileila Varella-Garcia and Akihiko Yoshida

7

Oncogenic activation of the *ROS1* gene in lung cancer by molecular fusion with an effector gene was first reported in lung adenocarcinoma by Rikova et al. and subsequently confirmed by other investigators (Rikova 2007). Patients with *ROS1*-rearranged lung tumors have had a good response to targeted therapy with tyrosine kinase inhibitors. Crizotinib was the first agent to successfully complete a multicenter, single-arm trial involving patients with metastatic ROS1-positive NSCLC, with an objective response rate of 72%, a median duration of response of 17.6 months, and a median progression-free survival of 19.2 months (Shaw 2014). Based on these results, crizotinib was approved by the US FDA on March 11, 2016. Other tyrosine kinase inhibitors, such as entrectinib, ceritinib, and PF-06463922, have been shown to be effective when tested clinically in ROS1-positive lung tumors and in preclinical models (Rolfo 2015, Subbiah 2016, Zou 2015).

The *ROS1* gene maps at 6q22.1 (genomic location: 117,288,300-117,425,855) and has a reverse-strand reading frame. The gene has been shown to be activated by multiple gene partners, 27 of which have been identified to date (Table 1). Among those partner genes, 22 are mapped in chromosomes other than 6 (chromosomes 1, 3, 4, 5, 7, 10, 12, 15, 17, 20 and X); thus, the molecular fusion between these 22 genes and *ROS1* occurs by some type of interchromosomal rearrangement, such as translocation or insertion. In contrast, five partner genes (*HLA-A*, *GOPC*, *CEP85L*, *TPD52L1*, and *EZR)* are located in chromosome 6, and expectedly, the molecular mechanisms generating the fusion are intrachromosomal rearrangements, such as deletions, duplications, and inversions. As discussed in Chapter 6, the break-apart FISH assay is a reliable method for diagnosis of gene fusions in interphase cells, covering the detection of multiple fusion partners and being effective even when partners are unknown. The ROS1 break-apart FISH assay was applied as confirmatory assay in the original description by Rikova et al. and has been frequently used since then, both in research and in clinical testing (Rikova 2007).

In NSCLC cohorts that are screened consecutively, *ROS1* rearrangements are present in approximately 1% to 2% of patients and are more common in tumors with adenocarcinoma histology compared with other histologic types (Table 2). Despite few studies in which

Table 1. Chromosomal and Genomic Location of ROS1 Fusion Partner Genes and Assessment of Specific Fusions by Fluorescent in Situ Hybridization (FISH)

Study	*ROS1* Fusion Partner	Tumor Type (at Discovery)	Chromosomal Location	Genomic Location	Reading Strand	Distance between *ROS1* and Partner Gene (BP)
Takeuchi et al., 2012	*TPM3*	Lung adenocarcinoma	1q21.3	154,155,304-154,194,648	Reverse	Interchromosomal
Lovly et al., 2014	*TFG*	Inflammatory myofibroblastic tumor	3q12.2	100,709,331-100,748,966	Forward	Interchromosomal
Rikova et al., 2007	*SLC34A2*	NSCLC	4p15.2	25,655,301-25,678,748	Forward	Interchromosomal
Rikova et al., 2007	*CD74*	NSCLC	5q13.2	150,401,637-150,412,929	Reverse	Interchromosomal
Wiesner et al., 2014	*PWWP2A*	Spitzoid neoplasia	5q33.3	160,061,801-160,119,423	Reverse	Interchromosomal
Wiesner et al., 2014	*HLA-A*	Spitzoid neoplasia	6p22.1	29,941,260-29,945,884	Forward	87,342,416
Charest et al., 2003	*GOPC (FIG)*[a]	Glioblastoma	6q22.1	117,560,269-117,602,542	Reverse	134,414
Giacomini et al., 2013	*CEP85L*[a]	Primary angiosarcoma	6q22.31	118,460,772-118,710,075	Reverse	1,034,917
Zhu et al., 2016	*TPD52L1*[b]	Lung adenosquamous	6q22.31	125,119,049-125,264,407	Forward	7,693,194
Takeuchi et al., 2012	*EZR*	Lung adenocarcinoma	6q25.3	158,765,741-158,819,412	Reverse	41,205,472
Ou et al., 2015	*TMEM106B*	Lung adenocarcinoma	7p21.3	12,211,241-12,243,367	Forward	Interchromosomal
Govindan et al., 2012	*KDELR2*	NSCLC	7p22.1	6,445,953-6,484,242	Reverse	Interchromosomal
Seo et al., 2012	*CCDC6*	Lung adenocarcinoma	10q21.2	59,788,763-59,906,656	Reverse	Interchromosomal
Crescenzo et al., 2015	*NFκB2*	Anaplastic large cell lymphoma	10q24.32	102,394,110-102,402,529	Forward	Interchromosomal
Wiesner et al., 2014	*KIAA1598*	Spitzoid neoplasia	10q25.3	116,883,377-117,126,586	Reverse	Interchromosomal
Wiesner et al., 2014	*PPFIBP1*	Spitzoid neoplasia	12p11.22	27,523,431-27,695,564	Forward	Interchromosomal
Wiesner et al., 2014	*ERC1*	Spitzoid neoplasia	12p13.33	990,509-1,495,933	Forward	Interchromosomal
Shaw et al., 2014	*LIMA1*	Lung adenocarcinoma	12q13.12	50,175,788-50,283,546	Reverse	Interchromosomal
Takeuchi et al. 2012	*LRIG3*	Lung adenocarcinoma	12q14.1	58,872,149-58,920,522	Reverse	Interchromosomal
Wiesner et al., 2014	*CLIP1*	Spitzoid neoplasia	12q24.31	122,271,432-122,422,632	Reverse	Interchromosomal
Wiesner et al., 2014	*ZCCHC8*	Spitzoid neoplasia	12q24.31	122,471,600-122,501,073	Reverse	Interchromosomal
Crescenzo et al., 2015	*NCOR2*	Anaplastic large cell lymphoma	12q24.31	124,324,415-124,567,589	Reverse	Interchromosomal
Wiesner et al., 2014	*MYO5A*	Spitzoid neoplasia	15q21.2	52,307,283-52,529,050	Reverse	Interchromosomal

Table 1 continued on next page

Lovly et al., 2014	YWHAE	Inflammatory myofibroblastic tumor	17p13.3	1,344,272-1,400,378	Reverse	Interchromosomal
Cancer Genomic Atlas Res Network, 2014	CLTC	Lung adenocarcinoma	17q23.1	59,619,689-59,696,956	Forward	Interchromosomal
Takeuchi et al., 2012	SDC4	Lung adenocarcinoma	20q13.12	45,325,288-45,348,424	Reverse	Interchromosomal
Shaw et al., 2014	MSN	Lung adenocarcinoma	Xq12	65,588,377-65,741,931	Forward	Interchromosomal
	ROS1		6q22.1	117,288,300-117,425,855	Reverse	

[a]*The fusion partner may be detected by commercially available break-apart FISH if genomic deletion involving the 5′ probe occurs.*
[b]*Detection of the fusion partner by commercially available break-apart FISH is challenging; if the fusion is generated by inversion, the 3′ and 5′ signals would commonly be split at a borderline interval.*

concurrent activating mutations in other dominant oncogenes, such as *EGFR*, *KRAS*, *BRAF*, and *MET*, were found (Ju 2016, Scheffler 2015), *ROS1* rearrangement generally occurs as a single major molecular driver. Therefore, the prevalence of ROS1-positive tumors increases when the patient cohorts are enriched or preselected for pan-negative NSCLC (Table 2).

The overall patient and disease characteristics of ROS1-positive NSCLCs are similar to those of ALK-positive NSCLCs. Thus, ROS1-positive NSCLC typically occurs in younger people with no or light smoking history. Histologically, most are adenocarcinomas that immunohistochemically express TTF-1.

Probe Design and Assay Requirements

Studies have involved the use of multiple types of FISH with break-apart probe sets for *ROS1* that include laboratory-developed and commercial analytes. Focusing on only commercial reagents, ROS1 break-apart probe sets typically comprise two individual analytes: one that recognizes the genomic sequences around the 5' (telomeric) part of the breakpoint and one that recognizes genomic sequences around the 3' (centromeric) part of the fusion breakpoint. Some variation exists among reagents from different manufacturers as to the specific genomic areas covered by the probes (Table 3). A common design is that the 3' end probe is labeled in green and encompasses much larger genomic areas than the 5' end probe, which is labeled in orange or red. The difference in design may have an effect on the ability to detect specific rearrangements, as will be discussed.

The preanalytic requirements for the ROS1 break-apart FISH assay, the strategies for quality assessment of the hybridized specimens, and the criteria for selection of tumor cells to be scored are similar to those described in Chapter 6 for the ALK break-apart FISH assay.

Cell Classification: Signal Patterns

The rules for cell classification are similar to those for the ALK break-apart FISH assays. In brief, each tumor cell is classified as having a fused pattern, split pattern, isolated 3' pattern, or isolated 5' pattern on the basis of the specific signal patterns. (*See Chapter 6 for details.*) In the fused pattern, orange or red and green spots from a single copy of the gene are seen as fused, touching, or adjacent because the genomic areas homologous to the 5' and 3' probes are close (Figure 1, A and B). In the split pattern, the 5' *ROS1* orange or red signal becomes

Table 2. *ROS1* Rearrangements in Lung Cancer: Frequency in Specific Cohorts and Detection Methods

Study	Population	No. of Patients	No. of *ROS1*-Positive Tumors (%)	Detection Method (Confirmation)
Zhu et al., 2015[a]	NSCLC and adenocarcinoma	9,898	193 (1.9)	FISH, IHC, RT-PCR, ARMS-PCR
Fu et al., 2015	NSCLC, stage IIIA, N2	204	4 (2.0)	FISH (IHC, direct sequencing)
Ha et al., 2015	Asian women, never-smokers, lung adenocarcinoma	198	2 (1.0)	RNA expression (NanoString)
Jang et al., 2015	Pan-negative lung adenocarcinoma (negative for *EGFR, KRAS, BRAF, ERRB2, MET, PIK3CA, AKT1, AKT2, KIT, JAK2*)	13	1 (7.7)	RNA sequencing
Karrison et al., 2015	Large cell with neuroendocrine features (LCNEC) and without (LC)	32 LCNE 41 LC	0 (0)	NGS
Rogers et al., 2015	NSCLC	317	3 (0.9)	FISH, IHC
Takeda et al., 2015	Lung adenocarcinoma (AD) and squamous cell carcinoma (SCC)	78 AD 22 SCC	1 AD (1.3) 0 SCC	NGS
Wu et al., 2015	Triple-negative adenocarcinoma (negative for *EGFR, KRAS, ALK*)	127	5 (3.9)	FISH
Lee et al., 2015	Adenocarcinoma negative for *EGFR, KRAS, ALK*	94	9 (10.0)	FISH (IHC, RT-PCR)
Zhong et al., 2015	NSCLC	302	12 (4.0)	RT-PCR
Sheren et al., 2015	NSCLC enriched for tumors negative for *EGFR, KRAS, ALK*	452	189 (4.2)	FISH
Cao et al., 2016	Resected adenocarcinoma	183	3 (1.6)	FISH, IHC, RT-PCR
Clave et al., 2016	NSCLC	283	5 (1.8)	FISH (IHC)
Lim et al., 2016	Adenocarcinoma negative for *EGFR, KRAS, ALK*	51	7 (13.7)	NGS
Scheffler et al., 2016	Adenocarcinoma	1,035	19 (1.8)	FISH (NGS)
Zhao et al., 2016	Resected squamous cell carcinoma	214	0 (0)	NanoString fusion assay

[a]*This study is a meta-analysis that includes 18 studies published from 2011 to 2015.*
ARMS = amplification-refractory mutation system.

split from the 3' *ROS1* green signal (Figure 1C). The physical gap between the orange or red and green signals for the pair must be longer than the diameter of the largest signal in the pair to be classified as a split pattern.

Scoring and Rearrangement-Positive Cell Rate

At least 50 tumor cells should be scored per specimen. A scoring worksheet as described in Chapter 6 may be helpful. The rate of rearrangement-positive cells is determined by combining the results of all scored cells as follows:

Rearrangement-positive cell rate (%) = [(number of cells with a split pattern + number of cells with an isolated 3' pattern) /Total number of cells evaluated] × 100

Table 3. Commercially Available DNA FISH Probes for Testing *ROS1* Rearrangements

	Orientation 3′ (Green)			
Manufacturer	**Analyte**	**Catalog No.**	**Genomic Position**	**Size (~Kb)**
Abbott Molecular	Vysis LSI ROS1 (Cen) SpectrumGreen Probe	08N07-020	UCSC GRCh37/hg19 117,177,382 117,733,849	557
Agilent Technologies/ Dako	SureFISH ROS1 3′ Break-Apart Probe	G100952G	UCSC GRCh37/hg19 116,510,648 117,642,558	1,132
Cytocell	ROS1-GOPC (FIG) Proximal Probe	LPS 510-A	Ensembl GRCh37/hg19 117,037,649 117,535,48	498
Leica Biosystems/ Kreatech	ROS1 (6q22) Proximal-XL Probe	06Q006V495	Undisclosed	450
ZytoVision	Zyto*Light* SPEC ROS1 Dual Color Break Apart Probe[a]	Z-2144	Ensembl GRCh37/hg19 116,912,298-117,627,255	715

	Orientation 5′ (Red)			
Manufacturer	**Analyte**	**Catalog No.**	**Genomic Position**	**Size (~Kb)**
Abbott Molecular	Vysis LSI ROS1 (Tel) SpectrumOrange Probe	08N05-020	UCSC GRCh37/hg19 117,761,413 118,078,260	317
Agilent Technologies/ Dako	SureFISH ROS1 5′ Break-Apart Probe	G100953R	UCSC GRCh37/hg19 117,642,558 117,888,013	245
Cytocell	ROS1-GOPC (FIG) Distal Probe	LPS 511-A	Ensembl GRCh37/hg19 117,665,515 117,871,721	206
Leica Biosystems/ Kreatech	ROS1 (6q22) Distal-XL Probe	06Q007V550	Undisclosed	260
ZytoVision	Zyto*Light* SPEC ROS1 Dual Color Break Apart Probe[a]	Z-2144	Ensembl GRCh37/ hg19 117,659,135 - 117,871,701	215

[a]*The 3′ and 5′ probes are available as a set.*

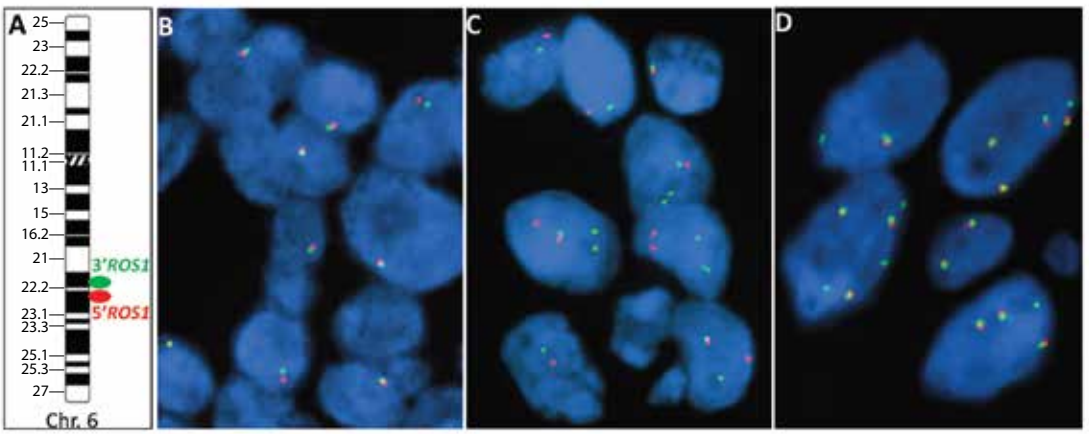

Figure 1. ROS1 break-apart FISH probe commonly has the 5' end labeled in red and the 3' end labeled in green (A). ROS1 FISH analysis of a ROS1-negative lung adenocarcinoma, showing fused 5' red and 3' green fluorescent signals in low copy number per nucleus (B). ROS1 FISH analysis of ROS1-positive lung adenocarcinomas showing split 5' red and 3' green signals (C) and isolated 3' green signals (D).

Because the ROS1 tyrosine kinase domain is encoded by the 3' part of the gene, the unpaired 3' signal indicates the oncogenic relevant fusion gene, whereas the unpaired 5' signal represents a likely nonfunctional reciprocal fusion product. Therefore, cells with an isolated 3' pattern (Figure 1D) are categorized as rearrangement-positive cells along with those with a split pattern. Approximately 30% of ROS1-positive NSCLCs predominantly show an isolated 3' pattern (Yoshida 2013). Conversely, as a rule, cells with an isolated 5' pattern should not be interpreted as rearrangement-positive cells, but if a predominant pattern with an unpaired 5' *ROS1* signal is seen, orthogonal testing is recommended to exclude cryptic rearrangements.

Cutoff Value and Specimen Classification

The distribution of ROS1-positive cell rates in NSCLC is continuous, and molecular laboratories should set a cutoff value to discriminate between specimens that are positive and negative for *ROS1* rearrangement. The cutoff value should be based on the laboratory's internal validation studies involving a large number of formalin-fixed paraffin-embedded specimens with normal and abnormal gene status, and the results should be confirmed in other laboratories or by other technologies in the same laboratory. The cutoff value may vary according to the accumulated laboratory experience, including the hybridization protocol, the ROS1 probe set used, and the correlation between the results of FISH and of other molecular methods. A practical cutoff value of 15% has been reported in most studies on clinical specimens, and this value has correlated well with the *ROS1* fusion status as determined by RT-PCR (Yoshida 2013). Thus, when the rearrangement-positive cell rate is 15% or more, the specimen is interpreted as being ROS1-positive; when the rate is less than 15%, the specimen is interpreted as being ROS1-negative.

In order to minimize technical bias, we recommend a two-step assessment with one or two trained FISH technologists. With two technologists, the first one scores 50 tumor cells and classifies the specimen as negative for *ROS1* rearrangement if the rate of rearrangement-positive cells is less than 10% (ie, rearrangement in fewer than five of 50 cells) or as positive for *ROS1* rearrangement if the rate is higher than 30% (ie, rearrangement in more than 15 of 50 cells). If the first technologist determines the rate to be 10% to 30% (ie, five to 15 of 50 cells), the second technologist should score an additional 50 tumor cells. In this latter scenario, the final rate of rearrangement-positive cells is calculated on the combined results of both scorers, and the specimen is classified according to the cutoff value of 15%.

