ALK Status Testing in Non–Small-Cell Lung Carcinoma by FISH on ThinPrep Slides with Cytology Material

Eugen C. Minca, MD, PhD, Christopher P. Lanigan, MS,* Jordan P. Reynolds, MD,* Zhen Wang, MD, PhD,* Patrick C. Ma, MD,† Joseph Cicenia, MD,‡ Francisco A. Almeida, MD,‡ Nathan A. Pennell, MD,† and Raymond R. Tubbs, DO**

Introduction: Oncogenic anaplastic lymphoma kinase (*ALK*) gene rearrangements in non–small-cell lung carcinomas (NSCLC) provide the basis for targeted therapy with crizotinib and other specific ALK inhibitors. Treatment eligibility is conventionally determined by the Food and Drug Administration–approved companion diagnostic fluorescence in situ hybridization (FISH) assay on paraffin-embedded tissue (PET). On limited samples such as fine needle aspiration–derived cytoblocks, FISH for *ALK* is often uninformative. FISH performed on liquid-based ThinPrep slides (ThinPrep-FISH) may represent a robust alternative.

Methods: Two hundred thirty cytology samples from 217 patients with advanced NSCLC, including a consecutive series of 179 specimens, were used to generate matched ThinPrep slides and paraffin cytoblocks. The same ThinPrep slides used for cytologic diagnosis were assessed by standard *ALK* break-apart two-color probe FISH, after etching of tumor areas. Ultrasensitive ALK immunohistochemistry (IHC) on corresponding cytoblocks [D5F3 antibody, OptiView signal amplification] served as the reference data set.

Results: ThinPrep-FISH *ALK* signals were robust in 228 of 230 cases and not compromised by nuclear truncation inherent in paraffin-embedded tissue–FISH; only two samples displayed no signals. Nine of 178 informative cases (5%) in the consecutive series and 18 of 228 informative cases (7.8%) overall were *ALK* rearranged by ThinPrep-FISH. In 154 informative matched ThinPrep-FISH and cytoblock-IHC samples, 152 were concordant (10, 6.5% *ALK* status positive; 142, 92.2% *ALK* status negative), and two (1.3%) were ThinPrep-FISH positive but IHC negative (sensitivity 100%, specificity 98.6%, overall agreement 98.7%).

Conclusion: Detection of *ALK* gene rearrangements in liquid cytology ThinPrep slides derived from patients with NSCLC can be confidently used for clinical ALK molecular testing.

Key Words: Anaplastic lymphoma kinase, Fluorescence in situ hybridization, ThinPrep, Non–small-cell lung cancer, Lung cancer, Adenocarcinoma.

(*J Thorac Oncol.* 2014;9: 464–468)

Lung cancer is the second most common overall malignancy in the United States and, despite improved detection and treatment, it remains the leading cause of cancer-related death in men and women worldwide.[1] Lung cancer is classically divided into small-cell and non–small-cell types based on morphology and clinical features. With recent advances in molecular pathogenesis and the discovery of specific targetable genetic alterations in certain lung carcinomas, molecular classification and genotyping have now become essential for optimal therapeutic management. Non–small cell lung carcinomas (NSCLCs) represent approximately 80% of lung cancers and are further classified into adenocarcinoma (40%–50%), squamous cell carcinoma (30%) and large-cell carcinoma (9%).[2] A subset of NSCLC, primarily adenocarcinomas, exhibit mutations of the epidermal growth factor receptor gene that confer sensitivity to tyrosine kinase inhibitors. Targeted therapies with such inhibitors have been shown to improve the otherwise dismal survival in patients with tumors harboring epidermal growth factor receptor gene activating mutations.[3]

The anaplastic lymphoma kinase (*ALK*) gene encodes a receptor tyrosine kinase that is normally not expressed in lung cells. Approximately 5% of NSCLCs harbor *ALK* gene rearrangements, most commonly in the form of a small intrachromosomal inversion, inv(2)(p21;p23), resulting in the fusion of *ALK* with echinoderm microtubule associated protein like 4 gene.[4] The *ALK*/echinoderm microtubule associated protein like 4 fusion produces an abnormal, constitutively active chimeric protein kinase with oncogenic properties.[5,6] Targeted ALK tyrosine kinase inhibitors have proven anticancer

*Departments of Molecular Pathology, Cytopathology, and Anatomic Pathology, Robert J. Tomsich Pathology and Laboratory Medicine Institute, Cleveland, Ohio; †Departments of Translational Hematology and Oncology Research, and Solid Tumor Oncology, Taussig Cancer Institute, Cleveland, Ohio; and ‡Respiratory Institute, Cleveland Clinic Foundation, Cleveland, Ohio.

