Review Article

Overview of clinicopathologic features of *ALK*-rearranged lung adenocarcinoma and current diagnostic testing for *ALK* rearrangement

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Abstract: Patients with non-small cell lung cancer (NSCLC) who harbor anaplastic lymphoma kinase (*ALK*) gene rearrangements can derive significant clinical benefit from ALK tyrosine kinase inhibitor. Accurate patient identification is absolutely crucial for successful using ALK inhibitor treatment. However, lung cancer patients with *ALK* gene rearrangement after ALK inhibitor therapy eventually develop acquired resistance to treatment. In this review, the authors summarize the clinicopathologic features of *ALK*-rearranged NSCLC and the pros and cons of current diagnostic testing. In addition, we discuss the current diagnostic flow of ALK testing and consideration of rebiopsy sample during disease progression in patients treated by ALK inhibitors.

Keywords: Anaplastic lymphoma kinase (*ALK*) gene rearrangement; histology; fluorescent in situ hybridization (FISH); immunohistochemistry (IHC); non-small cell lung cancer (NSCLC)

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Introduction

The echinoderm microtubule-associated protein-like 4 and the anaplastic lymphoma kinase (*EML4-ALK*) fusion genes were identified in non-small cell lung cancer (NSCLC) in 2007 (1). The presence of *ALK* fusion in NSCLC is the best predictor of response to crizotinib, an ALK tyrosine kinase inhibitor (2,3), and these data led to the accelerated approval of crizotinib by the U.S. Food and Drug Administration (FDA). The incidence of the *ALK* rearrangement in NSCLC has been reported to be approximately 5% in various studies (1,4-6). Several studies showed particular clinical characteristics of patients with *ALK*-rearranged NSCLC (7-9). In addition, *ALK*-rearranged tumors were associated with histomorphologic features and positive correlation with histologic subtypes using the new International Association for the Study of Lung Cancer, American Thoracic Society and European Respiratory Society (IASLC/ATS/ERS) lung adenocarcinoma (ADC) classification (4,10-13).

Currently, the three primary methods of detecting *ALK* rearrangements are fluorescent in situ hybridization (FISH), immunohistochemistry (IHC), and the reverse transcriptase polymerase chain reaction (RT-PCR). Each of these individual methods has both advantages and disadvantages. There are many efforts to improve the sensitivity of identifying *ALK* rearrangement and recently, the Ventana ALK assay is a new method of detecting *ALK* rearrangements with high sensitivity (14).

This review is focused on clinicopathologic features of *ALK*-rearranged lung ADC and current diagnostic testing for *ALK* rearrangement.

ALK gene rearrangement in NSCLC

The *EML4-ALK* fusion in NSCLC results from an inversion in the short arm of chromosome two, fusing the

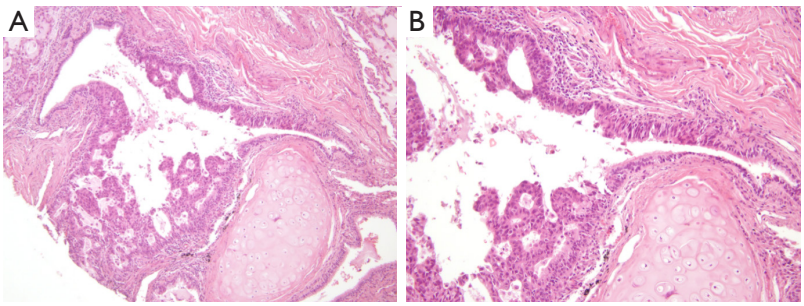

Figure 1 Relationship of *ALK*-rearranged tumors with the bronchiole. (A) Tumor cells invaded the adjacent bronchiolar epithelium (magnification, 10×); (B) at higher magnification, dysplastic epithelial lesions that resembled adjacent tumor cells continued the non-neoplastic bronchial epithelium (magnification, 20×). *ALK*, anaplastic lymphoma kinase.

N-terminal domain of *EML4* to the intracellular kinase domain of *ALK* (3' gene region), resulting in a constitutively active *ALK* tyrosine kinase (1). *EML4-ALK* fusion gene, by itself, is a potent oncogenic driver, reported in about 3-7% of all NSCLC patients. Other fusion partners for ALK have been discovered in NSCLC, such as *TFG-ALK* (15), *KIF5B-ALK* (16), and *KCL1-ALK* (17), and multiple *EML4-ALK* isoforms (18-20) have been identified, but their clinical significance still remains unknown.