Both manual microscope analyses and semiautomated analyses based on scanned images representing all section depths (z axis) in multiple microscopic fields are acceptable. However, the criteria for classifying the split pattern in each clinical laboratory must be based on its internal validation studies performed on FFPE tumor sections with the probe set of choice and the specific platform of analyses. This point is crucial because the size of the probe differs among probe set designs, and a larger probe usually results in both a larger signal and a shorter distance required for the definition of a split. Additionally, in scanned images, the signal size tends to be seen as larger than on actual examination under fluorescent microscopy, and consequently, the distance between signals may be underestimated, compromising the results. (*Details on internal laboratory validation are provided in Chapter 6.*)

Challenges

Interphase FISH is a robust technical platform for DNA testing in tumor tissues. The strengths of this platform include its in situ nature, high performance in long-term archived tissues, and high sensitivity and specificity. Yet, the ROS1 break-apart FISH assay faces challenges related to technical issues and tumor biology, as described in Chapter 6 for ALK break-apart FISH. Similarly, the technical artifacts may be minimized by adequate training in scoring signals and selecting tumor cells in FISH slides, by strict adherence to the enumeration rules and a careful correlation with morphology on H & E-stained slides, and by participation in external quality assurance programs, such as proficiency testing surveys, to improve the performance and maintain high standards of the test quality.

False-positive and false-negative results may also arise for biologic reasons. False-positive FISH results may be due to detection of nonfunctional *ROS1* fusions because the genomic breaks do not occur in the expected points to generate an active fusion or may be due to intervening post-transcription and post-translation phenomena that inactivate a fusion product. In one published example of false-positive results on FISH, an isolated 3' signal pattern was predominant on an *EGFR*-mutant adenocarcinoma, yet in-frame *ROS1* fusions were lacking when testing was done with multiplex RT-PCR (Yoshida 2014) and RNA sequencing (Yoshida, unpublished data available courtesy of Dr. Takashi Kohno, Tokyo). False-negative FISH results may be associated with complex or cryptic genomic rearrangements. A number of clustered rearrangements have been more recently identified in solid tumors by the novel and sophisticated technologic platforms available, some of which have been attributed to the phenomenon of chromothripsis, a massive chromosome reorganization resulting from a single disruptive event (Kass 2016). False-negative FISH results also may be associated with certain partner genes. Most *ROS1* partner genes are mapped outside of chromosome 6, and FISH analysis for such interchromosomal fusions typically demonstrates an easily visible physical gap between the orange or red and green signals. A readily discernible positive signal is also expected in tumors with fusions between *ROS1* and the genes that are mapped in chromosome 6 but are more than 40 kb from the *ROS1* locus (eg, *HLA-A* and *EZR*). However, a few *ROS1* partner genes may be difficult to detect with break-apart FISH assay. For example, *TPD52L1* is mapped very close to the *ROS1* locus in an opposite orientation (Table 1), and it will be a challenge to detect this fusion with the break-apart FISH assay if the fusion is generated by a chromosomal inversion. *CEP85L* and *GOPC* are located approximately 1 MB and 200 kb from *ROS1*, respectively, and their fusions with *ROS1* will be detectable as an isolated 3' signal pattern only if the molecular fusion is generated by genomic deletions that encompass the 5' *ROS1* component of the probe set. The glioblastoma cell line U-118 MG, which harbors *GOPC-ROS1* as a result of such genomic deletion (Charest 2003), is a useful reagent for determining if a given FISH probe set is suitable for detecting this rare fusion.

Other Relevant Issues

Alterations in the copy number of native *ROS1* are frequently detected in NSCLC, represented both by loss (Figure 1, B) and gain (Figure 2, A) in the mean copy number per cell (Jin 2015, Clavé 2016). There is no indication that copy number gain is associated with an increased level of protein expression (Clavé 2016); thus, at this time, *ROS1* copy number

is not recommended as a routine part of scoring. Interestingly, gain can also occur for the rearranged *ROS1* copy (Figure 2, B), and its biologic relevance (including association with resistance to targeted therapy) is currently unknown.

In the experience of one author of this chapter, atypical patterns, such as a predominance of an isolated 5' pattern and presence of signal doublets or clusters (Figure 2, C), occur in less than 1% of patients. For specimens with doublets and clusters of signals, the rearrangement-positive cell rate is usually close to the threshold of 15% because of the increased likelihood of signal separation or truncation in tissue sections. The predominance of an isolated 5' pattern may indicate a cryptic rearrangement. Therefore, we recommend orthogonal testing with use of other technical platforms for specimens with such atypical signals.

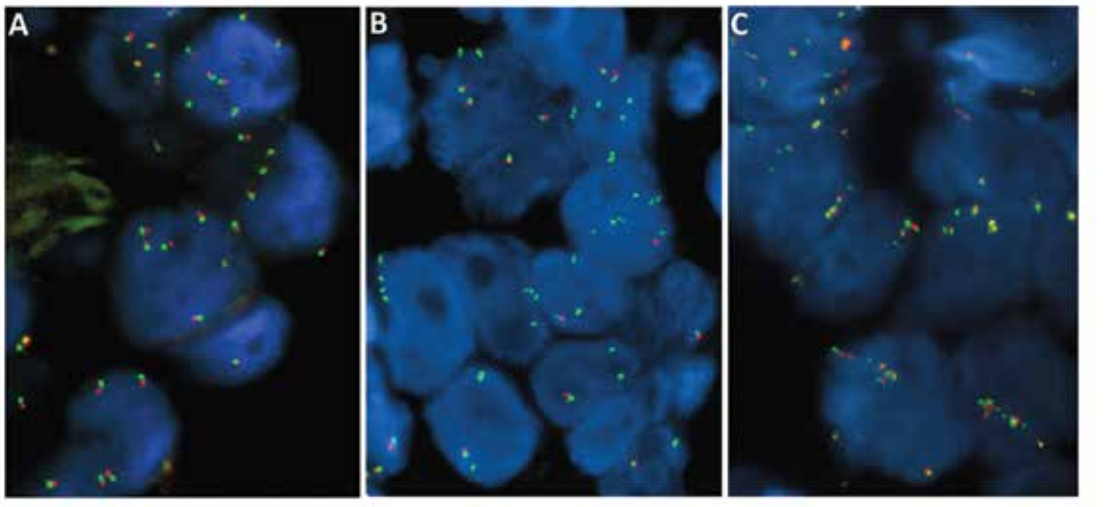

Figure 2. Lung adenocarcinomas hybridized with the ROS1 break-apart FISH probe showing copy number gain of the native gene (A), copy number gain of the isolated 3' green signals (B), and doublets and clusters of fusion signal (C). For the last specimen (C), the rearrangement-positive cell rate was 14% according to FISH, but the results were negative for *ROS1* rearrangement on next-generation sequencing panel testing.

No data are available to support the change in genomic status as a result of clonal evolution of the tumor, regardless of treatment. Also, no findings have suggested that *ROS1* rearrangement is heterogeneously distributed within lung tumor tissues, although such heterogeneity has been reported for other tumor types, such as colorectal cancer (Aisner 2014). These issues warrant attention and further investigation, as they may have significant clinical implications.

A four-color break-apart FISH probe set, which simultaneously detects rearrangements in *ALK* (3' *ALK* in red; 5' *ALK* in green) and *ROS1* (3' *ROS1* in aqua and 5' *ROS1* in yellow), has been used for other tumor types (Figure 3) (Aisner 2014). Multiplex FISH panels are commonly used in prenatal and hematologic neoplasias, and their incorporation in molecular testing of lung cancers is welcome as a way to minimize the limitation of tissue scarcity.

Conclusion

The ROS1 break-apart FISH assay is reliable for the diagnosis of *ROS1*-rearranged NSCLCs and has been used to select patients for treatment with tyrosine kinase inhibitors. The technique has unique strengths and limitations and should be used within an appropriate

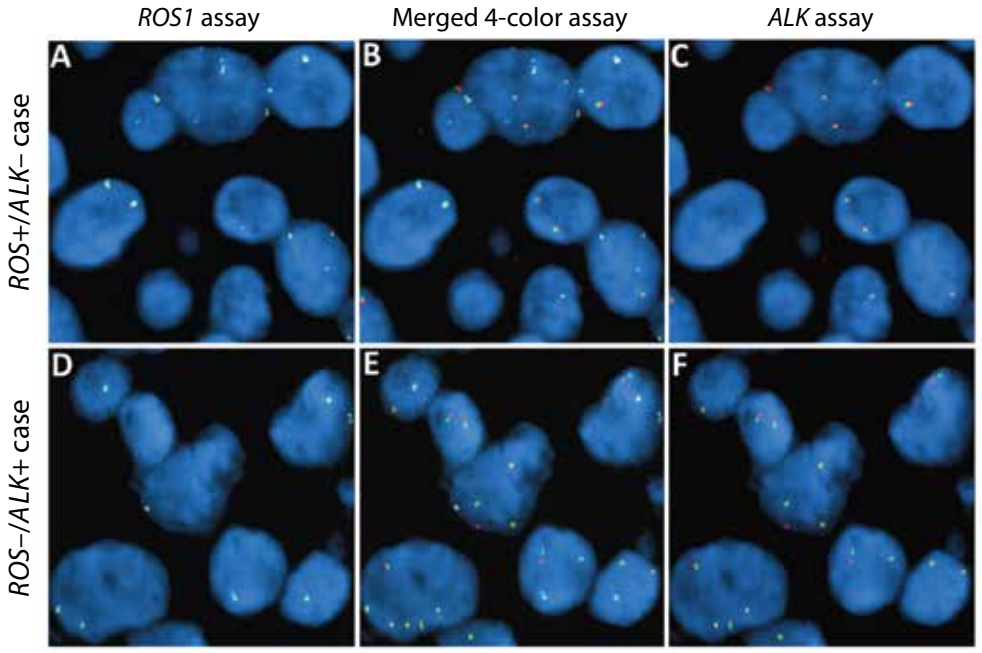

Figure 3. A four-color break-apart ALK and ROS1 FISH probe panel for a lung adenocarcinoma that is ROS1 positive and ALK negative (A, B, and C) and for a lung adenocarcinoma that is ROS1 negative and ALK positive (D, E, and F). The ROS1-positive, ALK-negative sample shows positive ROS1 results, with isolated 3' *ROS1* (aqua) signals in addition to fused 5' (yellow)/3' (aqua) signals (A); ALK assay shows negative results, with only fused 5' *ALK* (green) and 3' *ALK* (red) (C) and all channels merged in the 4-color assay (B). In contrast, the ROS1-negative, ALK-positive sample shows negative *ROS1* FISH results, with fused 5' ROS1 (yellow) and 3' *ROS1* (aqua) (D); ALK FISH shows positive results, with split 5' *ALK* (green) and 3' *ALK* (red) (F) and all channels merged in the four-color assay (E).

diagnostic context and after proper laboratory validation. Similar to other break-apart FISH tests, correct interpretation of the results depends on training and adherence to guidelines. FISH may produce equivocal or even erroneous results; to minimize technical variability, laboratories should periodically participate in proficiency testing surveys from approved vendors. Alternative assessment programs may include slide exchange with other accredited clinical laboratories and blind comparison with a complementary platform run in the same laboratory.

RT-PCR and Nonmulitiplex Platforms

By Yasushi Yatabe, Ming Sound Tsao, and Ignacio Wistuba

8

PCR is clinically feasible, as this technique is used in most assays to detect *EGFR* mutations in NSCLC. RT-PCR for detecting *ALK* rearrangements provides the most robust and detailed information about *ALK* fusion patterns. However, RT-PCR can be considered as an acceptable alternative to IHC or FISH for detecting *ALK* and *ROS1* rearrangement under some clinical circumstances. The risk of false-negative results and a high failure rate for RNA-based assays on FFPE samples cannot be neglected because high-quality RNA is required, which is difficult to obtain in clinical practice. In addition, more and more partner genes have been identified in the rearrangements. However, recent reports, some of which used recently developed techniques, support successful application of RT-PCR to clinical samples.

ALK RT-PCR

EML4-ALK shows many fusion variants (Table 1). The breakpoint of *ALK* is constantly located before the 5'-end of exon 20 (ENST00000389048) where the kinase domain starts, and this constant retention is seen in other cancers with *ALK* translocation, such as lymphoma, sarcoma, and thyroid cancer. In contrast, the breakpoint of *EML4* may distribute various exons. Following the discovery of the first two fusion variants (Soda 2007), more than 30 variants and eight fusion partners have been found (*see Chapter 1*). In the most common *EML4-ALK* variants, the exons of *EML4* and *ALK* are directly fused in an in-frame fashion without any insertion or deletion. The three major variants (v1: E13;A20, v2: E20;A20, and v3: E6;A20) account for more than 90% of lung cancers associated with *EML4-ALK*.

RT-PCR on fresh or snap-frozen tumor samples may be more sensitive than other methods in terms of the number of cancer cells required. However, high sensitivity is achieved only when the fusion pattern is within a detectable range of primer pairs. To detect all the possible *EML4-ALK* variants, primer sets should be designed in a comprehensive way (Figure 1), and even a comprehensive design may not detect irregular variants with deletions in the annealing site of the primers. Furthermore, RT-PCR systems designed for detecting *EML4-ALK* cannot detect *ALK* fusions with other partner sets such as kinesin family member 5B (*KIF5B*) and kinesin light chain 1 (*KLC1*) (Takeuchi 2009, Togashi 2012). To overcome this

limitation, primers for other fusion partners have been designed, enabling simultaneous one-tube RT-PCR detection of *EML4-ALK*, *KIF5B-ALK* (Figure 1), and ret proto-oncogene (*RET*) fusions (Takeuchi 2012). Despite these efforts, fusions with unknown partners have remained undetectable. Therefore, negative results with RT-PCR do not have any sense in screening of patients for treatment with an ALK inhibitor. From a different perspective, therapeutic efficacy with ALK inhibitors may vary according to fusion partners and/or fusion variants, as has been suggested by studies showing that L858R point mutation and *EGFR* exon 19 deletion affect transformation activity in vitro and clinical efficacy of treatment with EGFR tyrosine kinase inhibitors. Recently, Yoshida et al. reported that response rates to treatment with crizotinib differed between patients with variant 1 and nonvariant 1 *ALK* rearrangements (Yoshida 2016).

Table 1. *ALK* Fusion Variants in NSCLC

Study	Partner	Pattern	Fusion Details	Variant Name
Takeuchi et al., 2008	*EML4*	E2;A20		5b
			E2; ins117A20	5a
Takeuchi et al., 2009		E3;A20	E3; ins69A20	6
Doebele et al., 2012		E6; A19		"V5"a
Choi et al., 2008		E6;A20		3a
Rikova et al., 2007			E6ins11;A20	
Choi et al., 2008			E6ins33; A20	3b
Wang et al., 2012			E6ins18; A20	3a
Wang et al., 2012		E10;A20	E10del54 E13;A20	1a
Yoshida et al., 2016		E12;A20	E12ins51;A20	
Soda et al., 2007		E13; A20		1
Yoshida et al., 2016			E13ins60;A20	
Takeuchi et al., 2009		E14;A20	E14; del12A20	7
Fujimoto et al., 2013			E14; del36A20	7a
Takeuchi et al., 2008			E14; ins11del49A20	4
Koivunen et al., 2008		E15;A20	E15del19; del20A20	"V4"
Yoshida et al., 2016		E17;A20	E17ins27;A20	
Sanders et al., 2011			E17; ins30A20	8ab
Wang et al., 2012			E17del58ins39; A20	8a
Sanders et al., 2011			E17ins61; ins34A20	8b
Wang et al., 2012			E17ins65; A20	8a
Takahashi et al., 2010			E17ins68; A20	8a
Wong et al., 2009		E18; A20		"V5"
Soda et al., 2007		E20; A20		2
Takahashi et al., 2010			E20ins18; A20	2a
Takeuchi et al., 2009	*KIF5B*	KI24; A20		
Wong et al., 2011		KI15; A20		
Takeuchi et al., 2012		KI17; A20		
Takeuchi et al., 2009	*KLC1*	KL9; A20		
Rikova et al., 2007	*TFG*	T3; A20		

RT-PCR enables and is best suited for the examination of specimens that are not amenable to tissue blocks, such as bronchial washing fluid, sputum, blood, body cavity effusion, and other body fluids (Soda 2012). For patients with confirmed *ALK* rearrangement, RT-PCR using cell-free RNA or circulating tumor cells in fluid samples is a powerful and minimally invasive tool to monitor disease progression. However, the presence of tumor cells (or tumor-derived RNA) in fluid samples is often difficult to confirm, which increases the risk that specimens with no tumor cells will be misdiagnosed as negative for *ALK* rearrangement. In principle, if fluid samples are used for primary screening, only samples confirmed to be positive for cancer cells should be examined, but the high sensitivity of RT-PCR is then no longer advantageous.

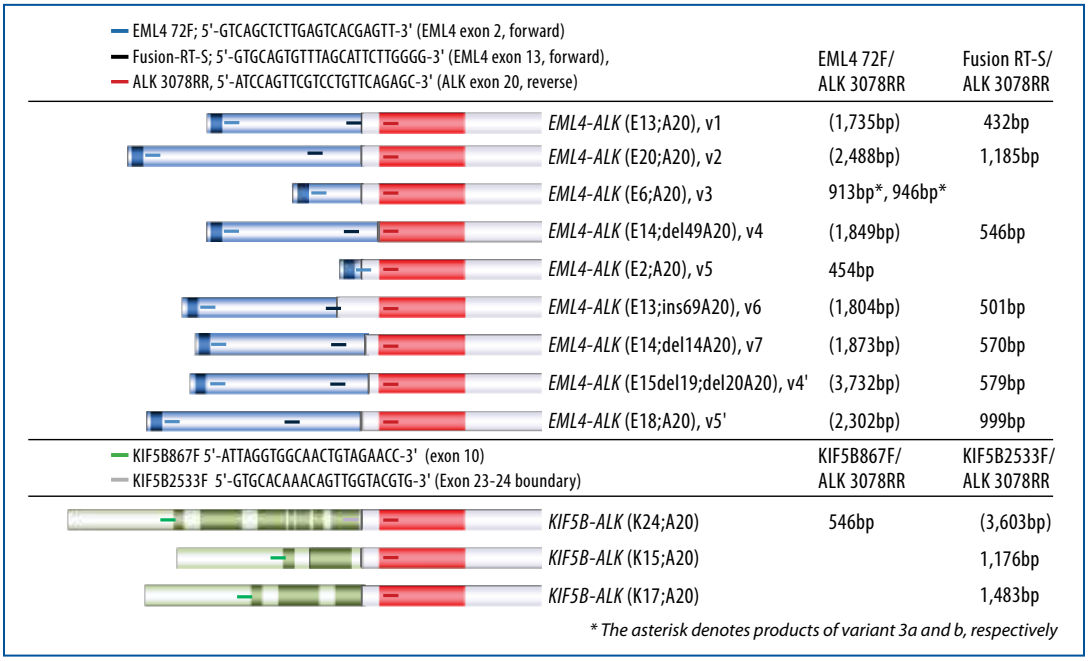

Figure 1. Primer sites and their expected product lengths of multiplex RT-PCR. Because the *ALK* kinase domain is fused with various exons of *EML4* and other partner genes, such as *KIF5B*, several primer sets are needed for successful detection of *ALK* fusion.