Disclosure: This research was supported in part by grants from three industry sponsors: Ventana Medical Systems, (immunohistochemistry detection reagents and partial salary support for the project); Cell Signaling Technologies (D5F3 primary antibody); and Abbott Molecular (anaplastic lymphoma kinase fluorescence in situ hybridization probe for the initial assay development and support for travel expenses). Dr. Tubbs has received honoraria for speaking on behalf of Ventana in the past (none in 2012 or 2013) and serves without compensation on the Ventana scientific advisory board. He does not own stock or other equities in the above three companies. The other authors declare no conflict of interest.

Address for correspondence: Raymond R. Tubbs, DO, Department of Molecular Pathology, Cleveland Clinic Foundation, 10300 Carnegie Avenue, Cleveland, OH 44106. E-mail: tubbsr@ccf.org

ISSN: 1556-0864/14/0904-0464

activity, with crizotinib showing a good clinical response in advanced NSCLC patients harboring *ALK* rearrangements.[7]

Although relatively uncommon in NSCLC, the presence of *ALK* rearrangements has major therapeutic implications, and thus needs to be assessed through an accurate, reproducible, and accessible diagnostic test. The U.S. Food and Drug Administration approved an in vitro diagnostic class fluorescence in situ hybridization (FISH) test as a companion diagnostic tool for crizotinib-based treatment eligibility (Abbott Molecular Vysis [AMV], Des Plaines, IL). As a consequence, FISH is currently considered the definitive standard for *ALK* status testing and it can be performed on paraffin-embedded tissue material (PET). The commercial break-apart FISH format consists of red and green probes that flank the highly conserved translocation breakpoint within *ALK* gene. Normal cells exhibit yellow fusion signals whereas neoplastic cells harboring *ALK* rearrangements show split red and green signals. A case is interpreted as *ALK*-positive by FISH when 15% or more tumor cell nuclei demonstrate isolated green and red signals, or isolated red signals, among 50 tumor nuclei scored.[8]

With the current rising availability and emphasis on minimally invasive diagnostic procedures such as endobronchial ultrasound (EBUS), testing for *ALK* rearrangements by FISH is increasingly attempted on small specimens, including biopsy samples or paraffin cytoblocks prepared from fine needle aspiration (FNA) samples. Indeed, at our institution, FNA samples are a common form of diagnostic material, especially for patients with locally advanced disease for which the EBUS FNA is performed for both diagnosis and staging—EBUS FNA specimens comprise approximately a third of the samples submitted for molecular testing at our institution. Notably, the data submitted by AMV to the Food and Drug Administration did not include any paraffin cytoblocks or other cytopathology material.[7] In certain cases, that is, those of patients with late-stage disease, small FNA specimens may be the only material available for testing. This issue has been recognized, and the latest guidelines from the College of American Pathologists stipulate that cytology specimens consisting of paraffin cytoblocks and smears are also suitable material for molecular testing, including ALK-FISH.[9] As previously reported by others, as well as from our own experience, *ALK*-FISH performed on paraffin cytoblock slides has a high rate of failure (>30%) because of either insufficient material for cytoblock preparation or insufficient tumor cells in the cytoblock for informative FISH results.[10] The latter seems to be the case even though abundant tumor cells are present on the ThinPrep slide used for cytopathologic diagnosis. Even with enough available material, *ALK*-FISH on paraffin samples may be difficult to interpret because of signal loss by section artifacts, target DNA integrity, or incomplete penetration of probes into the tissue.