Clinicopathologic characteristics of *ALK*-rearranged NSCLC

ALK rearrangements are more often found in never or light ex-smokers, in younger age patients and in lung ADC. Published studies have consistently reported that young age and history of never smoking are statistically different between patients with *ALK*-rearranged and *ALK*-negative lung ADCs (6,8,21,22). Although approximately 70-80% of *ALK*-rearranged patients are nonsmokers, the remaining 20-30% includes ex- or current smokers. Some previous studies, however, found that the *ALK* rearrangements were not associated with non-smoking (23,24). The age range of *ALK*-rearranged patients is commonly lower than NSCLC patients' and even younger than the *EGFR*-mutated population (25). A major challenge is that a younger age at presentation and a lack of smoking history of patients with tumors harboring *ALK* rearrangement are overlapped characteristic of those who harbor *EGFR* mutations.

In our previous study, *ALK*-rearranged tumors exhibited aggressive behavior such as nodal metastasis and advanced disease stage at diagnosis (25,26). In another study, they also observed a strong association of *ALK* rearrangement with advanced stage in NSCLC patients (27), which strengthened the importance of ALK testing in advanced stage disease.

Distinct histomorphologic features of *ALK*-rearranged lung ADC

Several studies have investigated the predictive value of pathological and morphological features in detecting *ALK*-rearranged tumors. Although the results of these studies have been inconsistent because of the limited number of *ALK*-rearranged tumors, solid signet-ring cell and cribriform pattern has been known to be associated with *ALK* rearrangement in lung ADC (7,9-12,28). In our previous study, *ALK*-rearranged lung ADC exhibited several histological characteristics that differentiated it from other genotypes: cribriform formation, presence of mucin-containing cells and presence of psammoma bodies (25). We also identified a close relationship to the adjacent bronchial epithelium is a unique feature of *ALK*-rearranged tumors. In some *ALK*-rearranged cases, tumor cells invaded the adjacent bronchiolar epithelium and showed the appearance of "budding off" of small epithelial cell clusters into the lumen. Furthermore, flat atypical lesions that resembled adjacent tumor cells infiltrated the non-neoplastic bronchial epithelium (*Figure 1*). *ALK*-rearranged tumors were more likely to be centrally located and easily obtained from the bronchoscopic biopsy procedure. Our findings suggest that *ALK*-rearranged tumors might be originated from different cell type, in contrast to *EGFR*-mutated tumors that is originated terminal respiratory unit (TRU) (29-31). In addition, frequent immunoexpression of p63 as well as TTF-1 in *ALK*-rearranged tumors has been described in a few studies (10,25) (*Figure 2*). Although the frequent reactivity to TTF-1 in *ALK*-rearranged tumors indicates

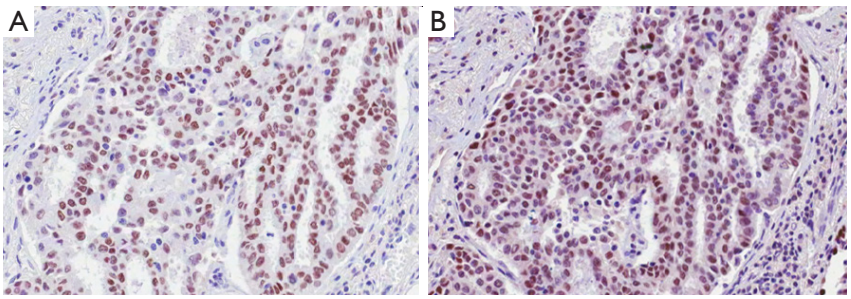

Figure 2 (A) Dual nuclear expression of TTF-1 (magnification, 40×) and (B) p63 in *ALK*-rearranged tumors (magnification, 40×). *ALK*, anaplastic lymphoma kinase.

derivation from TRU (31), type II pneumocytes or Clara cells native to that unit are typically negative for p63 (32). We proposed that a cell type that dually expresses TTF-1 and p63, as a cell of origin of *ALK*-rearranged tumors and overexpression of p63 might have functional roles related with carcinogenesis or tumor differentiation in specific subset of lung ADCs, however, a specific cell type of *ALK*-rearranged tumors has not yet been elucidated.