Recently, several methods using FFPE samples have been reported to detect *ALK* rearrangement successfully. Comparative quantitative RT-PCR (discussed in the next section) is the representative method, but simple detection of chimeric fusion transcripts has also been used, and no difference in the response rates to crizotinib were reported between patients who had testing with FISH or with RT-PCR (Li 2014, Wang 2015). In China, RT-PCR on FFPE specimens was found to be superior to FISH and IHC for detection of *ALK* rearrangement, and the AmoyDx EML4-ALK fusion gene detection kit (Amoy Diagnostics Co., LTD, Fujan, China), which can detect 21 fusion transcripts by changes in melting curve, was the best (Li 2016).

ROS1 RT-PCR

In contrast to *EML4-ALK* predominant rearrangements with *ALK*, *ROS1* has a more diverse range of fusion partners and patterns (Table 2). Two-thirds of *ROS1* rearrangements are distributed over three genes: *CD74*, *EZR*, and *SLC34A2*, each of which has two or more fusion patterns (*see Chapter 1*). Fusion partners have not been identified in more than 15% of *ROS1* rearrangements. These characteristics of *ROS1* rearrangements lead to a limited advantage of RT-PCR as an assay platform, and ROS1 RT-PCR requires complex primer designs. Therefore, similar to detection of *ALK* rearrangement, several attempts have been made to use IHC for screening of *ROS1* rearrangement. In contrast to ALK IHC, expression of *ROS1* is detected in normal lung tissue, and specificity of the positive results is not high enough to identify ROS1-positive lung cancer without FISH confirmation (*see Chapter 5*). However, RT-PCR was used to select the patients in a phase II trial of crizotinib for ROS1-positive metastatic NSCLC in East Asian countries, and the results demonstrated clinical benefit of crizotinib (Goto 2016).

Comparative Quantitative RT-PCR

IHC can be used to detect *ALK* rearrangements because ALK is not expressed in normal lung tissues; as such, a positive reaction of ALK on IHC always suggests aberrant ALK expression that is most frequently caused by rearrangement in lung cancer. This principle can be applied to expression of the *ALK* transcript. The breakpoint in *ALK* occurs before the oncogenic kinase domain (exon 20) that the chimeric transcript consistently retains. Therefore, when *ALK* is fused with *EML4* or other partners, the 3' and 5' regions of the ALK transcript are expressed differently (Figure 2).

The greatest benefit of these and 3' and 5'-comparative mRNA-based arrays is that they can simultaneously and independently detect various fusion partners. Although break-apart FISH offers a similar benefit, the comparative measurement of 3' and 5' regions of the transcript allows high-throughput analyses, which means that all fusion-type alterations, including those involving *ALK*, *ROS1*, and *RET*, are detectable. Furthermore, because quantitative RT-PCR generally requires short-length products (approximately 150 bp or less), which are usually retained, even in FFPE samples, several researchers have reported that this measurement for fusion genes was clinically feasible (Suehara 2012, Wang R 2012a, Wang R 2012b, Dama 2016, Lira 2014). The method is currently being shifted to a multiplex assay using NGS (*see Chapter 9*).

Table 2. *ROS1* Fusion Variants in NSCLC

Study	Partner	Pattern
Rikova et al., 2007 Takeuchi et al., 2012	*CD74*	C6;R32 C6;R34
Bergethon et al., 2012	*SLC34A2*	S4;R32
Takeuchi et al., 2012	*TPM3*	T8;R35
Takeuchi et al., 2012	*SDC4*	S2;R35 S4;R32 E4;R34
Takeuchi et al., 2012	*SLC34A2*	S12;E32 S12;E34
Takeuchi et al., 2012	*EZR*	E10;E34
Takeuchi et al., 2012	*LRIG3*	L16;E35
Rimkunas et al., 2012	*FIG*	F7R34
Takeuchi et al., 2012	*CCDC6*	C6;R34
Suehara et al., 2012	*GOPC*	G7;R35
Govindan et al., 2012	*KDELR2**	

**Precise KDELR2-FOS1 fusion breakpoints have not been published.*

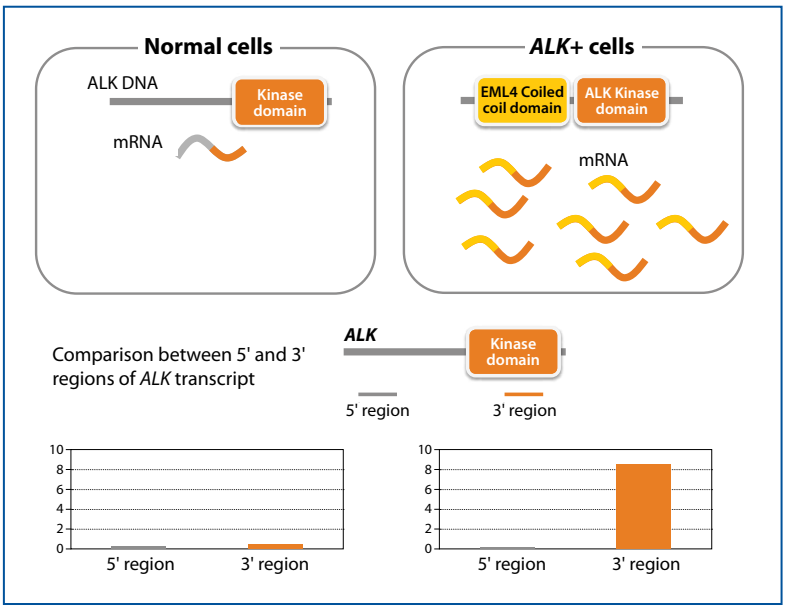

Figure 2. In normal lung cells, *ALK* is expressed at a very low or undetectable level of mRNA, whereas ALK-positive tumor cells produce abundant chimeric *ALK* transcript. When the 3' and 5' regions of *ALK* are analyzed separately, a high transcript of *ALK* 5' region is detected exclusively in ALK-positive cells. This method is not influenced by mixed normal cells and is independent of the fusion partners.

Conclusion

RT-PCR for fusion genes is a high throughput screening tool with rapid turnaround time, providing the most robust and detailed information about the fusion partners and patterns. However, this technique is unable to identify rearrangements involving unknown fusion partners, and the results depend on the quality of RNA, which is often difficult to obtain in clinical practice. To overcome the limitation, comparative quantitative RT-PCR has been developed and has been reported to be a clinically feasible method. Although real-time PCR has been applied to the technique, NGS is now rapidly replacing it and is expected to become a major method of molecular testing in clinical practice.

ALK and ROS1 Testing with NGS

By Ignacio I. Wistuba, Lynette M. Sholl, and Neal I. Lindeman

9

Since the completion of the human genome sequencing project in 2001, the field of genomics and diagnostic molecular pathology of cancer has grown exponentially, in part as a result of the advent of massively parallel sequencing (McPherson 2001, Venter 2001). This new technology enables large-scale nucleic acid sequencing, allowing the analysis of whole genome and exome DNA and whole transcriptome RNA (Wu 2013). A natural application of this NGS methodology is to assess the complex genomic alterations existing in nucleic acids extracted from tumor tissue specimens and other types of samples (eg, circulating cell-free [cf] DNA) from people with cancer, including lung cancers (Frampton 2015, Frampton 2013, Goswami 2016, Hovelson 2015).

In molecular diagnostic testing in the clinical cancer setting, NGS is most often implemented through targeted gene panels (Frampton 2015, Frampton 2013, Goswami 2016, Hovelson 2015, Kanagal-Shamanna 2014, Singh, 2013). Several NGS approaches have been used successfully, including multiplex PCR-based panels assessing scores of genes, hybrid capture-based panels targeting hundreds of genes, and comprehensive exome/genome/transcriptome sequencing (Frampton 2015, Frampton 2013, Goswami 2016, Hovelson 2015). In NGS assays, raw sequence reads are first aligned to the reference human genome, and variant calling is performed to identify mismatches in these alignments that may represent genomic changes in the specimens (Strom 2016). Technical validity and clinical utility are the two major issues that must be resolved for every variant identified via NGS somatic variant detection (Strom 2016).

Precision medicine approaches in lung cancer require rapid, inexpensive, and scalable NGS solutions capable of assessing all classes of clinically relevant targets (point mutations, short insertions/deletions [indels], copy number variations [CNVs], and structural variants such as chromosomal rearrangements) in routine FFPE tissues. Currently, the NGS approaches used in diagnostic laboratories vary in terms of sample requirements, nucleic acid assessed, cost, throughput, genes and alteration types assessed, and analytical performance characteristics. An important point in lung cancer is that most multiplex PCR-based approaches used in the clinical setting fail to assess CNVs and/or gene fusions,

such as those involving *ALK* and *ROS1* genes. The use of hybrid capture-based NGS assays, however, not only allows the identification of so-called hotspot mutations but can also interrogate the entire coding sequence of oncogenes and tumor suppressor genes and the introns of selected genes involved in gene fusions (such as *ALK*, *ROS1*, *RET,* and *NTRK*) for structural variant detection, as well as allow assessment of CNVs, all from a single FFPE specimen.

Methodologic Considerations

The sample requirements for successful use of NGS in diagnostic laboratories to assess genomic changes in tumors, including tissue and cytology specimens, have been reported in a number of studies (Goswami 2016, Kanagal-Shamanna 2014, Singh 2013). The following factors are needed for successful application of NGS in cancer molecular diagnosis: (1) careful screening and selection of tumor tissue (and avoidance of necrotic areas) by experienced pathologists to confirm that tumor cell content and cellularity meet minimum requirements based on NGS assay validation, (2) use of a properly validated amount of input DNA/RNA, and (3) establishment, during validation, of the minimum sequencing depth/coverage at several putative variant loci for precise and accurate interpretation (Goswami 2016, Kanagal-Shamanna 2014, Singh 2013).

An increasing number of studies are showing that cytology smears and liquid preparations (eg, from FNA or endobronchial ultrasound, or pleural fluid) can be used as specimens for NGS-targeted gene panel analysis (Goswami 2016, Hadd 2013, Karnes 2014, Wei 2016). The results of these studies suggest that NGS provides accurate sequence information on FNA cytology specimens that is indistinguishable from that obtained with FFPE tissue (Hadd 2013, Karnes 2014).

NGS-based Testing for *ALK* and *ROS1* Abnormalities

As discussed in other chapters, the current recommended methodologies to assay *ALK* and *ROS1* rearrangements using tissue specimens are IHC and FISH. However, the use of hybrid capture-based NGS gene panels to assess multiple clinically relevant genes and types of genomic abnormalities in NSCLC, particularly of adenocarcinoma histology, has shown the feasibility and value of NGS approaches to detect targetable gene rearrangements in lung cancer, including *ALK*, *ROS1*, *RET,* and *NTRK* (Ali 2016, Drilon 2015, Frampton 2015, Suh 2016).

Drilon et al. reported on hybrid capture-based NGS analysis of 4,557 exons of 287 genes and 47 introns of 19 frequently rearranged cancer-related genes in tumors from 31 patients with adenocarcinoma and a smoking history of 15 pack-years or less; the tumors tested negatively for 11 genes, including *ALK* and *ROS1* (Drilon 2015). Eight (26%) patients had tumors with actionable abnormalities, including two patients with FISH-negative results for *ALK* rearrangements (*SOCS5-ALK* and *HIP1-ALK*) and one with FISH-negative results for *ROS1* rearrangement (*CD74-ROS1*). Additionally, hybrid capture-based NGS approaches have been shown to detect complex *ALK* rearrangements, such as the one described by Peled et al. that included breakpoints in at least five different genomic loci, including *ALK* intron 19, giving rise to the canonical *EML4-ALK* translocation (Peled 2012). This complexity may provide an explanation for a negative signal on break-apart FISH testing.

More recently, Suh et al. reported on an analysis of 6,832 NSCLC tumors using a comprehensive genomic profiling panel (3,320 exons from two sets of genes; 236 genes in set 1 and 315 genes in set 2), including *ALK* and *ROS1* (Suh 2016). In this study, 280 (4.1%) tumors harbored *ALK* alterations and 100 (1.5%) harbored *ROS1* alterations. As expected, most of the *ALK* (3.9%) and *ROS1* (1.3%) abnormalities corresponded to gene rearrangements. Using the same NGS methodology and a similar gene panel, Ali et al. reported on an analysis of 1,070 tumors from patients with advanced NSCLC and identified 47 tumors (4.4%) with *ALK* rearrangements (Ali 2016). Of the 47 tumors, 41 had an *EML4-ALK* fusion, and six had other fusion partners, including three previously unreported rearrangement events (*EIF2AK-ALK*, *PPM1B-ALK*, and *PRKAR1A-ALK*). Among the 41 tumors with *ALK* rearrangements, prior FISH testing results were available for 31 tumors; 20 (65%) were FISH-positive for *ALK* and 11 (35%) were FISH-negative for *ALK*. Nine patients with an ALK FISH-negative tumor were treated with crizotinib on the basis of the NGS results, and seven of them had a response, with a median duration of 17 months. These data indicate that hybrid capture-based NGS can detect *ALK* canonical rearrangements as well as rearrangements with non-canonical fusion partners in a subset of patients with NSCLC that had previously tested negative for *ALK* on FISH. In this series of patients in the study by Ali et al., a subset of such patients had durable responses to ALK inhibitors, comparable to historical response rates for *ALK* FISH-positive tumors. Another advantage of NGS-based testing is the ability to identify *ALK* gene-related mechanism of resistance to ALK inhibitors, including secondary *ALK* mutations (*L1196M*, *G1269A*, *C1156Y*, *G1202R*, *I1171T*, and *E1210K*) (Gainor 2016) and gene amplification (Doebele 2012).

DNA-based NGS methods can detect a rearrangement only if its breakpoints are adequately covered in the assay design. Hybrid capture-based NGS, but not amplicon sequencing methods, is amenable to detection of most genomic breakpoints, which can occur over areas spanning several kilobases within introns. Large targeted panels that capture introns for rearrangement detection do so to some extent at the expense of broader and/or deeper exonic coverage (Rizzo 2012). Indeed, cost-effective assay design often demands relatively low intronic coverage. This factor, coupled with reduced hybridization quality as a result of the high AT and repeat-element content that characterizes noncoding regions, can compromise the analytic sensitivity (eg, ability to detect a rearrangement in a specimen with low tumor content) of hybrid capture-based NGS relative to other more targeted approaches, such as RT-PCR (Aird 2011). Sensitive and specific rearrangement detection by NGS requires sophisticated bioinformatics software, often requiring customization by a local informatics team, as well adequate genomic knowledge of laboratory personnel to ensure accurate interpretation of the sequencing calls (Abel 2014).

Currently, NGS-targeted gene panels are also being used to test genomic changes in cfDNA of blood from patients for whom tumor tissues are not readily available (Lanman 2015, Schwaederle 2016). In one of these studies, sequencing of 54 cancer-related genes in plasma cfDNA led to the identification of *ALK* rearrangements in four (10%) of 40 patients with NSCLC (Schwaederle 2016). The application of NGS testing to liquid biopsy strategies provides new opportunities for the diagnosis and monitoring of patients with *ALK*- and *ROS1*-rearranged tumors.

Conclusion

NGS represents a practical and reliable *ALK* and *ROS1* testing approach for use with routine lung cancer tissue specimens, enabling patients to receive optimal therapies. This novel methodology can also assess genomic-related mechanism of resistance to ALK-targeted therapies and guide the appropriate selection of the next generation of inhibitors.

Comparison of Different Assay Platforms for ALK Testing

10

By Sylvie Lantuéjoul, Marileila Varella-Garcia, Erik Thunnissen, and Yasushi Yatabe

As noted earlier, FISH has been universally accepted as a reference standard in the assessment of *ALK* gene rearrangement, and it was initially validated and approved for testing to select patients for treatment with an ALK inhibitor (crizotinib). FISH with break-apart probes can detect *ALK* gene rearrangement regardless of the gene partner and variant and can be performed on archived FFPE specimens. However, the FISH assay requires a minimum of 50 tumor cells, and this requirement may be the reason FISH cannot be used in as many as 20% of lung cancer biopsies (Camidge 2010, McLeer-Florin 2012). FISH has many other limitations, including low throughput technology and the need for specialized training to interpret results.

In 2015, ALK protein expression by IHC was also approved by the US FDA as a companion diagnostic test for ALK inhibitors, and these assays are now routinely used as a screening method in pathology laboratories worldwide. The assay is relatively inexpensive, is applicable to FFPE specimens, offers rapid training and optimization, and usually requires a small number of tumor cells to detect the presence of the fusion protein. In theory, IHC detects expression of all ALK fusion proteins, but some variants and fusion partners may generate low protein levels that are difficult to detect. Therefore, many IHC assays now include an amplification process to enhance the protein signal. IHC remains the most popular and cost-effective platform.

Many studies have compared FISH with IHC with use of various antibodies and multiplex and quantitative RT-PCR. Although correlation of results of ALK IHC and ALK FISH is excellent, IHC as a predictive marker alone for response to ALK inhibitor therapy has only recently been validated with the D5F3 IHC assay. IHC also does not directly demonstrate the *ALK* gene rearrangement, and some false-negative and false-positive results (compared with FISH results) have been reported (*see section on discrepancies of results later*).

Guidelines for standardization of the IHC assay for ALK testing have been developed (Lindeman 2013) and recommend ALK IHC for screening, with or without verification of positive results by FISH for eligibility for ALK inhibitor therapy.

RT-PCR is a highly specific and reliable technique, as it allows for the precise identification of the 5' partners and breakpoint variants. But atypical *ALK* variants or fusion partners (such as an irregular variant with insertion or deletion) may be undetected. RT-PCR of *EML4-ALK* rearrangement is highly sensitive because the primers will not amplify a product in normal cells, but intact mRNA is poorly preserved in an archived FFPE specimen. The cost of RT-PCR is still being evaluated and depends on the laboratory and the test used, but the use of RT-PCR in routine screening of *ALK*-rearranged lung cancer remains limited. Chromogenic in situ hybridization (CISH), a conventional bright-field light microscopy method developed for detection of ALK rearrangement with the aim of overcoming some of the disadvantages of FISH (Kim 2011) gave promising results with a concordance with FISH of 97.4 to 100% (Schultheis 2014, Yoshida 2011b, Rogers 2015) and sensitivity and specificity of 93 and 100% versus IHC, respectively. However, interpretation remains challenging due to the different chromogenic substrates used and further clinical and/or commercial development has not been pursued for a routine practice.

IHC versus FISH

Several studies have compared IHC with FISH, and a variety of antibodies have been used for ALK IHC (*see Chapter 4*). In most studies, FISH has been performed with the ALK FISH Break Apart Probe Kit (Abbott Molecular), which was approved by the US FDA as the first companion diagnostic for ALK testing to determine patient eligibility for treatment with crizotinib.

ALK1 IHC versus FISH

Initially used for diagnosing anaplastic large cell lymphoma, the ALK1 antibody has also been evaluated for the detection of *ALK* rearrangement in NSCLC. ALK1 IHC and FISH were compared in three large series of adenocarcinoma of the lung or NSCLC (Rodig 2009, Mino-Kenudson 2010, Yi 2011); ALK1 was associated with good specificity but a lower sensitivity than 5A4 and D5F3 clones, possibly because the ALK fusion protein is expressed at lower levels in NSCLC. Therefore, ALK1 antibody is not currently recommended for ALK testing in lung cancers.