Recent reports, including one from our institution, have described the value of ultrasensitive immunohistochemistry (IHC) in detecting *ALK* status. ALK-IHC with novel engineered antibodies and secondary signal amplification showed high concordance with *ALK*-FISH on both tissue[11–13] and cytology material.[14,15] Although IHC is more frequently informative on cytoblocks than FISH, it is still not an alternative in cases with limited or no PET material. In such cases, the only available material may be the slide used for cytopathologic diagnosis in the form of smear or liquid-based preparation such as ThinPrep, which may represent a valuable resource for testing *ALK* status.

Here we assessed the diagnostic value of *ALK*-FISH performed on ThinPrep slides prepared from FNA biopsies for detecting *ALK* status in an NSCLC case series at our institution. We demonstrate that ThinPrep-FISH can reliably detect *ALK* gene rearrangements in NSCLC and has a very high concordance with ultrasensitive ALK-IHC, warranting its routine use for clinical *ALK* molecular testing in NSCLCs.

MATERIALS AND METHODS

Specimens

The study included 230 samples derived from transbronchial FNA (164), extrapulmonary FNA (chest wall, neck, other sites; 16), bronchial brush (20), pleural fluid (24), pericardial fluid (5), and peritoneal fluid (1) from 217 patients with advanced NSCLCs that were clinically referred for *ALK* testing at our institution (Cleveland Clinic Foundation). The cohort included a consecutive series of 179 samples collected from 174 patients between December 2012 and August 2013. The samples were collected in CytoLyt (Cytyc Corp., Boxborough, MA) and centrifuged to generate cell pellets. Material from the cell pellets was divided for preparation of ThinPrep slides by using T200 or T500 processors (Hologic, Bedford, MA) and cytoblocks using a Cellient cell block processor (Hologic).

Fluorescence In Situ Hybridization

FISH for *ALK* rearrangements was performed on the ThinPrep slides using the AMV *ALK* Break Apart FISH Probe Kit with preanalytical and assay modifications previously detailed for ThinPrep-FISH to monitor recurrence of urothelial carcinoma.[16,17] Specific areas with abundant tumor cells were etched with a diamond tip pen on the reverse side of the slide before removal of the cover slip and probe application. All areas of the ThinPrep slide were screened qualitatively and signals enumerated selectively for tumor cells based on 4′,6-diamidino-2-phenylindole (DAPI) fluorescence pattern. To interpret the ThinPrep-FISH, the same cutoff of 15% positive cells was used as is specified for the in vitro diagnostic AMV probe set. Similarly, signal pattern classification was also based on the same criteria used for PET-FISH.

Immunohistochemistry

Automated IHC for ALK expression was performed on material from 211 available cytoblocks using the D5F3 antibody (a generous gift from Cell Signaling Technology, Danvers, MA), coupled with OptiView DAB IHC Detection Kit with Optiview signal amplification (a generous gift from Ventana Medical Systems, Tucson, AZ), and interpreted as either negative (no staining present) or positive (membranous staining present) as described previously.[11,12]

Statistical Analysis

The χ^2 test was used to compare the proportions of uninformative samples between ThinPrep-FISH and IHC (GraphPad Prism, GraphPad Software).

RESULTS

Our study included a total of 230 cytology samples from 217 patients with advanced NSCLC clinically referred for *ALK* testing in the Cleveland Clinic Health System, including a consecutive series of 179 specimens. ThinPrep-FISH was performed on the ThinPrep slides used for the cytopathology diagnosis for all samples (Fig. 1*A*). Matched cytoblocks were available in 211 of 230 samples (92.6%) and were used for comparative *ALK* status testing (Table 1). ThinPrep-FISH was overall informative for 228 of 230 samples (99.1%) (178 of 179 cases, 99.4%, in the consecutive series), with two samples displaying no signals for enumeration on the ThinPrep slides. *ALK* rearrangements were detected by ThinPrep-FISH on nine of 178 informative samples (5%) in the consecutive series and 18 of 228 informative samples (7.8%) overall. Of the 18 *ALK*-rearranged samples, seven demonstrated a typical positive pattern (isolated green and red signals accompanied by juxtaposed normal signal pairs) and 11 showed an atypical positive pattern (isolated red signals only accompanied by juxtaposed normal signal pairs). A representative ThinPrep-FISH image is shown in Figure 1*B*.