A few studies have reported a positive histological correlation with *ALK* rearrangement in lung ADC using the new IASLC/ATS/ERS classification that was published in 2011 (4,10-13). The solid subtype was significantly more frequent in the *ALK*-rearranged cancers, however, an *ALK*-positive rate is about 8% among the solid subtype ADCs that is similar with 9% in acinar subtype (SNUBH unpublished data). In our study, *ALK*-rearranged lung ADCs were also significantly associated with solid predominant subtype and not with acinar or papillary predominant subtypes (33). Another study showed that the existence of a minor mucinous component was independently associated with a relatively high prevalence of *ALK* rearrangement (34). However, no morphological characteristics could identify a specific genetic subtype, suggesting that genetic alterations are associated with a spectrum of morphological features.

Diagnostic methods for detecting *ALK* gene rearrangement

Currently three main methods of detecting *ALK* rearrangement are FISH, IHC, and the RT-PCR.

FISH has been considered the gold standard method for detecting *ALK* rearrangement. The FDA in the USA approved the Abbot Vysis *ALK* Break Apart FISH Probe Kit for companion diagnostic testing for *ALK*-rearranged NSCLC. Although FISH can detect rearrangements regardless of the fusion partners, it is expensive, generally requires specialized technical resources and expertise and thus cannot be applied in all pathological laboratories. In clinical practice, it is important to determine the presence of an *ALK* rearrangement in small biopsy samples with advanced stage NSCLC patients. Therefore, FISH analysis may not be available for screening all NSCLC patients.

Alternatively, IHC is less expensive and less time-consuming than *ALK* FISH, and is a well-established method in the routine work of every pathology department. IHC is less sensitive than FISH analysis to variations in handling or pretreatment of specimens, and a diagnosis can be established with a smaller number of tumor cells than required for FISH analysis. Several antibodies and detection systems have been investigated for overcoming the low expression level of the ALK fusion protein (35-37). 5A4 and D5F3 are known to be high affinity antibody clones (*Figure 3*) (35-38). Recently, the novel fully automated ALK IHC assay developed by Ventana company has been introduced that uses D5F3 antibody and relies on the tyramide amplification technique bound to the Ventana automated BenchMark XT for high sensitivity (*Figure 3B*). Several studies have demonstrated that there is a high concordance between the Ventana IHC and FISH (14,39,40). In September 2013, this automated IHC of Ventana Company has received China's FDA approval as a companion diagnostic identifying ALK protein expression in lung cancer patients.

The RT-PCR is a more sensitive and rapid method that can identify specific variants of the *ALK* rearrangements. However, RT-PCR requires *ALK* fusion variants to be known so that primers to all variants are included in the reaction. Although with an ever-expanding list of *ALK* fusion variants, all the reported variants require skillful application. In addition, majority of current *ALK* fusion variants were detected by RT-PCR in fresh frozen tumor tissue. However, in daily clinical practice, most of the tumor tissue available for molecular profiling is from

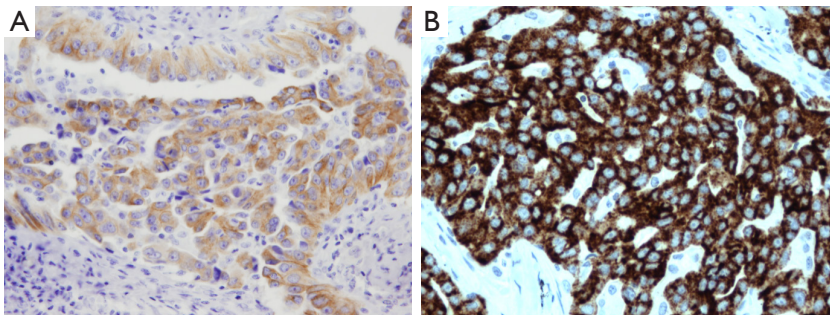

Figure 3 (A) ALK protein expression on immunohistochemistry using 5A4 antibody (magnification, 40×) and (B) D5F3 antibody (magnification, 40×). ALK, anaplastic lymphoma kinase.

FFPE tissue, where the integrity of RNAs is likely to be greatly compromised compared with fresh frozen tissue. Although FISH and IHC can be performed on a single FFPE slide, RT-PCR requires multiple slides in order to extract sufficient RNA for a successful reaction. Therefore, detecting *ALK* rearrangements using RT-PCR continues to be challenging in routine practice.