D5F3 IHC versus FISH

IHC with the D5F3 clone has been compared with FISH in at least 12 studies (Table 1) (Mino-Kenudson 2010, Martinez 2013, Minca 2013, Ying 2013, Tantraworasin 2014, Wang 2014, Zhou 2014, Shan 2014, Ali 2014, Wynes 2014, Ilie 2015). The sensitivity ranged from 81% to 100%, and the specificity, from 82% to 100%. In a study in which an international panel of pathologists evaluated D5F3 IHC in a series of lung adenocarcinoma with known *ALK* genotype, the inter-observer concordance in scoring was high (Wynes 2014).

5A4 IHC versus FISH

Numerous studies have compared IHC with the 5A4 clone and FISH in large series (Table 2) (Jokoji 2010, Paik 2011, Kim 2011, McLeer-Florin 2012, Lopes 2012, Park 2012, Sholl 2013b, To 2013, Blackhall 2014, Cabillic 2014). Across studies, the sensitivity and specificity of the 5A4 antibody versus FISH ranged from 93% to 100% and 96% to 100%, respectively.

Table 1. Staining Features of IHC with D5F3 Antibody

Study	No. of Specimens	D5F3 Dilution	Antigen Retrieval	Detection and Amplification System	Scoring	IHC Positive Threshold	IHC Sensitivity (vs. FISH)	IHC Specificity (vs. FISH)
Mino-Kenudson et al., 2010	153	1:100	EDTA (pH 8.0) in pressure cooker	EnVision+	0, 1+, 2+, or 3+ and % of tumor cells	>10% positive tumor cells	100%	99%
Martinez et al., 2013	79	1:50	Standard on BenchMark XT	ultraView	0 vs. +	≥10% positive tumor cells	83%	100%
Minca et al., 2013	231[a]	1:100	Heat mediated with BenchMark XT	OptiView	0 vs. +	Positive	94%	100%
Ying et al., 2013	193	1:50	Not specified	OptiView and ultraView	0, 1+, 2+, or 3+	1+, 2+, or 3+	100%	95%
Tantraworasin et al., 2014	267	Not specified	iH Not specified	OptiView	0 vs. +	Positive	Not specified	95%
Wang et al., 2014	430	Not specified	Not specified	OptiView	0 vs. +	Positive	100%	98%
Zhou et al., 2014	410	1:100	Not specified	REAL EnVision-HRP and DAB	0, 1+, 2+, or 3+	2+, or 3+	100%	98%
Shan et al., 2014	297	1:150	EDTA (pH 9.0) solution	Not specified	0, 1+, 2+, or 3+	1+, 2+, or 3+	100%	82%
Wynes et al., 2014	103	Pre-diluted	Not specified	OptiView and OptiView Amplification	0 vs. +	Not specified	90%	95%
Ali et al., 2014	523	Not specified	Not specified	OptiView and OptiView Amplification	0 vs. +	Positive	Not specified	Not specified
Ilie et al., 2015	176	Pre-diluted	CC1 retrieval solution	OptiView and OptiView Amplification	0 vs. +	Positive	81%	99%

[a]*Two false-negative results on IHC testing of FFPE specimens were subsequently corrected by testing with a ThinPrep processor (positive results).*

[b]*Cases selected for the study included 43 specimens that were ALK positive by FISH and 55 that were ALK negative by FISH.*

BenchMark XT, ultraView (Universal DAB Detection Kit), OptiView (DAB IHC Detection Kit), and OptiView Amplification Kit are products of Ventana Medical Systems, Inc. EnVision+ and REAL EnVision-HRP and DAB are products of Dako.

Comparison of Antibody Clones

Some authors have directly addressed concordance among antibody clones using the same sample sets, and concordance has been antibody dependent (Table 3) (Takeuchi 2009, Rodig 2009, Mino-Kenudson 2010, Murakami 2012, Conklin 2013, Selinger 2013, Cutz 2014, Zwaenepoel 2014, Condé 2014, Gruber 2015, Lantuéjoul 2015). Takeuchi et al. reported comparable results with the ALK1 and 5A4 antibodies using an intercalated antibody-enhanced polymer (iAEP) method to amplify signals (Takeuchi 2009). However, discordance has been found in other studies. For example, Murakami et al. reported one discordant case among 12 specimens with *ALK* gene rearrangement; the result was negative with 5A4 IHC but positive with D5F3 IHC (Murakami 2012). Conklin et al. compared five combinations of antibody

Table 2. Staining Features of IHC with 5A4 Antibody

Study	No. of Specimens	5A4 Dilution[a]	Antigen Retrieval	Detection and Amplification System	Scoring	IHC Positive Threshold	IHC Sensitivity (vs. FISH)	IHC Specificity (vs. FISH)
Jokoji et al., 2010	254	1:100	Target Retrieval Solution, high pH	EnVision FLEX+ and iAEP	0 vs. +	No score	100% (FISH done on IHC+ specimens only)	100%
Paik et al., 2011	640	1:30	CC1 solution	iVIEW	0, 1+, 2+, or 3+	0, or 1+: negative 2+: equivocal 3+: positive	100%	96%
Kim et al., 2011	465	1:30	CC1 solution	iVIEW	0, 1+, 2+, or 3+	0, or 1+: negative 2+: equivocal 3+: positive	100% when score of 2+ considered positive	98%
McLeer-Florin et al., 2012	441	1:50	CC1 solution	UltraView Universal DAB detection kit	0, 1+, 2+, or 3+ and % of positive tumor cells	>10% positive tumor cells	95%	100%
Lopes et al., 2012	62	1:200	Microwave	Novolink	No score	>10% positive tumor cells	100%	100%
Park et al., 2012	262	1:50	Leica microsystem solution (pH 9.0)	Bond Polymer Refine Detection system	0, 1+, 2+, or 3+	1+, 2+, or 3+	100%	98%
Sholl et al., 2013b	186	1:50	Citrate buffer, (pH 6) in pressure cooker	EnVision FLEX+	0, 1+, or 2+	1+, 2+	93%	100%
To et al., 2013	373	1:100	EDTA buffer (pH 8.0) in pressure cooker	Bond Polymer Refine Detection system	% of positive cells (0, none; 1, ≤10%; 2, 10%–25%; 3, >25%–50%; 4, > 50%) x intensity score (0, none; 1, weak; 2, moderate; 3, strong); final scores 0-12	Final score ≥ 7	100%	Not specified
Cabillic et al., 2014	3,244	1:50	Not specified	ultraView Bond Polymer Refine Detection system	0, 1+, 2+, or 3+	1+, 2+, or 3+	69%	99%

The 5A4 antibody used in the studies by Jokoji et al., McLeer-Florin et al., and Lopes et al. were from Abcam ; the antibody used in the other studies were from Novocastra.

iView (DAB Detection Kit) and OptiView (DAB IHC Detection Kit) are products of Ventana Medical Systems. Novolink (Polymer Detection System) and Bond Polymer Refine Detection System are products of Leica Biosystems.

CC1 (Tris/borate/ EDTA) is a product of Roche (Basel, Switzerland). ultraView (Universal DAB Detection Kit) is a product of Ventana Medical Systems, Inc. , and HRP (horseradish peroxidase) complex is a product of Dako.

Table 3. Comparison of Antibody Clones Used with IHC

Study	No. of Specimens (% of ALK+)	Antibody Clone	Antigen Retrieval	Detection/ Amplification System	Scoring	Standard	IHC Sensitivity (vs. Standard)	IHC Specificity (vs. Standard)
Takeuchi et al., 2009	21 (52%)	5A4	Target Retrieval Solution (pH 9.0) (for all)	iAEP	0 vs. + (for all)	RT-PCR	100%	100%
		5A4		EnVision+			27%	100%
		ALK1		iAEP			100%	100%
		ALK1		EnVision+			9%	100%
		SP8		iAEP			20%	100%
		SP8		EnVision+			100%	18%
Rodig et al., 2009	239 (4%)	ALK1	EDTA in pressure cooker (for both)	EnVision+	0 vs. + (for both)	FISH	40%	100%
		ALK1		Tyramide amplification			80%	100%
Mino-Kenudson et al., 2010	37 (59%)	ALK1	EDTA in pressure cooker (for both)	EnVision+ (for both)	Score >2.7 on image analysis is positive	FISH	67%	97%
		D5F3					100%	99%
Murakami et al., 2012	361[a] (5%)	ALK1	Target Retrieval Solution (pH 9.0) (for all)	ABC (no enhancement)	0 vs. + (for all)	RT-PCR/ FISH	81%	100%
		5A4		EnVision FLEX+			100%	100%
				EnVision FLEX+			100%	99.7%
Conklin et al., 2013	377 (3%)	5A4	According to manufacturer's instructions (for all)	iAEP	0, 1+, 2+, or 3+ (for all)	FISH	100%	62.5%
		ALK1		EnVision FLEX+			66%	100%
		ALK1		ADVANCE			66%	87.5%
		5A4		ADVANCE			100%	87.5%
		D5F3		ADVANCE			100%	75%
Selinger et al., 2013	594 (11%)	ALK1	Buffer (pH 9.0) in pressure cooker	EnVision FLEX+	0, 1+, 2+, or 3+ (for all)	FISH	100%	99%
		5A4	According to manufacturer's instructions	ultraView and ultraView Amplification			100%	98%
		D5F3	According to manufacturer's instructions	OptiView and OptiView Amplification			100%	99%
Cutz et al., 2014	28 (79%)	5A4	Not reported	ultraview	H-score	Pearson correlation between antibodies	5A4 vs D5F3 : 0.89 - 0.97 5A4 vs ALK1 : 0.84	
		D5F3	Not reported	ultraview EnVision FLEX+				
		ALK 1	Not reported	EnVision FLEX+				

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Table 3. (cont.)

Study	No. of Specimens (% of ALK+)	Antibody Clone	Antigen Retrieval	Detection/ Amplification System	Scoring	Standard	IHC Sensitivity (vs. Standard)	IHC Specificity (vs. Standard)
Zwaenepoel et al., 2014	53	5A4	High pH buffer (dilution 1:50)	EnVision FLEX+	0, 1+, 2+, 3+	1+, 2+, 3+	96%	87-96%
		D5F3	High pH buffer (EnV)	En Vision FLEX+ (D5F3 EnV) OptiView Amplification	0, 1+, 2+, 3+	1+, 2+, 3+ (D5F3 EnV) 3+ (D5F3 Ov)	96% (D5F3 EnV) 93% (D5F3 Ov)	87-96% (D5F3 EnV) 100% (D5F3 Ov)
Condé et al. 2014	79	5A4		OptiView and OptiView Amplification	0, 1+, 2+, 3+	2+, 3+	98%	100%
		D5F3	According to manufacturer's instructions	OptiView and OptiView Amplification			98%	100%
Gruber et al., 2015	218	1A4	pH 6.0 (30 min)	OptiView and OptiView Amplification	0, 1+, 2+, 3+	1+, 2+, 3+	100%	95%
		D5F3	According to manufacturer's instructions	OptiView and OptiView Amplification	0, 1+, 2+, 3+	3+	99%	95%
Lantuéjoul et al., 2015	547	5A4		OptiView or ultraView Amplification	% of + cells and intensity (1+, 2+, 3+)	10% 1+	87%	89%
		D5F3	According to manufacturer's instructions	OptiView and OptiView Amplification	% of + cells and intensity (1+, 2+, 3+)	10% 1+	92%	76%
Savic et al., 2015	72	5A4		OptiView Amplification Bond Polymer Refine Detection	0, 1+, 2+, 3+	1+, 2+, 3+	96%	100%
		D5F3	According to manufacturer's instructions	OptiView and OptiView Amplification	0, 1+, 2+, 3+	3+		

SP8 is a product of Abcam. Target Retrieval Solution, buffer, EnVision, EnVision FLEX+, and ADVANCE are products of Dako. ultraView (Universal DAB Detection Kit), OptiView (DAB IHC Detection Kit), and ultraView and OptiView Amplification kits are products of Ventana Medical Systems, Inc. Bond Polymer Refine Detection is a product of Leica Biosystems.

ABC = avidin biotin complex.

clones and detection systems, and the concordance was highest with 5A4 and D5F3 with the ADVANCE system (Dako), but a heterogeneous positive reaction was detected in a specimen that was ALK negative on FISH (Conklin 2013). Several studies have also emphasized the superiority of the Optiview Amplification Kit (Ventana). In most studies, the results with both 5A4 and D5F3 have been similar (Cutz 2014, Condé 2014, Marchetti 2016), but in others, the sensitivity of D5F3 has been slightly higher and the specificity of 5A4 has been slightly higher (Lantuéjoul 2015).

Multiplex or Quantitative RT-PCR versus FISH (with or without IHC)

EML4-ALK transcripts were detected in the normal lung tissue in one study (Martelli 2009), but these results were not confirmed in other studies, which has led to the questioning of those data (Mano 2010, Sasaki 2010). RT-PCR is a highly sensitive method associated with high specificity and no false-positive results; however, there is a risk of false-negative results because of the difficulty in obtaining high-quality RNA from FFPE specimens. Although the rate of false-negative results with RT-PCR has not been addressed in detail, successful detection of *ALK* transcripts in a prospective manner has been reported (Soda 2012). In that study, 108 (12%) of 916 specimens were excluded because RNA was of poor quality. *EML4-ALK* transcripts were detected in 36 specimens, 15 of which were available for IHC; all 15 specimens were ALK positive by IHC. In a 2015 study, nearly 39% (50 of 127) of the cases that were positive by FISH and IHC were negative with RT-PCR (Lantuéjoul 2015).

Overall, the sensitivity and specificity of RT-PCR for detection of ALK transcripts, compared with IHC and FISH, are good, ranging from 94% to 100% (Table 4) (Inamura 2008, Takeuchi 2008, Takeuchi 2009, Soda 2012).

Table 4. Comparison of Results of RT-PCR with the Results of Other Methods of ALK Testing

Study	Type of RT-PCR	IHC Antibody	FISH	RT-PCR Sensitivity (vs. FISH and/or IHC)	RT-PCR Specificity (vs. FISH and/or IHC)
Inamura et al., 2008	Multiplex RT- PCR	ALK1	None	100% (vs. IHC)	100% (vs. IHC)
Takeuchi et al., 2008	Multiplex RT-PCR	–	FISH-based fusion assay	100% (vs. FISH)	100% (vs. FISH)
Takeuchi et al., 2009	Inverse and multiplex RT-PCR	5A4 ALK1	*EML4* and *KIF5B* fusion assay	100% (vs. IHC)	100% (vs. IHC)
Soda et al., 2012	Multiplex RT-PCR	5A4	ALK break-apart probe kit	100% (vs. IHC) 94% (vs. FISH)	100% (vs. IHC) 100% (vs. FISH)

The ALK1 antibody in the studies by Inamura et al. and Takeuchi et al. (2009) is a product of Dako. The 5A4 antibody in the study by Takeuchi et al. (2009) is a product of Abcam and, in the study by Soda et al., is a product of Nichirei Biosciences, Inc. In the study by Soda et al., the break-apart probe kit is a product of Abbott Molecular.

Discrepancies between IHC, FISH, and RT-PCR

The biggest limitation in comparing platforms is the absence of IHC and FISH standardization (antibody clones, antigen retrieval, detection systems, and scoring methods). Overall, false-negative and false-positive results with IHC and FISH have been widely described in the literature since 2012 (Table 5). The reasons for false-negative results have mainly been related to the paucity of tumor cells present on specimens (fewer than 50 cells) (Sholl 2013b), presence of hyperplastic (reactive) normal cells considered as malignant (Sholl 2013b), tissue preservation, and fixation with variations of ALK protein expression among specimens, perhaps associated with heterogeneity of *ALK* abnormalities (Conde 2014, Wynes 2014). Complex rearrangements can also be negative on FISH testing but positive on IHC (Peled 2012, Ren 2014). In contrast, false-positive results on FISH are very often due to atypical patterns, such as multiple fusion signals and solitary green signals with split 5' centromeric probe, misinterpreted as positive (Sholl 2013b, Cutz 2014). In addition, discrepancies between FISH and IHC seem to occur when the percentage of rearranged cells ranges from

Table 5. Discordances between IHC, FISH, and RT-PCR

		Number of Cases				
Study	No. of Specimens (% of ALK+)	IHC+ / FISH -	IHC- / FISH+	FISH NI	RT-PCR + / FISH- and/or IHC -	RT-PCR – / FISH+ and/ or IHC+
McLeer et al., 2012	100 (21%)	1	0		19	
Park et al., 2012	262 (9.5%)	3	0			
Yang et al., 2012	225 (10%)	0 (ALK01)	0			
Sholl et al., 2013b	186 (7%)	1	0			
Wu et al., 2013	312 (10%)	215[a]	0	5		
Ying et al., 2013	196 (3%)	2	0		5 IHC- ; 7 FISH-	1
Minca et al., 2013	318 (10%)	0	2	83 (IHC NI 14)		
To et al., 2013	373 (6%)	2			2	4
Ali et al., 2014	523 (8%)	0	2			2
Blackhall et al., 2014	1,281 (6%)	52 (6 at 2+ and 3+)	0	3	1	1
Cabillic et al., 2014	3,244 (5%)	19	36	481		
Conde et al., 2014	103 (4.5%)	0 (2+, 3+)	1 (SCC)			
Cutz et al., 2014	411 (4.8%)	0	0	38		
Tontraworasin et al., 2014	267 (4%)	13	2			
Wang et al., 2014	430 (10%)	7	0		20	2
Zhou et al., 2014	368 (9.5%)	7				
Shan et al., 2014	286 (12.5%)	8	0		2 (FISH and IHC -)	3 (CST Ab 1+)
Wynes et al,. 2014	691 (5.6%)	2	4			
Zwaenepoel et al., 2014	53 (56%)	3	1			
Gruber et al., 2015	218 (9%)	0 (2 "critical" cases, negative according to manufacturer's recommendation)	0			
Ilie et al., 2015	176 (15%)	2 (ALK-amplified cases)	5			
Jurmeister et al., 2015	473 (5%)		4			
Lantuéjoul et al., 2015	547 (23%)	100 (39 5A4,56 D5F3, 5 both) (1+ mainly)	7 (FISH > 20%)	7	0	50

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Savic et al., 2015	72 (35%)[b] 303 (10%)	 2	1 3			
Gao et al., 2015	1,614 (5%)	0	3			
Pekar-Zlotin et al., 2015	51 (8%)	5	1			
Von Laffert et al., 2015	753 (2%)	1	5			
Marchetti et al., 2016	1,031 (3%)	2 (D5F3) 17 (5A4)	3 (D5F3) 3 (5A4)			

[a]*Clone ZAL4 was used.*
[b]*The study consisted of 72 patients in a retrospective cohort (comparison of two antibodies on three autostainers), and 303 patients in a prospective cohort (the 5A4 antibody versus FISH).*
NI = not interpretable.