Because of the high failure rate of ALK-FISH on paraffin cytoblock slides (>30% in our preliminary experience before the initiation of this study, data not shown), we chose to use ultrasensitive ALK-IHC as previously described[11] on the available corresponding cytoblocks as the reference data set. Of the available 211 cytoblocks, 57 (27%) proved to have no material or insufficient tumor cells for evaluation despite abundant tumor cells being present on the ThinPrep slide.

Of 154 samples with informative paired ALK ThinPrep-FISH and cytoblock-IHC results, 10 of 154 samples (6.5%) were ThinPrep-FISH positive and IHC positive for ALK rearrangements/expression (Fig. 1*C*), two of 154 samples (1.3%) were ThinPrep-FISH positive but IHC negative, and 142 of 154 samples (92.2%) were ThinPrep-FISH negative and IHC negative. Overall, ThinPrep-FISH showed 100% sensitivity, 98.6% specificity, and 98.7% overall agreement when compared with ultrasensitive D5F3 ALK-IHC (Table 2).

Importantly, ThinPrep-FISH was the sole informative analysis in a significant proportion of samples (74 of 228, 32.4%) and revealed additional *ALK*-positive cases (6 of 74), which were missed by cytoblock analysis because of lack of material or insufficient tumor cells for interpretation.

DISCUSSION

The detection of *ALK* rearrangements in NSCLC is currently accomplished by most clinical laboratories using PET material testing by FISH as an Food and Drug Administration–approved companion in vitro diagnostic tool for determining treatment eligibility with the ALK inhibitor