ALK testing in routine practice

Currently, crizotinib therapy is indicated only in *ALK*-rearranged NSCLC patients who are either inoperable or have residual or recurrent disease after surgery. However, the majority of lung cancer patients present with advanced stage and at the time of initial biopsy many patients have not been fully-staged or assessed for surgery. In this context, the guidelines for molecular testing in lung cancer recently published by the College of American Pathologists (CAP), IASLC, and Association for Molecular Pathology (AMP) has recommended performing *ALK* FISH testing at the time of diagnosis for patients presenting with advanced-stage NSCLC who are suitable for medical therapy or at a time of recurrence (41). That is, reflex ALK testing in all lung cancer patients would be encouraged but is only possible if it can be performed in a cost effective and timely manner.

As a companion diagnostic test, reflex *ALK* FISH in all lung cancer patients would be desirable, however, this strategy in the routine practice is difficult due to several limitations such as cost, resource and time constraints. Recently, many retrospective studies have suggested that ALK IHC can be used as a screening test for *ALK* gene rearrangements in lung cancer (6,35,38,42-45). Thus, reflex ALK IHC followed by confirmatory FISH testing can be readily integrated into the routine clinical setting and represents a cost effective and practical approach to screening for this druggable gene rearrangement. For the successful reflex test of ALK, we caution that ALK IHC should be fully validated in individual laboratories, performed with appropriate lung specific protocols when applied in clinical setting and controlled based on the results of the test. Even if IHC result is negative, FISH studies can still be performed on patients with a high clinical suspicion of *ALK* gene rearrangement.

ALK testing of rebiopsy samples during disease progression in patients treated by ALK inhibitor

Many of advanced NSCLC harboring *ALK* gene rearrangement treated with ALK inhibitors eventually relapse due to acquired resistance. Identifying the various mechanisms of resistance is critical to developing new treatment strategies in the acquired resistance setting. Several studies have identified several resistance mechanisms to crizotinib in rearranged *EML4-ALK* NSCLC and more studies are needed to fully understand the resistance mechanisms and to define new targeted strategies (46-48). This resistance has been associated with various tumoral genetic changes, such as other mutations in the *ALK* gene, *ALK* gene amplification or activating mutations of other genes (49). These changes may guide the selection of further treatments in these patients with resistant tumors. Therefore, it is widely accepted that rebiopsy is useful at the time of progression. However, this depends on the feasibility of rebiopsy at this time. Bosc *et al.* evaluated the percentage of patients who underwent rebiopsy with mutant *EGFR* or *ALK*-rearranged NSCLC and acquired resistance to tyrosine kinase inhibitors (50). A rebiopsy was considered as feasible in 72% while a biopsy was in fact performed in 46%. When rebiopsy was performed, there was sufficient

tumor material in the vast majority of cases (more than 85%) in several studies (50,51).

There were few contraindications to biopsy, reflecting the fact that patients with activating mutations are often nonsmokers or former light smokers and therefore less prone to tobacco related comorbid conditions such as COPD and heart disease. The most frequent constraint was poor physical condition, probably associated with cancer progression.

It should be considered that some degree of heterogeneity may occur between the primary tumor and its metastases. We previously found ALK protein expression in 11.9% (8/67) of primary NSCLCs and 25.4% (17/67) of their matched metastatic lesions, indicating that metastatic progression can be associated changes in ALK expression (52). Regarding the biopsy site, some authors consider that the highest failure rates are observed when the tissue is obtained from bone samples (53). These high failure rates are mostly observed when a decalcification process is needed. Despite significant improvements using EDTA (54), we have to recognize that bone biopsies are still not recommended for molecular testing. Patients and physicians may be reluctant to accept a surgical brain biopsy, even a minimally invasive stereotactic biopsy.

Understanding the molecular mechanisms of resistance and personalizing the treatment accordingly justify the need for rebiopsy. Although a vast majority of patients may undergo a second biopsy procedure, in one third of cases a biopsy was either not feasible, contraindicated or not suitable for molecular analysis. This emphasizes the need for the development of less invasive techniques.

Clinical impact and conclusions

The codevelopment of drug with a companion diagnostic assay has accelerated rapid development in the area of diagnostic assays in lung cancer. This led to the most sensitive, specific, and cost-effective assay for the screening of *ALK* rearrangement. As well as diagnostic testing, understanding distinct clinical and histomorphological characteristics of *ALK*-rearranged lung cancer may improve diagnostic accuracy and help us to detect all patients with *ALK*-rearranged lung cancer.

With the advances in acquired resistance after crizotinib therapy, the importance of repeat tissue acquisition and molecular testing during disease progression and the need for close collaboration between pathologists and clinicians are increasing.

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