10% to 20% (Ilie 2015, Lantuéjoul 2015) and appear center dependent in multicentric studies (Wynes 2014, Lantuéjoul 2015).

Examples of Diagnostic Algorithms for ALK Testing

Based on the advantages and characteristics of individual methods for detecting *ALK* gene rearrangement, several investigator groups or pathology societies have proposed diagnostic algorithms for conducting ALK testing (Figures 1-4) (Yatabe 2015, Paik 2011, Thunnissen 2012a, Shiau 2015, Melosky 2016). During that period, ALK IHC was recommended for screening with further confirmation by FISH (Lindeman 2013). However, the approval of the ALK (D5F3) CDx Assay (Ventana) in many countries may have significantly altered the algorithm of patient selection for ALK inhibitor therapy. Considering the common use of either a laboratory developed test with the 5A4 antibody or the D5F3 IHC CDx assay, the algorithm proposed by Marchetti et al. or a variation of it is quite reasonable (Figure 5) (Marchetti 2016). Furthermore, more extensive use of NGS in the future may additionally alter this algorithm in some institutions or parts of the world. In fact, an unreported rearrangement partner gene was detected with this method in a FISH-negative, IHC-negative tumor, and treatment with an ALK inhibitor led to a significant response. (Ali 2016).

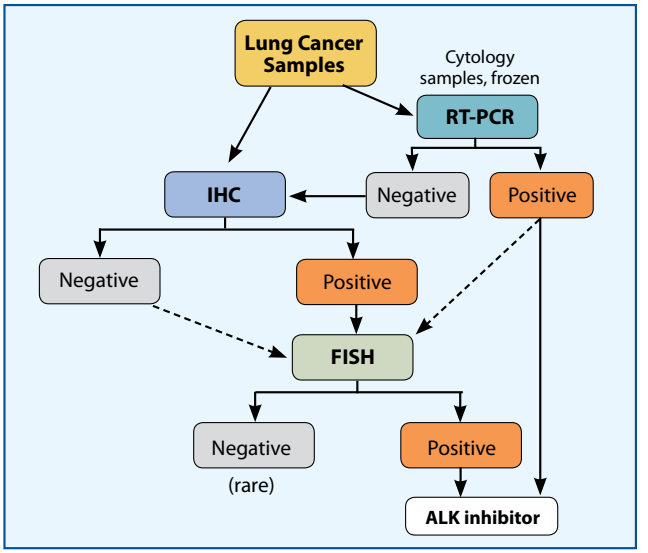

Figure 1. Algorithm proposed by the Japanese Lung Cancer Society, with screening by IHC and confirmation by FISH; RT-PCR is used for cytology specimens. Dotted lines represent possible duplicate examination according to clinicopathologic features. Dotted lines indicate possible duplicated examination according to clinicopathologic features. Modified, with permission from, Yatabe Y. ALK FISH and IHC. You cannot have one without the other. *J Thorac Oncol.* 2015;10(4):548-550.

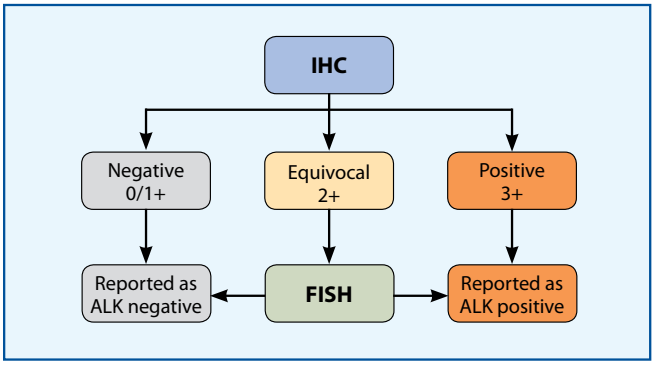

Figure 2. Diagnostic algorithm using ALK IHC and FISH in NSCLC. Modified, with permission, from Paik J, Choe g, Kim H, et al. Screening of anaplastic lymphoma kinase rearrangement by immunohistochemistry in non-small cell lung cancer: correlation with FISH. *J Thorac Oncol.* 2011;6(3):466-472.

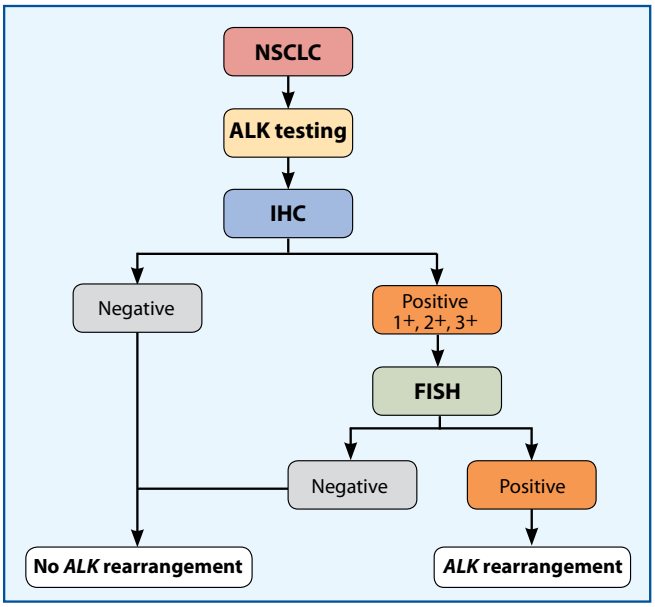

Figure 3. Proposed algorithm for ALK testing in NSCLC if ALK IHC becomes fully validated. Modified, with permission, from Thunnissen E, Bubendorf L, Dietel M, et al. EML4-ALK testing in non-small cell carcinomas of the lung: a review with recommendations. *Virchows Arch.* 2012;461(3):245-257.

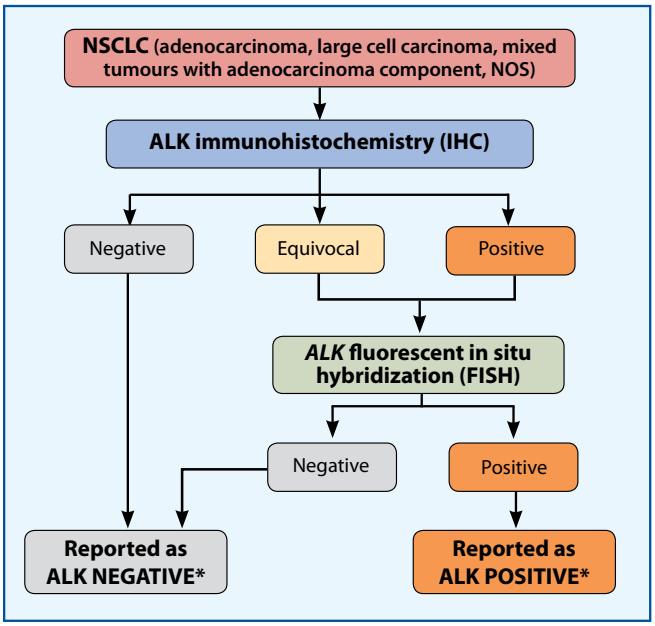

Figure 4. ALK testing algorithm commonly practiced in Canada using the 5A4 laboratory developed test for screening followed by FISH confirmation. Modified, with permission from, Shiau CJ, Tsao MS. Impact of molecular testing for non-small cell lung cancer: a pathologist's perspective. *Can J Pathol.* 2014;6 (Supplement 1): 166-173.

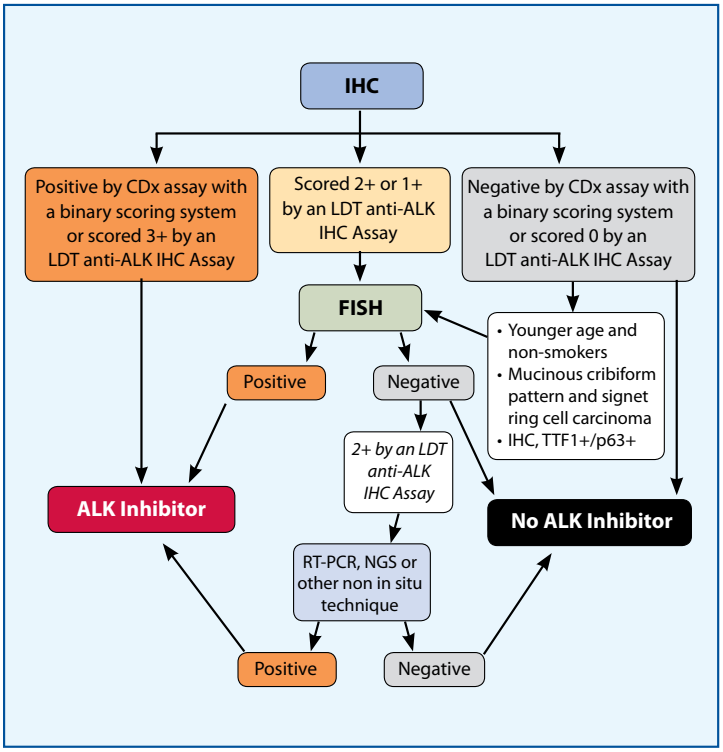

Figure 5. Comprehensive algorithm for the selection of patients with NSCLC to be treated with an ALK inhibitor. A first step by IHC staining with a companion diagnostic assay (CDx) based on a binary scoring system or IHC with a laboratory-developed test based on a four-level score is suggested. FISH analysis is reserved for cases scored 2+ or 1+ by a laboratory-developed test assay and for IHC-negative cases with clinicopathologic parameters more frequently reported in ALK-positive tumors (large green box). Further confirmation by RT-PCR, NGS, or other non–in situ techniques is recommended in cases that are scored 2+ on a laboratory-developed test assay and are negative on FISH (small green box). TTF1 = thyroid transcription factor 1. Modified, with permission, from Marchetti A, Di Lorito A, Pace MV, et al. ALK protein analysis by IHC staining after recent regulatory changes: a comparison of two widely used approaches, revision of the literature, and a new testing algorithm. *J Thorac Oncol*. 2016;11:487-495.

Conclusion

Screening for *ALK* gene abnormalities with IHC is now common practice around the world because of its cost-effectiveness for mass screening, with or without verification of ALK-positive results by FISH. IHC has demonstrated a strong correlation with response to ALK tyrosine kinase inhibitors, and several diagnostic algorithms for ALK testing are routinely used in many countries. The role of NGS, which could replace FISH as the reference technique, needs to be further evaluated, but promising results are emerging.

ALK and ROS1 Analysis in Cytology

By Lukas Bubendorf, Sylvie Lantuéjoul, and Yasushi Yatabe

11

Role of Cytology in NSCLC

FISH testing for detection of *ALK* rearrangement as a predictive marker in NSCLC was initially approved for testing of biopsy material (Kwak 2010). Biopsy material is often preferred for translational studies in clinical trials because paraffin blocks are routinely processed in pathology laboratories and these blocks provide multiple sections for various analyses. However, as many as 40% of all advanced NSCLCs are diagnosed by cytologic evaluation alone, with no concurrent histologic examination of biopsy material. Thus, reliance on histology as the only source for ALK testing would require repeat biopsy in a large proportion of patients, emphasizing the necessity to expand ALK analysis to cytologic specimens.

FISH analysis of cytologic specimens has a long tradition, and FISH technology was used to evaluate cell lines or disaggregated intact nuclei from histologic tumor specimens before it became applicable to tissue sections. FISH is also an established method in several fields of diagnostic cytology. From an analytic point of view, there is no rationale against applying ALK FISH to cytologic specimens. In fact, cytologic specimens have several advantages; for example, in contrast to the situation with histologic sections, the nuclei on cytology smears are not truncated, which allows for the detection of the true number of FISH signals in a nucleus.

Cytology is an attractive, minimally invasive method to collect tumor material for repetitive biomarker analysis on recurrent or metastatic disease. Cytologic diagnosis of NSCLC is typically based on specimens obtained by endobronchial ultrasound-fine-needle aspiration (EBUS-FNA), transthoracic FNA, bronchial secretions or brushes, bronchoalveolar lavages, and pleural effusions or FNA from other metastatic sites. Processing such specimens for FFPE cell blocks has become the preferred method in many laboratories, as cell blocks can be handled in the same way as histologic specimens, and the same protocols for biomarker analysis can be applied (Figure 1) (Alici 2013, Kalhor 2013). Aside from the ability to generate more material with repeated sectioning, cell blocks also have the advantage of long-term preservation of protein or DNA quality. In addition to commercial products, published protocols for cell block construction are available, and three

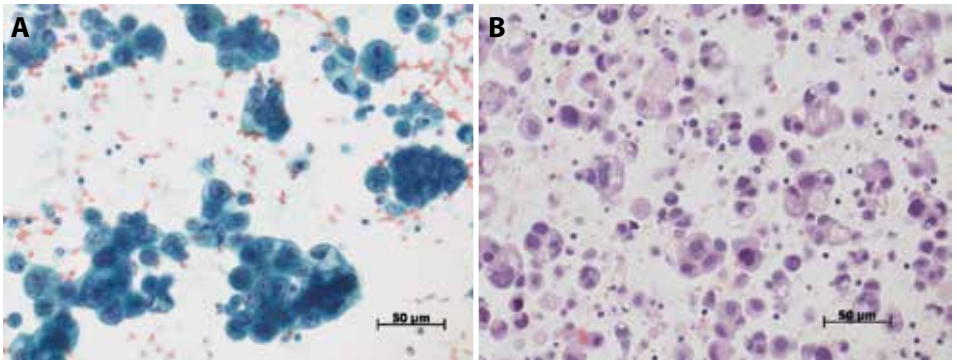

Figure 1. Papanicolaou-stained conventional smear (A) and H & E-stained section of a cell block (B) of a pleural effusion of a pulmonary adenocarcinoma (x400).

commonly used ones are the so-called cell button method, the sodium alginate method, and the plasma-thrombin method (Box 1) (Noda 2010, Orell 2011, Kalhor 2013, Jing 2013, Jain 2014).

Box 1. Protocols for Preparation of Cytology Cell Blocks

Method 1. "Cell button" method (Orell 2011, Kalhor 2013)

a. Gently expel a drop of aspirated or cytospinned material on to a glass slide without spreading or smearing.
b. After a few seconds for adhering, carefully immerse the slide in ethanol for fixation.
c. Gently detach the fixed drop (like a "button") with a scalpel blade and process in the same manner as a small biopsy specimen.

Method 2. Sodium alginate method (Noda 2010)

a. Suspend centrifuge-corrected fluid material and fix in 10% buffered formalin for 2-3 hours.
b. Correct the solution with fixed cells by centrifuge and decant off supernatant formalin and wash with distilled water.
c. Correct the solution again by centrifuge and resuspend the pellet with 0.5mL of 1% sodium alginate.
d. Add sodium calcium (1 M) to the solution and allow to gelatinate.
e. Remove the gelatinated material with forceps and processin the same manner as a small biopsy specimen.

Method 3. Plasma-thrombin method (protocol used at the University Hospitals of Basel and Zurich, Switzerland)

a. Centrifuge the cytologic material for 10 minutes at 2,500 rpm.
b. Remove the supernatant.
c. Pipette two drops of the sediment into a small tapered tube.
d. Add 200 µl plasma, and vortex the specimen briefly.
e. Add 50 µl thrombin, and vortex the specimen briefly.
f. Incubate the specimen for 5 minutes.
g. Put the clot into an embedding cassette (between two filter pads), and close the cassette.
h. Fix the material in 10% buffered formalin.
i. Take out the fixed material and process it in the same manner as a small biopsy specimen.

ALK and ROS1 FISH Analysis in Pulmonary Cytology

FISH is a robust technology that is applicable to almost all types and formats of cytologic specimens. The consensus in the literature is that cytologic and small biopsy specimens are equally suitable for ALK FISH testing. Globally, cell blocks appear to be the most commonly

applied cytology format for ALK testing, as the protocols and criteria for ALK FISH analysis on cell blocks are identical to those for histology. However, a subset of cell blocks contain too few or no cancer cells for molecular analysis (Knoepp 2013, Proietti 2014, Wang 2015), and differentiating tumor cells from adjacent reactive cells is more challenging than in conventional cytology, especially during FISH analysis. Therefore, ALK analysis of cytologic specimens is another valid option preferred in some laboratories (Figure 2) (Betz 2013, Savic 2013, Bozzetti 2015, Li 2015). An important advantage of conventional cytology is the ability to select the optimal cytologic slide among all previously stained slides for FISH analysis, with no need for additional unstained slides. In addition to a lack of nuclear truncation and related artifacts, the DNA quality in air-dried or alcohol-fixed cytologic specimens is better than that after formaldehyde fixation, which leads to crosslinking and chemical modification of nucleotides. This fact provides an explanation for a success rate as high as 100% for ALK FISH analysis in conventional cytology and a failure rate of up to 19% for histologic specimens (McLeer-Florin 2011, Savic 2013, Proietti 2014).

FISH is applicable to almost all types of cytologic specimens, including conventional smears, cytospins, or liquid-based preparations (eg, ThinPrep, Hologic; or SurePath, BD Diagnostics) regardless of fixation type (air-dried and alcohol-based fixatives). For cytologic examination of lung specimens, the use of adhesive-coated or positively charged slides is recommended, as the adherence is improved and the cells are prevented from floating off during technical FISH procedures. FISH works equally well on unstained specimens as well as those processed with Papanicolaou, hematoxylin, or a modified Giemsa stain, and a separate procedure is not usually required, except with a modified Giemsa stain, for which destaining with an acid-alcohol technique is recommended before FISH analysis (Betz 2013).

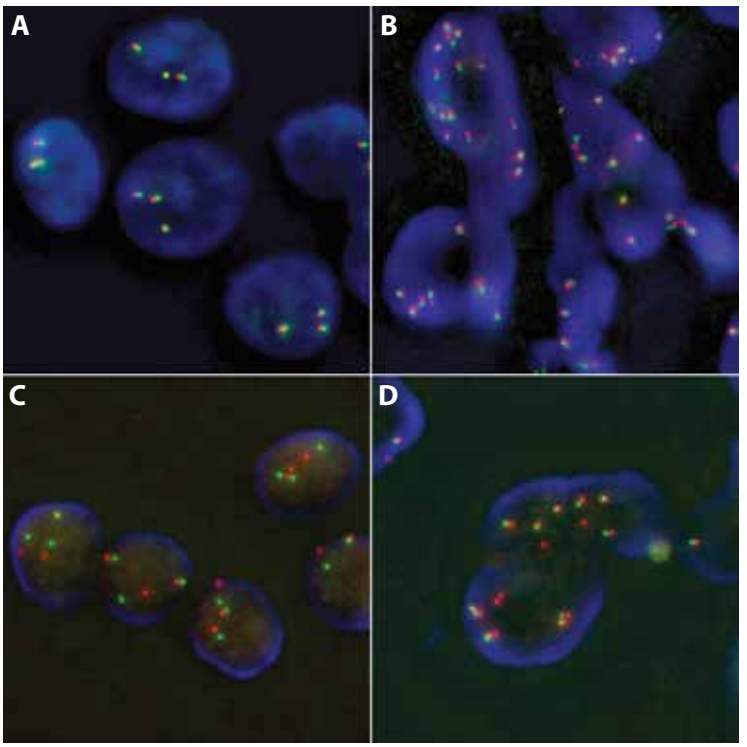

Figure 2. Representative findings on ALK FISH testing of previously Papanicolaou-stained conventional cytologic slides (compressed z-stacked images showing the projection of all FISH signals in the intact cell nuclei). Two ALK-negative cancers with three normal (fused) *ALK* signals (A), and with higher numbers of normal *ALK* signals per tumor cell nucleus (B). Two ALK-positive cancers with one or two break-apart signals (a distance between 5′ green and 3′ red signals of at least two times the diameter of the signal) (C) or a single 3′ red signal with no corresponding 5′ green signal, in addition to several normal signals per tumor cell nucleus (D).