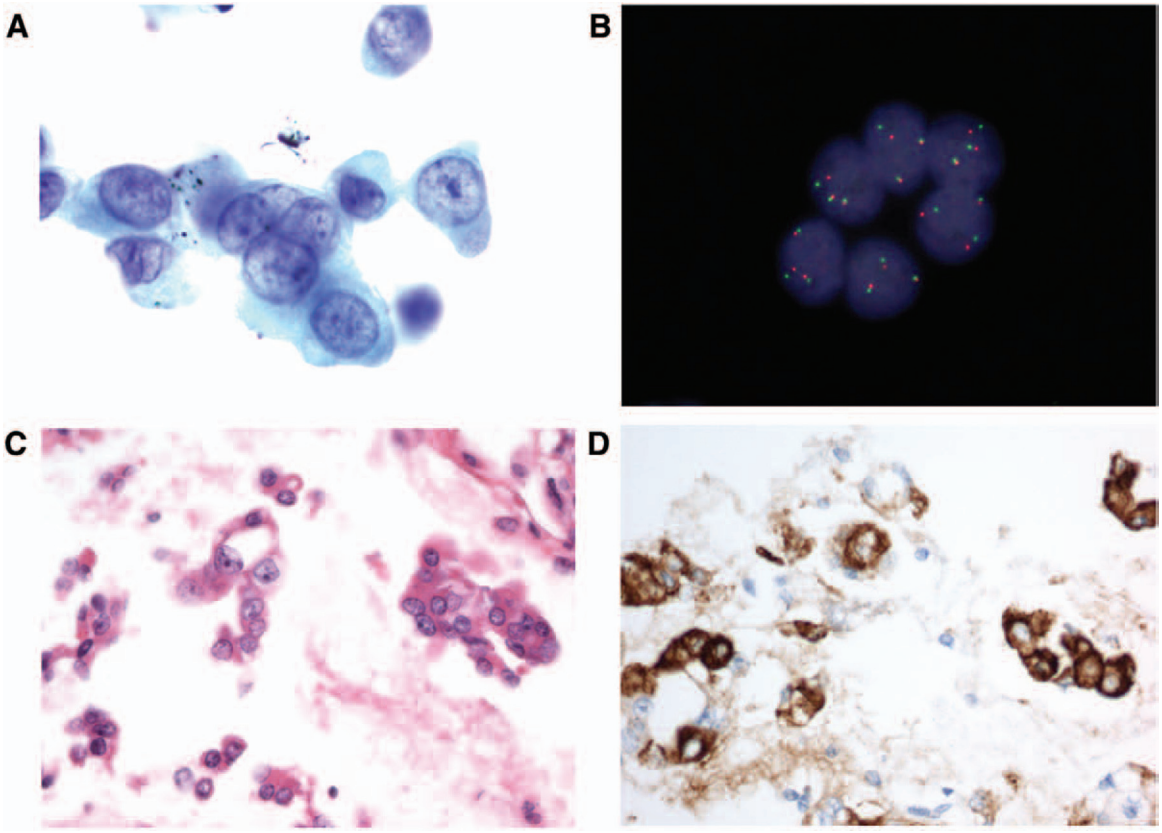

FIGURE 1. Representative images of non–small-cell lung carcinoma positive for *ALK* rearrangements by ThinPrep-FISH showing a typical positive pattern (*A*, ThinPrep-Papanicolaou 1000×; *B*, ThinPrep-FISH 1000×), and positive for ALK expression by D5F3-IHC on the paraffin cytoblock (*C*, hematoxylin and eosin 400×; *D*, D5F3-IHC, 400×). FISH, fluorescence in situ hybridization; ALK, anaplastic lymphoma kinase; IHC, immunohistochemistry.

TABLE 1. Breakdown of Available and Informative Non–Small-Cell Lung Carcinoma Samples by Type and Test

Sample Type	Total Available	Informative by ThinPrep-FISH	Informative by IHC	Informative by ThinPrep-FISH and IHC
ThinPrep slide	230	228	N/A	154
Cytoblock	211	N/A	154	

FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

TABLE 2. Correlation between ThinPrep-FISH and D5F3-IHC on 154 Available Dual-Informative Non–Small-Cell Lung Carcinoma Samples

		D5F3-IHC		
		Positive	Negative	Total
ThinPrep-FISH	Positive	10	2	
	Negative	0	142	
				154
Sensitivity		100% (95% CI: 0.65–1.00)		
Specificity		98.6% (95% CI: 0.94–0.99)		
Positive predictive value		83.3% (95% CI: 0.50–0.97)		
Negative predictive value		100% (95% CI: 0.96–1.00)		
Overall agreement		98.7%		

FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; CI, confidence interval.

crizotinib. Although considered a robust definitive standard test, ALK-FISH on PET material is not free of shortcomings, including: challenging interpretation because of probe design truncation artifacts and frequent uninformative results resulting from insufficient tumor cell number. IHC with novel antibodies and modified detection protocols is emerging as a potential alternative for FISH, with sensitivity and specificity results approaching those of the latter.[11–13] However, as with FISH, IHC may be of little use in cases with limited material, such as that obtained from cytology preparations derived from FNA biopsies.

Indeed, ALK status testing is requested with increasing frequency on small specimens or FNA cytopathology biopsy samples procured through minimally invasive procedures such as EBUS transbronchial needle aspiration. These procedures are generally successful in rendering enough diagnostic material,[18,19] and are preferred in cases with late-stage cancer not amenable for surgical treatment because of lower rate of complications. At our institution, the ThinPrep liquid-based cytology platform (Hologic, Bedford, MA) is the method of choice for processing FNA and body fluid specimens. Some of the advantages of this method include: minimal artifacts derived from air-drying and smear-crushing and homogenous cell enrichment with nearly 100% cell transfer to slide,[20] whereas criticism for this method is based on shrinkage artifacts[21] and higher cost. The protocol involves generation of a cell pellet from the FNA sample collected in CytoLyt, which is then divided for the preparation of a ThinPrep slide used for diagnosis and of a paraffin cytoblock used for IHC and molecular testing.[22] In a significant proportion of cases, the material obtained from an FNA procedure is not sufficient for cytoblock preparation and further molecular testing. Even more frequently in our experience, a significant proportion of the cytoblocks contain few if any recognizable tumor cells, despite the presence of a seemingly adequate initial cell pellet and abundant tumor cells on the ThinPrep slide used for diagnosis. This issue has also been reported by others[23] and has been attributed to the inherent variability in the cytoblock preparation process likely resulting in nonuniform cell clumping. Exhaustion of the cytoblock material after IHC workup for diagnostic purposes further limits additional molecular testing. Inadequacy of paraffin cytoblocks for molecular testing is a problematic issue that can lead to undesirable treatment delays or mistakes in a subset of cases and result in repeat procedures necessary to obtain additional material.