Notably, FISH also applies well to immunocytochemically stained specimens if 3-amino-9-ethylcarbazol (AEC) is used as a chromogen; use of 3, 3' diaminobenzidine (DAB) can interfere with the FISH signals because of autofluorescence. A protocol for FISH on stained cytologic specimens has been published (Thunnissen 2012a). There may be concerns about using diagnostic cytologic slides for FISH analysis because of a legal requirement that cytology laboratories archive diagnostic slides for several years and the potential need to review slides related to rare cases even years after diagnosis. These concerns can be addressed by capturing representative images or by scanning the whole slide before analysis. It is also possible to stain slides again after FISH analysis (Betz 2013).

The threshold for a positive ALK FISH result was established on the basis of analysis of histologic samples (a signal pattern typical for *ALK* rearrangement in at least 15% of cancer cells), but each individual laboratory needs to determine its own threshold for ALK-negative cytologic specimens until consensus recommendations become available. (*See Chapter 3 for more information on cutoff values.*) Nonoverlapping tumor cells should be selected for ALK FISH, but most three-dimensional clusters remain amenable to ALK analysis, as the detection of rearrangement signals is not hampered by three-dimensionality. For specimens with a low proportion of tumor cells, use of an automated stage guided by appropriate software to reposition the cancer cells increases the precision of analysis and facilitates review of the FISH result.

For ROS1 FISH analysis of cytology specimens, the preanalytic and analytic considerations and standards do not differ from those for FISH ALK analysis. ROS1 FISH analysis poses no specific challenges, and the same scoring rules for histologic specimens apply (as outlined in Chapter 7). The commercially available ROS1 FISH probes have a green 3' (centromeric) part of the fusion breakpoint that allows ROS1 and ALK (an orange 3' fluorochrome) tests to be distinguished, particularly if the two tests are to be run on the same slide simultaneously. Data on ROS1 FISH analysis in cytology are still scarce. In the only published study so far, all 12 cytologic samples, including eight unstained and four destained smears, were analyzed successfully (Bozzetti 2015).

ALK and ROS1 IHC in Cytology

IHC to detect overexpression of the ALK protein has recently emerged as a valuable method to screen NSCLC for subsequent FISH analysis and for further evaluation of uncertain FISH findings (as described in detail in Chapters 4 and 5). As with histologic specimens, ALK IHC is promising with cytologic specimens, including cell blocks and conventional or liquid-based cytologic preparations (Moreira 2012, Martinez 2013, Savic 2013, Tanaka 2013, Wang 2015). ALK IHC on cell blocks using the ALK (D5F3) CDx Assay on the Benchmark XT automated immunostainer (Ventana) was 100% concordant with FISH in a study published in 2015 (Wang 2015). The accuracy of ALK IHC on Papanicolaou-stained cytologic slides has been equally high (Savic 2013, Tanaka 2013). This accuracy was achieved with the 5A4 antibody (Novocastra; *see Chapter 4*) and the Bond-Max automated immunostainer (Leica Biosystems) (Figures 3 and 4). Additional studies are needed to validate this antibody on other platforms in cytology.

ROS1 IHC is highly accurate for prescreening of histologic specimens for *ROS1* rearrangement (*see Chapter 5*). In the experience of one of us (L.B.), ROS1 IHC is also highly accurate

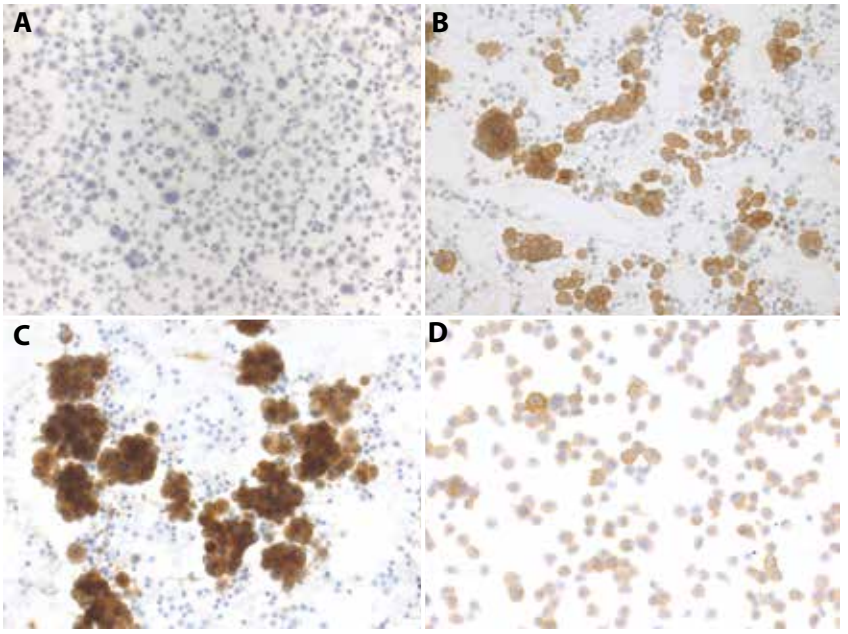

Figure 3. IHC on conventional cytologic slides from malignant pleural effusions of pulmonary adenocarcinoma (x200), showing ALK-negative (A) and ALK-positive (B, C) tumors, with an *ALK*-rearranged H2228 cell line used as a positive control (D). The 5A4 antibody (Novocastra) and an automated immunostainer (Bond-Max; Leica Biosystems) were used, and all results were confirmed by FISH.

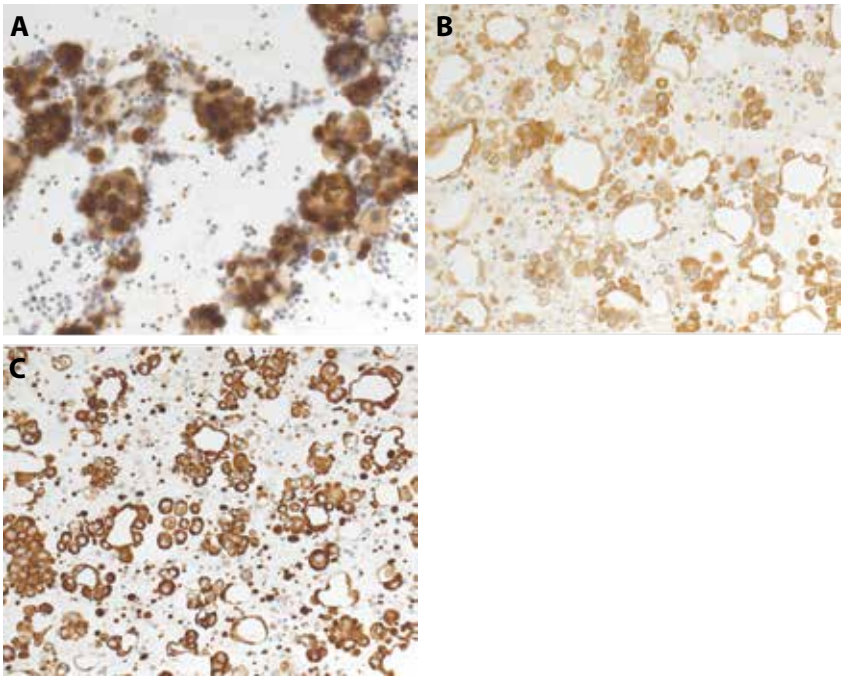

Figure 4. IHC of an ALK-positive pulmonary adenocarcinoma (x200), was done with the 5A4 antibody (Novocastra) on a slide with previously Papanicolaou-stained tissue (A) and corresponding cell block sections stained with the 5A4 antibody (B), and the D5F3 antibody (Ventana Medical Systems, Inc.) (C).

when analyzing cytology smears and cytospin specimens with use of the D4D6 rabbit monoclonal antibody (Cell Signaling Technology) on the Bond-Max automated immunostainer (Leica Biosystems) (Figure 5). However, published studies on ROS1 IHC in cytology are still missing.

Because IHC is broadly available, the number of pathology laboratories using IHC as a first means of ALK and ROS1 testing may also increase in cytology and limit FISH analysis to specimens with equivocal or positive findings on IHC. However, IHC performed on cytologic smears and/or cytospin slides may be influenced much more by various factors during the preanalytic phase because of high variability in preparation, fixation, and staining methods of cytologic specimens (Fischer 2014, Kirbis 2011). Thus, IHC should be performed only in laboratories with experience and appropriate external quality-control programs in place.

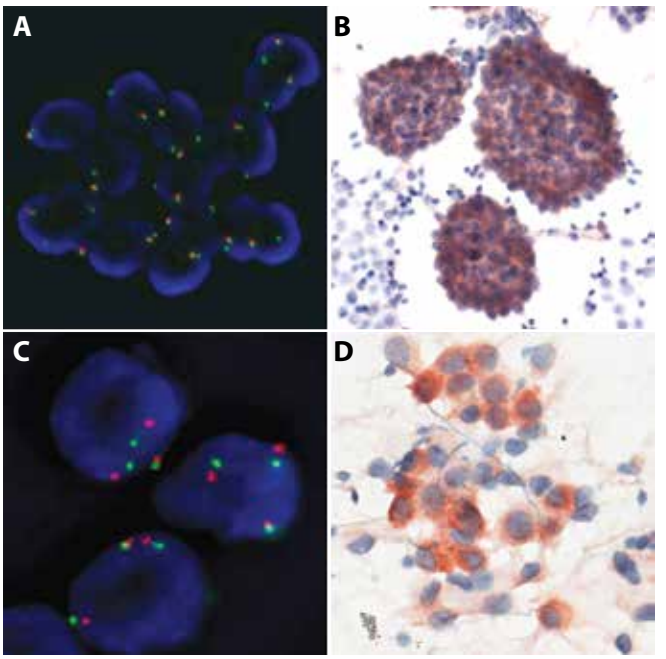

Figure 5. Representative findings on ROS1 testing of previously Papanicolaou-stained conventional cytologic slides of two ROS1-positive pulmonary adenocarcinomas. One single 3′ green signal without corresponding 5′ red signal in addition to one fused signal (A) or two break-apart signals in addition to one fused signal (C) (compressed z-stacked images showing the projection of all FISH signals in the intact cell nuclei). Matched positive ROS1 immunohistochemistry (B and D) (x400). The D4D6 antibody (Cell Signaling Technology) and an automated immunostainer (Bond-Max; Leica Biosystems) were used.

RT-PCR and Other Platforms

RT-PCR may be used to analyze cytologic specimens for *ALK* rearrangements (Betz 2013, Mitiushkina 2013, Wang 2016) and the same has been shown for *ROS1* rearrangements (Zhao C 2014,). RT-PCR may be adequate for confirming the results of ALK IHC or FISH, but is less appropriate for primary screening for *ALK* rearrangement (*see Chapter 8*). Further studies are needed to determine the utility of other platforms (eg, multiplex gene expression assays and RNA-based NGS gene fusion panels) for cytologic material.

Conclusion

Cytologic preparations, including conventional slides and cell blocks, can serve as a useful alternative to biopsy specimens for predictive ALK and ROS1 analysis.

Reporting of ALK and ROS1 Testing

By Erik Thunnissen, Marileila Varella-Garcia, Lynette Sholl, Yashushi Yatabe, Andrew Nicholson, and Ming Sound Tsao

12

Consistent with the standard reporting of the predictive molecular pathologic testing of NSCLCs, ALK and ROS1 testing reports should include four sections: preanalytic, analytic, results, and interpretation (or conclusion), regardless of the diagnostic method used (FISH, IHC, RT-PCR, or NGS).

Preanalytic Section

In addition to patient identifiers, this section of a standard report should include a summary of the specimen type and diagnosis, if the molecular diagnosis is not part of the pathologic report being issued at the same time. The following details should be reported.

Specimen features

- Specimen size and type: surgical resection (lobectomy, pneumonectomy, segmentectomy, wedge), biopsy (bronchial/transbronchial biopsy, core-needle biopsy), FNA cytology or fluid (pleural, cerebral spinal fluid) cell block
- Tissue preservation: snap-frozen (storage temperature) or FFPE
- Tissue fixation: only buffered formalin is recommended as the fixative for paraffin-embedded tissue samples (Optionally, if tissue has been processed with decalcification solution, this should be documented, as well as the reagent used.)

Tumor histologic diagnosis

When ALK or ROS1 testing is reported as a stand-alone test separate from the histologic diagnosis report, the original histologic diagnosis of the tumor should be recorded. If there is a significant discrepancy in the tumor classification, the revised diagnosis should be documented with an explanation for the discrepancy. For adenocarcinoma, the type and subtype should follow the 2015 World Health Organization (WHO) lung cancer classification (Travis 2015). Tumors with more than one type should be described as such: adenosquamous carcinoma, combined small cell lung cancer with adenocarcinoma, and large cell neuroendocrine carcinoma (pure or combined with adenocarcinoma).

Tumor assessment

- Estimate of tumor cellularity of the whole section (percentage of tumor cell nuclei compared with all nuclei present on the section), to determine whether the sample has sufficient tumor cells for FISH and/or RT-PCR, as dictated by laboratory requirements
- For ALK and ROS1 IHC, when the number of tumor cells in the section is low, the estimated number of tumor cells
- Percentage of tumor cells on initial section or block, and, optionally, after cancer-cell enrichment, such as manual dissection, to reflect the tumor cellularity of the sample part used for DNA/RNA isolation
- Extent of necrosis, inflammatory cell infiltrates, anthracosis, and tissue artifacts
- Test results of additional diagnostic IHC markers, such as thyroid transcription factor-1 (TTF-1), p63/p40, and mucin stains, if available, to support the histologic diagnosis

Overall specimen adequacy

- Documented as "adequate for testing" (relative to the findings noted here, as well as for ALK testing) or as "suboptimal" (with notation of the reason or reasons)

Other information

- Past drug treatment, if available (optional)

Analytic Section

This section should include the basic methodology for each assay used, along with the assay sensitivity and threshold. The information should be adequate enough for another laboratory to understand what was done, in the event of a discrepancy between laboratories, or if asked to repeat testing. In the end, a possible disclaimer of potential limitations according to the method used should be noted (eg, small sampling size, cryptic rearrangements, and low integrity of RNA).

- FISH: probe set (manufacturer, type) and threshold used to define a positive result
- IHC: antibody type (source), antibody concentration, incubation time and temperature, and secondary signal enhancement system
- RT-PCR: method used, primers, probes and their references, and analytic sensitivity of the assay
- NGS: method used; eg, platform, type of panel (amplicon, hybridization), exome, WGS; sensitivity of the method (percentage variant alleles detectable in a background of wild-type DNA); reference sequences for genes tested; results (using HGVS mutation nomenclature); how/where additional information about the analysis can be obtained (Deans 2016)

Results Section

This section should report the outcome of the test, including incidental findings and variants of uncertain significance. Inconclusive results should be clearly reported as such. The results should be reported as positive or negative for *ALK* or *ROS1* rearrangement so that oncologists and nonspecialist pathologists can readily understand the results. In addition, specific elements should be reported according to the testing method used.

FISH: The number of cells analyzed and the percentage of cells displaying positive patterns should be documented. If an atypical pattern was seen, it should be noted (eg, "negative for *ALK* rearrangement, see Interpretation"); if the International System for Human Cytogenetic Nomenclature (ISCN) is used, it should be accompanied by an easily understandable discrete result.

IHC: To ensure the validity of the technique, an external positive control is required for all tests. For ALK testing, preferably this control is a cross-section of an appendix to show positive staining of the myenteric plexus ganglion cells, and ALK-positive NSCLC specimen (*see Chapter 4*). For *ROS1*, preferably weak (U118-MG) and strong (HCC78) *ROS1*-expressing cell lines are included. Depending on the signal-enhancement method used, either a two-tiered approach (positive, negative) or a four-tiered approach (negative, positive: 1+, 2+, 3+) should be reported (*see Chapters 4 and 5*). In addition, a test is described as "equivocal" if uncertainty exists about the IHC result and FISH confirmation is required (eg, 1+ or 2+ in a four-tiered approach or weak positive staining in a two-tiered approach). A test is described as "indeterminate" when the test fails and new material (repeat biopsy specimen or a separate block) is required.

RT-PCR: The fusion patterns should be reported with gene names and exon numbers before the breakpoints, such "*EML4-ALK* (E13;A20)" for *EML4-ALK* variant 1 and "*CD74-ROS1* (CD6;R34) in case of the fusion involving exon 34 of ***R****OS1* fused to exon 6 of *CD74*, according to the recommendation for the descriptions (Soda 2012). (Additional information is available at http://atlasgeneticsoncology.org/Tumors/inv2p21p23NSCCLungID5667.html. The range of detectable fusions with the assay should also be clarified.

NGS: Standards for NGS reporting of structural variants have not been fully established, but a proposal recently published (Deans 2016). For the detected mutations it is essential to use Human Genome Variation Society (HGVS; http://www.hgvs.org/mutnomen/) mutation nomenclature and to include the appropriate reference sequence including version number used for gene or transcript number if locus-specific genome sequences (LRGs) are used. For reference purposes, the HUGO Gene Nomenclature Committee (HGNC) approved that gene symbols should be used at least once. In addition, it is strongly recommended to include genomic coordinates in order to ensure uniform bioinformatics analysis and consistent documentation of identified variants. If genomic coordinates are used, then the appropriate genome build must be stated.

Interpretation or Conclusion Section

The interpretation should be readily understandable by nonspecialists, and include the following.

- Specimen type and diagnosis (primary or after treatment with an ALK or a ROS1 inhibitor)
- Clinical interpretation, including outcome of the molecular test and an overall statement of the likelihood that the cancer will respond to or resist treatment with an ALK or a ROS1 inhibitor (considering also the clinical evidence)

- Explanation (as best as is known) for an indeterminate result, equivocal result, or discrepant results with multiple testing, whether due to assay failure, insufficient specimen, or another reason (eg, atypical FISH patterns) and suggestion of requirements for testing a different specimen that would be more likely to yield a successful result

Conclusion

Reports on the results of ALK and ROS1 testing should include sufficient details for both laboratory physicians and clinicians to understand the origin and features of the sample tested, the nature of the test performed, and the accuracy and potential clinical utility of the results.

Guidelines and Standardization Studies

By Yasushi Yatabe, Sylvie Lantuéjoul, Erik Thunnissen, Keith Kerr, and Ming Sound Tsao

13

With the development of ALK-targeted therapy for NSCLC, several oncology and pathology organizations around the world have established recommendations for ALK testing, either as stand-alone documents or as part of broader guidelines on molecular testing. In addition, several regional or international multicenter studies have been conducted to standardize ALK testing protocols across laboratories. In contrast, a therapeutic agent against *ROS1* rearrangement was approved in 2016, thus establishment of the guidelines and attempts for standardization of the assays are in progress.

Guidelines

Guidelines for molecular testing on lung cancer specimens may include clinical and methodologic recommendations. The most comprehensive guideline was the result of a collaborative effort by experts representing CAP, IASLC, and AMP (Lindeman 2013). The guideline was developed to select patients with lung cancer for EGFR and ALK tyrosine kinase inhibitors, and contained 15 evidence-based recommendations with the following major messages: (1) use molecular testing for *EGFR* mutations and *ALK* rearrangements to guide patient selection for EGFR and ALK inhibitors, respectively, in all patients with advanced-stage adenocarcinoma, regardless of clinical features, (2) to prioritize EGFR and ALK testing over other molecular tests, and (3) to address how the testing should be performed. After the publication, the guideline was reviewed by a panel of the American Society of Clinical Oncology, in terms of developmental rigor and the contents of the recommendations, and it was endorsed with the advice to update the guideline (Leighl 2014). Subsequently, the three organizations continued to collaborate in updating the guideline and made the draft public for review and open comments. The draft update includes new recommendations for other targetable genes (*ROS1, BRAF*, *RET*, *ERBB2*, and *MET*), as well as recommendation statements that have been reaffirmed since the initial guideline and that are consistent with the Institute of Medicine's Clinical Practice Guidelines You Can Trust. The updated CAP/IASLC/AMP guideline will be published after the publication of this Atlas, but we have included here some of the essence of the guideline related to *ALK* and *ROS1*, according to the draft

that was made available for review and open comments. The recommendation remains that all patients with advanced NSCLC be tested for *ALK* gene rearrangement if the tumor is of adenocarcinoma subtype or has an adenocarcinoma component. Testing for *ALK* gene rearrangement should also be performed if the specimen is small and an adenocarcinoma component cannot be excluded. Similar to testing for the *EGFR* mutation, it is necessary for a pathologist to be involved in the selection of tissue samples to determine the adequacy of specimens. According to the US FDA approval of the ALK (D5F3) CDx Assay (Ventana), it is recommended that physicians use IHC as an equivalent alternative to FISH for ALK testing. Regarding *ROS1*, IHC is recommended as a screening test for patients with lung adenocarcinoma, and a molecular or cytogenetic method should be used to confirm positive ROS1 results on IHC.

Standardization Studies

In the United States, the label for crizotinib notes the requirement for ALK testing with a US FDA-approved assay; the ALK FISH Break Apart FISH Probe Kit (Abbott Molecular) and the ALK (D5F3) CDx IHC assay (Ventana) are the only such approved tests for ALK. In other parts of the world, regulatory agencies do not mandate the use of a specific method but they do require the use of a validated assay. There is, however, no guidance on what that validation should be. Furthermore, the emergence of multiple ALK inhibitors makes the situation complex because the manufacturers of individual ALK inhibitors can develop their own companion diagnostic tests and patients should be tested with corresponding companion tests by regulation. Several multicenter studies have been conducted across different countries or regions of the world to implement standardization (Table 1). Because of differences in regulations across countries, biomarker testing for patients with lung cancer should be done according to the recommendations in the CAP/IASLC/AMP guideline. The different regulations and the results of quality assessment show that various modalities and methods are currently used in actual clinical practice. Some institutes still use ALK1 antibodies for lung cancer testing, despite the low sensitivity that has been confirmed with multiple studies. FISH and IHC are currently common methods worldwide to detect *ALK* gene rearrangement or fusion protein expression, while RT-PCR is most commonly used

Table 1. Multicenter Standardization Studies for ALK Testing Methods

Study	Region/ Country	Number of Participating Laboratories	Methods	Material
von Laffert et al., 2014	Germany	8	IHC and FISH	Tissue microarray
Cutz et al., 2014	Canada	13	IHC and FISH	1 x 1-cm tumor tissue slides
Marchetti et al., 2014	Italia	37	FISH	Tissue microarray
von Laffert et al., 2014*	Europe	16	IHC	Tissue microarray
Tembuyser et al., 2014	Europe	173	IHC and FISH	Tissue microarray
Li Y et al., 2016	China	94	IHC, FISH, RT-PCR	Tissue microarray
Ibrahim et al., 2016	30 countries	156	IHC	Tissue microarray

in China (Table 2). Even among National Cancer Institute-designated cancer centers in the United States, various combinations of ALK detection modalities are used (Schink 2014). Given these circumstances, it is important to recognize that each modality and method has advantages and disadvantages and that quality control is indispensable to ensuring that ALK testing is performed appropriately, especially now that effective treatment options are available. Unfortunately, quality control systems also vary among countries and regions; in one survey, nearly half of laboratories in Asia did not participate in quality assurance schema for EGFR testing (Yatabe 2015).

Table 2. Companion Diagnostic Tests for ALK and ROS1 Inhibitors

Country/ Region	ALK Inhibitor			ROS1
	Crizotinib	Alectinib	Ceritinib	Crizotinib
United States	Vysis ALK FISH	Vysis ALK FISH	Vysis ALK FISH[a]	Not available[b]
	Ventana ALK IHC	Ventana ALK IHC		
Japan	Vysis ALK FISH	Vysis ALK FISH	Vysis ALK FISH[a]	Not approved yet[c]
		Nichirei iAEP IHC		
Europe	No requirement of positivity on a specific companion diagnostic kit			
China	Vysis ALK FISH	Not approved yet	Not approved yet	Not approved yet[c]
	Surexam ALK FISH			
	Ventana ALK IHC			
	AmoyDx EML4-ALK Real Time PCR			
Korea	Vysis ALK FISH	Not approved yet	Vysis ALK FISH (No description related with diagnosis method in label)	Not approved yet[c]

[a] *Approved for patients who have already been treated with crizotinib.*
[b] *The NCCN guideline for NSCLC refers to the article using ROS1 FISH (National Comprehensive Cancer Network, 2017).*
[c] *The clinical trial for ROS1-positive lung cancer in Asia was conducted using AmoyDx ROS1 Real Time PCR for patient selection.*

External Quality Assessment Program

For optimal ALK testing in NSCLC, the quality of the sample, the validation status of the analytic procedure, and the reliability of the reporting of test results are crucial. However, individual laboratories may have difficulty developing and validating ALK IHC testing and sourcing the relevant tissue for use as positive controls in daily internal quality control because of the rarity of *ALK* gene rearrangements in lung cancer.

In 2012, the European Society of Pathology proposed an external quality assessment scheme to promote high-quality biomarker testing in NSCLC for *EGFR* mutation analysis and *ALK* gene rearrangement detection. Beginning in 2014, ROS1 testing has also been included (Tembuyser 2014). In parallel, the UK National External Quality Assessment Scheme and Nordic Immunohistochemical Quality Control (NordiQC) also organized ALK IHC external quality assessment schemes (Ibrahim 2016).

In Canada, a multicenter study, the Canadian Anaplastic Lymphoma Kinase (CALK) study, was conducted to optimize and standardize ALK IHC and FISH (Cutz 2014). In this study, samples were initially used to optimize local IHC protocols, followed by a repeat IHC study to assess the results of standardization. Post-optimization concurrent IHC/FISH testing

achieved 100% sensitivity and specificity for IHC versus FISH, suggesting a good model for multicenter standardization and optimization of laboratory developed tests. A similar attempt for ROS1 IHC and FISH is planned in Canada. In addition to the standardization, the Canadian Immunohistochemistry Quality Control (CIQC) provides ALK (lung cancer) proficiency testing for IHC laboratories in the country (Cheung 2015). Participating laboratories have used the proficiency testing exercise either to confirm that their testing was properly calibrated or to improve their protocols, which was confirmed by the achievement of significantly better results in repeated testing.

To conduct quality assurance studies, sample size and statistical methods should be considered carefully (Mahe 2014, Sabour 2016). Because *ALK* gene rearrangement is found in about 4% to 5% of NSCLC (primarily adenocarcinoma), more than 2,000 samples are needed to test the accuracy of a novel modality. When sensitivity, specificity, and accuracy are used to evaluate the validity of a single test compared with the standard test, sample size (preferably more than 50), number of positive and negative samples, and satisfactory levels should be carefully discussed. It is also important to determine the standard method to be compared. Multiple large-scale studies have already shown a non-negligible number of discordant results across modalities (*see Chapter 10*).

Conclusion

Since the first ALK inhibitor, crizotinib, was introduced into clinical practice more than 5 years ago, ALK testing has been integrated into clinical practice worldwide. During this period, various screening algorithms and guidelines were proposed, but they were not described here because of great differences in regulations across countries. More recently, ROS1 testing has also become a routine test, and accurate and timely detection of *ROS1* gene rearrangements is clinically required. An international molecular testing guideline is being updated for ALK and ROS1 testing, and various modalities are used in accordance with the emergence of new inhibitors and developments in technology. Under these circumstances, standardization and quality assurance of the methods become a clinically crucial issue.

Testing Practice and Algorithms

By Keith M. Kerr, Jin-Haeng Chung, Andrew Nicholson, Fred R. Hirsch, Yasushi Yatabe, and Ming Sound Tsao

14

The preceding chapters in this Atlas have discussed *ALK* and *ROS1* gene rearrangements, their biology, and various techniques that may be used for detecting these alterations in patients with NSCLC. In this chapter, we consider a number of issues related to how testing may be organized and implemented. Among the topics addressed are a comparison of sequential and parallel molecular testing and of reflex and bespoke testing, the development of a contemporary testing algorithm, and the potential impact of NGS on practice.

Sequential vs. Parallel Testing

The concept of sequential testing is based on the premise that, for all practical purposes, many addictive oncogenic targets that may be sought to inform treatment in advanced-stage lung adenocarcinoma are mutually exclusive. With sequential testing, the most common molecular aberration is tested for first, then, if testing is negative, the next most common aberration is sought, and so on; testing costs are saved, as unnecessary tests are not carried out. When sequential testing was first proposed, testing began with *KRAS* mutation, followed by *EGFR* mutation, then *ALK* gene rearrangement, and so on as required. In Asian countries, this testing sequence started with *EGFR* mutation because of the high frequency of that abnormality in the Asian population. Although the sequential approach may appear attractive in terms of cost-savings, and to some extent, the sparing of tumor tissue, it is probably a false economic perspective and is not recommended. The extended time required to run a series of sequential tests may be too long for a sick patient with advanced lung cancer, and many have found that this approach is inefficient for laboratories. In addition, because some biopsy or cytology samples are small, this sequential testing strategy is associated with a risk of using up all available tissue before FISH or IHC can be performed for ALK or ROS1 testing. On the basis of these factors, parallel testing is recommended. With this approach, all molecular tests that are relevant for a particular patient, at a particular time, are carried out contemporaneously. This approach is usually more efficient and, if a patient's tumor does harbor a targetable alteration, especially a less frequent one such as *ALK* or *ROS1* rearrangement, then the team treating the patient learns the mutation status

much more quickly. This approach will also identify those extremely rare patients who have more than one molecular alteration in their tumor, although the clinical significance of this finding remains uncertain.

Reflex vs. Bespoke Testing

It is difficult to make a clear recommendation on the issue of reflex vs. bespoke testing—or testing initiated by the pathologist immediately after diagnosis compared with testing initiated by the oncologist or multidisciplinary team only when needed. Different approaches to testing work for different multidisciplinary teams treating people with lung cancer and, indeed, both approaches may be appropriate for the same team, depending on patient circumstances.

In most circumstances, however, reflex testing is recommended. In the present context, reflex testing refers to testing for a patient with a diagnosis of nonsquamous NSCLC, de facto adenocarcinoma, although there are important caveats. As a result, testing would be done for patients with definite adenocarcinoma, with adenocarcinoma suggested by the results of IHC, or with NSCLC for which the IHC results are not conclusive. This process will be greatly facilitated by having appropriate tissue sections precut and available for testing, as discussed in Chapter 3. Reflex testing has the advantage of a short time between testing and the delivery of a molecular profile to inform a treatment decision for the patient. In fact, emerging evidence indicates that the availability of biomarker test results at the time of the initial oncology consultation is associated with a shorter median time from consultation to treatment decision and time to the start of treatment (Lim 2015). Another advantage of reflex testing is that the process becomes routine in the laboratory; carrying out extra diagnostic tests during the initial period when the case is in the so-called active stage is more efficient than starting another investigation at a later date, even if only a matter of days or a few weeks in the future. In addition, with routine testing, positive cases are less likely to be missed for one reason or another.

Advocates of bespoke testing assert that this practice eliminates testing that may not be needed for treatment of certain patients (eg, a patient is not suitable for or does not wish to have targeted therapy). At the time of diagnosis, the pathologist is rarely aware of these matters and often will not know the clinical stage of the disease. During the time when ALK tyrosine kinase inhibitors were approved or licensed only for second-line use, it was argued that the ALK status was not required until second-line therapy was being considered. However, many oncologists prefer to know the ALK status during the period of first-line chemotherapy, as this information may influence treatment decisions for patients who have a limited response to chemotherapy or in cases with other difficulties with first-line treatment. This issue will no longer be a factor as ALK inhibitors move into a routine first-line indication.

Bespoke testing will work adequately for some multidisciplinary teams if the timing of the test decision does not delay treatment and if the pathology laboratory can handle the extra processing steps efficiently. As already discussed, having to return to the retrieved tissue block and cut it again for ALK testing can waste tissue and compromise the chance of a successful test if the last remaining tissue is lost during repeat facing of the tissue block. Some of the issues about which patients should have testing for *ALK* and *ROS1* rearrangements

have already been discussed elsewhere in this Atlas. The cohort of patients recommended for testing are those who have a definite, probable, or possible diagnosis of adenocarcinoma, or for whom this diagnosis cannot be reasonably excluded (Lindeman 2013, Kerr 2014). Testing is not recommended for patients with definite or possible squamous cell carcinoma. Clinical features, such as sex, age, and smoking status should not be used as primary determinants of whether or not to test. The caveat to this practice is a diagnosis of squamous cell carcinoma for a never-smoker or a very long-term ex-smoker. In these very uncommon instances, guidelines recommend that testing should be considered (Peters 2012, Lindeman 2013); (*also, see Chapter 13*). In such a situation, a team that uses reflex testing would also initiate bespoke testing once the squamous cell carcinoma has been diagnosed. It is unlikely that the pathologist would be aware of these clinical features at the time of initial diagnosis. This situation emphasizes the need for good communication among all members of the multidisciplinary team and of providing adequate clinical information to the pathologist when samples are sent for diagnosis.

Testing Algorithms

Testing algorithms will depend to some extent on which tests are being performed and where they are being carried out. For this discussion, the availability of a treatment for a particular identified target is assumed. Some laboratories, especially in academic centers, may test for other molecular targets in NSCLC, but the context would be research, with the aim of entering any identified patients into clinical trials. Although trials are vital, testing for other molecular targets in NSCLC must remain outside of a core recommendation for clinical practice.

Some laboratories will outsource their molecular testing for NSCLC, by choice or need. This practice will vary greatly in different parts of the world, and it is difficult to generalize. For small laboratories, it may not be practical to develop testing in-house, or the required technology or expertise may not be available. Any recommended testing algorithm should still apply in this scenario; it will mean, however, that certain steps in the process will take longer. Generally, time will be needed to transfer the tissue from the pathology laboratory to the molecular testing laboratory and to communicate the test outcome(s) to the requesting oncologist, multidisciplinary team, or pathologist. For ALK and probably also ROS1 testing, where IHC is used either as an initial screening test to identify cases to be submitted for confirmatory testing by FISH or multiplex PCR or as the primary test to select patients for treatment with a tyrosine kinase inhibitor, it is unclear whether IHC testing should be conducted in the laboratory at which the initial diagnosis of NSCLC was made or in the laboratory providing the molecular testing service. Typically, this decision will be made at the local level, depending on the availability of suitable resources and expertise at each respective site.

In the algorithm presented here (Figure 1), it is assumed that appropriate steps are taken to diagnose and subtype NSCLC and that the use of IHC is limited to assist in subtyping (eg, testing for p40, TTF1). The cohort of patients with tumors suitable for ALK and ROS1 testing is the same group for whom EGFR testing is mandatory, and other mutations may also be sought. It is therefore not helpful to think about ALK or ROS1 testing in isolation. The algorithm must accommodate **all** of the required tests on the same, usually very limited,

tissue sample. Currently, in most health systems, drugs are available only for patients with *EGFR*-mutated and *ALK*-rearranged NSCLC; as such, testing for these abnormalities is considered mandatory, and parallel testing is recommended. Some centers still use FISH as the primary screening test for *ALK* rearrangement but, as discussed in earlier chapters, substantial evidence supports the use of IHC for ALK testing, and either patients with tumors that test positively are treated or the result is confirmed by another technique. IHC has advantages over FISH testing; it is cheaper, is rapid, and can be easily integrated into the pathologic assessment of the case, especially when IHC is already used for subtype diagnosis in a significant number of cases. This integration is greatly facilitated by so-called reflex cutting of extra tissue sections at the time the block is initially cut, in cases where lung cancer is a possibility. Obviously, this practice depends on local arrangements and relies on good communication when the sample is submitted to the laboratory for diagnosis. ALK IHC results can be available as a next-day service, which means that an ALK inhibitor can be prescribed only days after the NSCLC diagnosis is made.

There is generally less experience with ROS1 testing, although with the recent approval of crizotinib for patients with ROS1-positive tumors, experience will increase. ROS1 testing may be considered after other testing results are negative or it may become an upfront test to be done in parallel with ALK IHC testing (Figure 1). Some laboratories may choose to perform ROS1 FISH testing as the initial screening test, but there is a strong argument for using ROS1 IHC as the initial screening test and using FISH as a confirmatory test in the same way that testing is often done for ALK (*see Chapter 7*). Evidence suggests that ROS1 IHC has not reached the specificity needed, at least with currently available reagents, to be used as the primary prescription-determining test (*see Chapter 5*).