To our knowledge this is the first report showing the feasibility of *ALK*-FISH testing directly on ThinPrep liquid-based cytology slides. In keeping with the most recent recommendations of the College of American Pathologists,[9] several reports described the use of cytologic material derived from minimally invasive procedures like EBUS for molecular testing in NSCLC.[14,15,22,24–27] These studies involved limited series of cases with some making use of paraffin cytoblocks for *ALK* testing by FISH or IHC,[22,24,25] whereas others used Pananicolaou-stained smears directly for similar testing.[14,15,26,27] In these studies, IHC was highly concordant with FISH for ALK status, when performed on either cytoblock material or previously stained direct smears. The reported proportion of cases with uninformative FISH seemed to be higher for cytoblocks than that for the ThinPrep-FISH in our work.[22,24,25] Our study included a large NSCLC cohort with available liquid-based cytology material for *ALK* status testing by ThinPrep-FISH. The College of American Pathologists guidelines recommend that for ALK-FISH testing on cytology material, cytoblock-derived slides are preferred over smears for concerns of overlapping cells complicating the signal enumeration in the latter.[9] Using ThinPrep slides for *ALK*-FISH testing can eliminate this concern as the cells are distributed in a monolayer, and may result in a higher proportion of informative cases.

Several advantages can be envisioned for performing *ALK*-FISH directly on ThinPrep slides as compared with slides derived from paraffin cytoblocks. In addition to the already mentioned superior adequacy for sufficient tumor, compared with PET sections, cytologic preparations such as ThinPrep allow assessment of the entire tumor cell nucleus, thus avoiding signal loss by section truncation artifacts or incomplete penetration of probes into the tissue, and providing more accurate signal counts. Furthermore, the etching of areas with abundant tumor directly on the ThinPrep slides at the time of diagnosis allows precise FISH probe enumeration in tumor cells without interference from normal cells. Indeed,

consistent with the previous report,[27] we observed robust FISH signals on the ThinPrep slides, with representation of both described FISH patterns (separated green and red signals as well as isolated red signals) in the positive cases.

Our consecutive case subseries included nine NSCLC specimens positive for *ALK* rearrangements by ThinPrep-FISH. The percentage of *ALK*-positive samples in our study (9 of 179, 5% in the consecutive series; 18 of 228, 7.8% overall) was within the cited range (2%–13%).[4] The increased proportion of positive cases overall compared with the consecutive subseries is likely because of the fact that for several patients with *ALK*-positive tumors biopsied before December 2012, multiple unique specimens were analyzed.

Because of the high failure of *ALK*-FISH on cytoblocks before the initiation of this study, we used paired ultrasensitive IHC as the reference data set. Previous studies conducted by us and others demonstrated that ultrasensitive D5F3-IHC for ALK has near-perfect correlation with *ALK*-FISH[11,12] and is informative in cases with limited tumor cells on the paraffin cytoblocks that are otherwise inadequate for FISH testing. In the current study, ThinPrep-FISH showed a high correlation with ALK-IHC in assessing ALK status, with 100% sensitivity and 98.6% specificity.

In our study, there were significantly fewer uninformative ThinPrep-FISH results because of sample quality or quantity when compared with ALK-IHC (0.8% versus 27%, $p<0.001$). Furthermore, in 74 samples with uninformative ALK-IHC and successful ThinPrep-FISH, the latter test was positive in six cases (8.1%). These data indicate that ThinPrep-FISH can be successfully used on cytology samples that are limited or suboptimal for cytoblock analysis by FISH or IHC, and may effectively detect a subset of positive cases that would be otherwise missed by FISH or IHC analysis on PET material alone.

CONCLUSION

Our results demonstrate that ThinPrep-FISH is feasible and can reliably detect *ALK* gene rearrangements in NSCLC. The high concordance between ThinPrep-FISH and ALK-IHC on available cytoblock material warrants the routine use of ThinPrep-FISH for the clinical *ALK* molecular testing in NSCLC, especially in cases with limited cytology material.

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