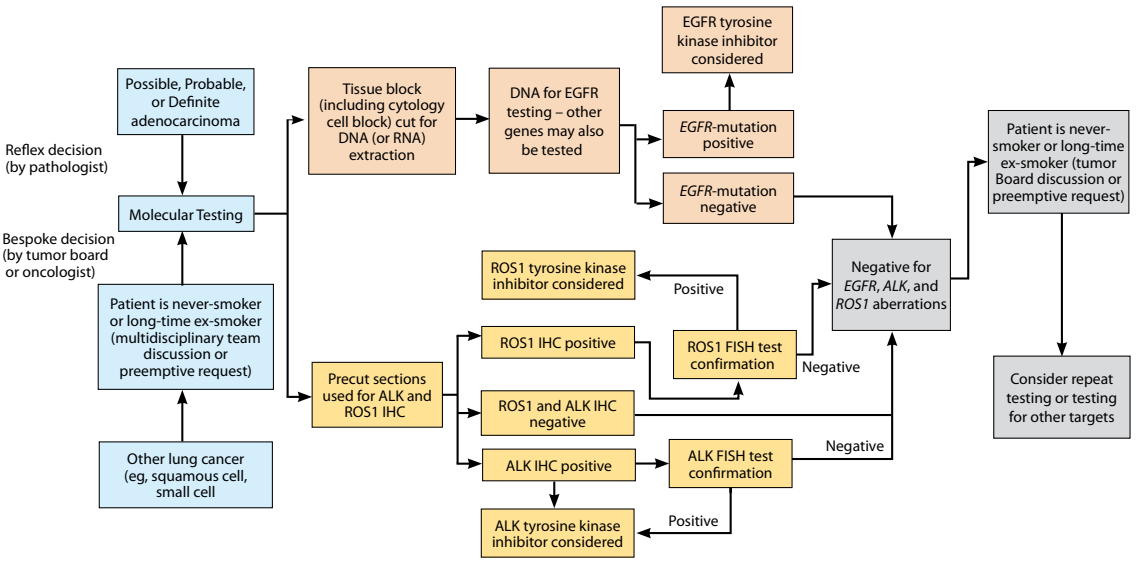

Figure 1. One possible algorithm for *ALK* and *ROS1* testing, incorporating into the standard of care *EGFR* mutation testing in NSCLC. This depends on the availability of drugs, a highly variable matter between countries. (Adapted from Kerr 2016).

Impact of NGS

NGS is rapidly emerging as a viable option for the delivery of multiplex genomic testing in NSCLC. (*See Chapter 9 for details of this approach.*) When a laboratory decides to use

this approach as its initial tool for molecular testing, the dynamics of testing change in a number of ways. Whether using laboratory-developed panels of genes or a commercially available panel, NGS testing potentially provides data on a large number of genes, many of which the treating oncologist would not necessarily have requested. Also, NGS may detect some alterations for which no treatment is available or for which treatment is available only through a clinical trial. Thus, the distinction between routine testing for approved drug use versus clinical research becomes blurred. Individual teams must decide how an NGS approach will be used. In some centers, NGS may still be a supplementary test approach, used in special circumstances when initial testing fails to provide a more routine druggable target, and treatment options being considered include those available in trials. Many academic centers are moving toward using NGS as their primary, upfront testing approach, especially in health systems where more than *EGFR*, *ALK*, and perhaps *ROS1* are considered routine targets with available drugs. NGS is still relatively costly and its use will depend on whether it is considered cost-effective compared with several stand-alone tests. As ROS1 testing joins EGFR and ALK testing as routine and mandatory, the argument for NGS will be strengthened. Triage of nonsquamous tumors for molecular testing may become void if NGS screening of squamous tumors is considered worthwhile and cost-effective. However, we have not reached that point at the time of writing.

NGS approaches are becoming available for the identification of fusion genes involving *ALK* and *ROS1*, but experience of the clinical significance of these aberrations is still limited. Given the controversy about the expected responses to treatment for tumors with different results of ALK testing on IHC and FISH (positive on ALK IHC but negative on ALK FISH or negative on ALK IHC but positive on ALK FISH), will a second confirmatory test by IHC or FISH be needed before treatment is considered when a fusion gene is identified by NGS? Anecdotal evidence suggests that NGS may identify *ALK* rearrangements in tumors that do not test positively on either FISH or IHC.

Other Issues

Testing in the context of disease relapse after treatment with an ALK inhibitor is another emerging area, and experience is limited. Among the proposed mechanisms of resistance that emerge during treatment with an ALK inhibitor are mutations in the *ALK* gene (Lovly 2012). These and other mechanisms, such as apparently acquired *KRAS* mutation, may be of little more than academic interest in the absence of a therapeutic intervention determined by a specific resistance mechanism. Current practice, either routinely or in a clinical trial setting, is to use a second-generation ALK inhibitor when disease relapses during treatment with crizotinib. However, emerging data suggest that different *ALK* mutations may confer different sensitivity to different second-generation ALK inhibitors (Gainor 2016; Liao 2015). These data raise the possibility of repeat biopsy at the time of relapse to allow testing for *ALK* mutations so that an appropriate second-generation ALK inhibitor can be selected.

Quality assurance in ALK and ROS1 testing is another important issue. As discussed in Chapter 13, all biomarker testing should be carried out in certified laboratories and should be subject to appropriate and rigorous internal quality assurance procedures. In addition, laboratories should perform satisfactorily in external quality assurance programs (Lindeman 2013, Kerr 2014, Tembuyser 2014, Cheung 2015). One of the ongoing challenges

for laboratories is access to good positive control material for ALK and ROS1 testing. If appropriate clinical case material cannot be sourced locally, commercially available cell lines are available to serve as suitable positive control testing material. Access to external quality assurance schemes should be sought, according to geographic and other factors that may influence participation. These schemes are available for ALK testing in some parts of the world and are in development for ROS1 testing.

Conclusion

Current practice of biomarker testing, including testing for *ALK* and *ROS1* aberrations, varies across institutions and countries in accordance with local conditions and funding mechanisms. Parallel testing of *EGFR*, *ALK*, and *ROS1* offers substantial advantage in terms of maximizing tissue use for personalized treatment, but the adoption of reflex testing is more controversial. As more targeted therapies are anticipated to emerge rapidly during the coming years, the testing algorithm likely will continue to evolve accordingly. The testing algorithm will also be considerably influenced by the more widespread adoption of panel biomarker testing using NGS technology.

Molecular testing for patients with early-stage NSCLC is not routinely recommended. However, many academic (and some nonacademic) institutions have implemented molecular testing for patients with early-stage disease, either for research reasons or for the purpose of having a molecular profile on hand if relapse occurs.

Summary and Future Perspectives

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15

Treatment results for patients with advanced NSCLC harboring *ALK* gene rearrangement continues to be encouraging, and molecular testing for the rearrangement is crucial for the optimal choice of therapy for patients with advanced NSCLC, particularly tumors that are an adenocarcinoma or have an adenocarcinoma component. With the approval of ALK IHC as a diagnostic test to determine eligibility for treatment with an ALK inhibitor, access of effective drugs becomes easier and better. Most recently testing for *ROS1* gene rearrangement has also become a crucial part of the routine molecular testing for patients with advanced NSCLC of adenocarcinoma or mixed histologies, with approvals in both the United States and Europe. However, several questions still await definite answers.

1. Which patients should be screened for *ALK* and *ROS1* gene rearrangements?
2. What is the most cost-effective screening method?
3. What is the most optimal screening-diagnosis paradigm for selecting patients for treatment with ALK and ROS1 inhibitors in order to capture all the patients who may potentially benefit from this therapy?
4. What is the role of NGS in the screening and diagnostic paradigm?
5. What are the therapeutic differences between the different fusion patterns and partners?
6. What is the most optimal treatment paradigm based on the current clinical evidence?

Although the last two questions are beyond the scope of this Atlas, the first four questions have been addressed.

Which Patients Should Be Screened for *ALK* and *ROS1* Gene Rearrangement?

There seems to be consensus that, at minimum, screening for *ALK* and *ROS1* gene rearrangement should be done for all patients with advanced NSCLC that is an adenocarcinoma or has an adenocarcinoma component. Depending on resources and academic interest, screening of patients with advanced NSCLC of other histologies should be considered, especially patients

with one or more of these features: younger patient age, never/light smoking history, or negative results on testing for *EGFR* and *KRAS* mutations. *ALK* or *ROS1* gene rearrangement may be found in tumors with non-adenocarcinoma histologies, although this finding is rare. If the diagnostic specimen is small and an adenocarcinoma component cannot be excluded, ALK testing is recommended.

Although treatment with an ALK or ROS1 inhibitor is not currently recommended for patients with early-stage NSCLC (I-IIIA), molecular testing, including ALK and ROS1 testing, on the surgical specimen is recommended to provide information if disease relapse or advanced disease subsequently occurs.

Today, multiplex testing with NGS is the preferred testing platform in many places, but it is crucial that the platform include detection of *ALK* and *ROS1* gene arrangements. However, it is still not clear whether this detection platform qualifies for treatment eligibility, and the issue should be clarified as soon as possible.

What Is the Most Cost-effective Screening Method?

In the United States, eligibility for crizotinib and other ALK-targeted therapies requires the diagnosis of *ALK* gene rearrangement on an ALK assay that is approved by the US FDA. Currently, two assays are US FDA approved: the ALK FISH Break Apart assay (Abbott Molecular) and the ALK (D5F3) IHC (Ventana). For *ROS*1 gene rearrangement, detection by FISH is the approved method. However, screening with ROS1 IHC verified by ROS1 FISH seems to be more cost-effective (as is the case for HER2 testing in breast cancer). Studies have confirmed a high diagnostic sensitivity for ROS IHC but lower specificity when referenced to FISH; thus, FISH confirmation is necessary to validate IHC results. Therefore, further work may be necessary to improve the ROS1 IHC assay.

As already mentioned, many institutions have implemented multiplex testing with NGS, and the regulatory aspects of this platform need to be clarified. RT-PCR has been approved only in select countries and is not currently recommended for widespread ALK or ROS1 screening.

Where Are We Going in the Future?

From a therapeutic point of view, several second-generation and third-generation ALK inhibitors are currently in clinical development, with very encouraging results. With the detection of acquired resistant mutations—some of them with therapeutic implication—a biopsy at the time of progression from first-line therapy seems to be clinically important for the choice of subsequent therapy. Although biopsy at the time of progression may represent challenges, the development of plasma-based testing is rapidly under clinical development. Studies are beginning to tell us whether different fusion patterns and partners represent different therapeutic outcomes (Yoshida 2016). It is assumed that multiplex testing with NGS will become more common in the primary diagnostic paradigm, and the validation and the regulatory aspects of this testing will need to be addressed in different countries. With the recent approval of several immune checkpoint therapies, their role in the treatment of patients with ALK- and ROS1-positive lung cancer remains to be determined.

Conclusion

As illustrated throughout this Atlas, the diagnostic aspects of *ALK* and *ROS1* gene rearrangements are still developing. In the next couple of years, the diagnostic schema may change, and emergence of new targeted drugs may facilitate that transition. However, we know that we can now properly treat patients who have advanced NSCLC harboring these gene alterations. All professionals in this field should ensure that all patients who may potentially benefit from ALK-targeted therapy receive optimal treatment. Ongoing studies will tell us whether these agents also will have a role in the treatment of early-stage NSCLC in the future. With the detection of several acquired resistant mechanisms, including gate keeper mutation, which might in the future be decisive for subsequent therapies, a culture change toward specimen analysis at the time of progression seems relevant.

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Manufacturers

The following manufacturers and their products are noted in this Atlas. The locations given for each manufacturer is not the only location; most manufacturers have offices worldwide.

Abbott Molecular
Abbott Park, Illinois, USA
Vysis LSI ALK Break Apart FISH Probe Kit; Vysis LSI ROS1 (Tel) SpectrumOrange Probe; Vysis LSI ROS1 (Cen) SpectrumGreen Probe

Abcam
Cambridge, UK
5A4 antibody

Agilent Technologies/Dako
Carpineteria, California, USA
EnVision+; EnVision Flex+; REAL EnVision-HRP and DAB; Dako Autostainer; SureFISH ROS1 3' Break-Apart Probe; SureFISH ROS1 5' Break-Apart Probe

Amoy Diagnostics Co., LTD
Fujan, China
AmoyDx EML4-ALK fusion gene detection kit; AmoyDx EML4-ALK Real Time PCR

BD (Becton, Dickinson and Company) Diagnostics
Franklin Lakes, New Jersey, USA
SurePath

Cell Signaling Technology
Danvers, Massachusetts, USA
D3F3 antibody; D4D6 rabbit monoclonal antibody; SignalStain Boost

Cytocell
Tarrytown, New York, USA
ROS1-GOPC (FIG) Distal Probe; ROS1-GOPC (FIG) Proximal Probe

Hoffmann La Roche
Basel, Switzerland
Alecensa (alectinib)

Hologic, Inc.
Bedford, Massachusetts, USA
ThinPrep

Leica Biosystems/Kreatech
Buffalo Grove, Illinois, USA
Novolink (Polymer Detection System); Bond Polymer Refine Detection System; Bond-Max automated immunostainer; ROS1 (6q22) Proximal-XL Probe; ROS1 (6q22) Distal-XL Probe

NanoString Technologies
Seattle, Washington, USA
NanoString assay

Nichirei Biosciences, Inc.
Tokyo, Japan
5A4 antibody; iAEP IHC

Novartis International AG
Basel Switzerland
Zykadia (ceritinib)

Novocastra
Newcastle, UK
5A4 antibody

Pfizer Oncology
New York, New York, USA
Xalkori (crizotinib)

Surexam Bio-Tech Co., Ltd
Guangzhou, China
ALK FISH

Ventana Medical Systems, Inc.
Tucson, Arizona, USA
ALK (D5F3) CDx Assay; Benchmark XT automated immunostainer, iView (DAB Detection Kit), ultaView (Universal DAB Detection Kit), OptiView (DAB IHC Detection Kit), OptiView Amplification Kit

ZytoVision GmbH
Bremerhaven, Germany
ZytoLight SPEC ROS1 Dual Color Break Apart Probe

Appendix 1

Summary of Published Studies on *ALK* Gene Rearrangement Testing in Lung Cancer since 2013[a]

Study	Country	Study Period	No. of Study Centers	Testing Platform	No. of Pts.	No. of ALK+	% ALK+	Patient Selection
Asia								
Hong et al., 2014	China	2012-2014	Single	FISH	1,016	94	9.2	NSCLC
Serizawa et al., 2014	Japan	2011-2013	Single	RT-PCR/ IHC/FISH	411	12	2.9	Adenocarcinoma
Kim et al., 2014	Korea	2011-2013	Multiple	FISH/IHC	510	47[b]	9.2	NSCLC
Fu et al., 2015	China	2012-2013	Single	FISH	487	44	9.0	NSCLC
Doval et al., 2015	India	2010-2014	Single	FISH	500	15	3.0	Adenocarcinoma
Hsu et al., 2015	Taiwan	2011-2013	Multiple	IHC (D5F3)	295	29	9.8	*EGFR*-wild-type adenocarcinoma
Zheng et al. 2016	China	2007-2013	Single	RT-PCR/ FISH	1,407	74	5.3	Adenocarcinoma
Lee et al., 2016	Korea	2006-2014	Single	IHC/FISH	4,870	281	5.8	NSCLC
Australia								
Rogers et al., 2015	Victoria	NA	Single	FISH	429	3	0.7	NSCLC
Europe								
Hutarew et al., 2014	Austria	NA	Single	IHC/FISH	303	14	4.6	Adenocarcinoma
Ali et al., 2014	Italy	2007-2013	Single	FISH	523	20	3.8	NSCLC
Vidal et al., 2014	Spain	2010-2014	Multiple	FISH	1,092	35	3.2	NSCLC
Barlesi et al., 2016	France	2012-2013	Multiple	FISH	8,134	388	2.1	NSCLC
Scarpino et al., 2016	Italy	NA	Single	FISH/IHC	637	47	4.7	*EGFR*-wild-type adenocarcinoma
North America								
Gainor et al., 2013a	United States	2009-2012	Three	FISH	1,683	75	4.5	NSCLC
Sacher et al., 2016	United States	2002-2014	Single	FISH/IHC/ NGS	1,783	84	4.7	NSCLC
Kris et al., 2016	United States	2009-2012	Multiple	FISH	1,007	80	7.9	Nonsquamous NSCLC
Total					25,087	1,342	5.3	

[a]*Includes only screening studies involving a large sample (>250 patients). For studies with apparent overlaps, only those with the latest and largest datasets are included.*

[b]*There was a large discrepancy between the number ALK-positive cases on FISH testing (47) and IHC testing (29).*

Appendix 2

Summary of Published Studies on *ROS1* Gene Rearrangement in Lung Cancer[a]

Study	Country	Study Period	No. of Study Centers	Testing Platform	No. of Pts.	No. ROS1+	% Positive by FISH	Patient Selection
Asia								
Rimkunas et al., 2012	China	NA	Single	IHC	556	9	1.6	NSCLC
Go et al., 2013	Korea	1997-2008	Single	FISH	515	16	3.1	NSCLC
Cai et al., 2013	China	2003-2011	Single	RT-PCR	392	8	2.0	NSCLC
Cha et al., 2014	Korea	2005-2012	Single	FISH	330	13	3.9	Adenocarcinoma
Chen et al., 2014	Taiwan	NA	Single	RT-PCR	492	12	2.4	Adenocarcinoma
Yoshida et al., 2014	Japan	1997-2009	Single	FISH	346	17	4.9	*EGFR*-wild-type adenocarcinoma
Lee et al., 2015	Korea	NA	Single	NanoString	94	9	9.6	*EGFR*-/*KRAS*-/*ALK*-wild-type adenocarcinoma
Shan et al., 2015	China	2009-2013	Single	FISH	681	13	1.9	Adenocarcinoma
Jin et al., 2015	Korea	2006-2008	Single	FISH	375	3	0.8	NSCLC
Wang et al., 2015	China	2007-2013	Single	Sequencing	1,356	11	0.8	Resected adenocarcinoma
Wu et al., 2016	China	2013-2015	Single	FISH	238	10	4.2	NSCLC
Australia								
Rogers et al., 2015	Victoria	NA	Single	FISH	317	3	0.9	NSCLC
Europe								
Mescam-Mancini et al., 2014	France	2012-2013	Single	FISH	121	9	7.4	*EGFR*-/*KRAS*-/*ALK*-wild-type adenocarcinoma
Scheffler et al., 2015	Germany/Spain	2012-2014	Multiple	FISH	1,035	19	1.8	Adenocarcinoma
Scarpino et al., 2016	Italy	NA	Single	FISH	637	8	1.3	*EGFR*-wild type
Viola et al., 2016	UK	NA	Single	FISH	35	1	2.9	*EGFR*-/*KRAS*-/*BRAF*-wild-type adenocarcinoma/adenosquamous cell carcinoma
North America								
Bergethon et al., 2012	United States	NA	Four	FISH	1,073	18	1.7	NSCLC
Sholl et al., 2013a	United States	2006-2012	Single	FISH	167	2	1.2	Adenocarcinoma (unselected)
Total					8,760	181	2.1	

[a]*Includes only studies that may be regarded as screening studies, without "known positive cases" added to the cohort.*

The *IASLC Atlas of ALK and ROS1 Testing in Lung Cancer* is a resource designed to help pathologists, laboratory scientists, and practicing physicians better understand the background, protocol, and interpretation of results of ALK and ROS1 testing for patients with advanced non-small cell lung cancer.

ALK and *ROS1* gene rearrangements occur in approximately 4% and 2% of lung adenocarcinoma, respectively. Although the frequency of these genomic aberrations is low, their diagnosis offers patients with lung cancer the opportunity to receive highly effective targeted therapies. The story of ALK and ROS1 reflects the current exciting state in lung cancer research.

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