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Original Article

Concordance of immunohistochemistry and fluorescent in situ hybridization for anaplastic lymphoma kinase (ALK) rearrangement in non-small cell lung cancer: results from a multicenter study in China

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Abstract: Crizotinib, a small molecular anaplastic lymphoma kinase (ALK) inhibitor, was approved by the Chinese Food and Drug Administration for ALK positive non-small cell lung cancer (NSCLC) patients in 2013. Although clinical trials have used the Vysis fluorescent in situ hybridization (FISH) ALK Break-Apart Assay to determine ALK status, the test is only available in limited laboratories and is technically challenging. A standardized and cost effective methodology is therefore in high demand. The aim of this study was to compare the diagnostic accuracy of Vysis FISH ALK Break-Apart Assay and VENTANA ALK (D5F3) immunohistochemistry (IHC) assay for detection of echinoderm microtubule associated protein like4-anaplastic lymphoma kinase (EML4-ALK) rearrangement in NSCLC. FISH and IHC analyses were performed on 1174 cases collected from three provincial cancer hospitals in China. The patient cohort included cases of NSCLC, small cell lung cancer (SCLC), normal lung specimens and other tumor types. We found that IHC assay is highly concordance with FISH assay, showing a positive percent agreement rate of 99.30%, negative percent agreement rate of 99.22%, and overall agreement rate of 99.23%. Our results thus suggest that the VENTANA ALK (D5F3) IHC assay is a valuable tool for the screening of patients with ALK rearrangement in clinical practice.

Keywords: Non-small cell lung cancer, EML4-ALK gene fusion, immunohistochemistry, fluorescent in situ hybridization

Introduction

The anaplastic lymphoma kinase (ALK) protein is a member of the insulin receptor superfamily of receptor tyrosine kinases [1]. ALK is a type I membrane glycoprotein that is normally expressed only in the nervous system, and cells of neuroendocrine origin [2]. In some tumor types, including lymphoma and lung cancer, the molecular pathogenesis of ALK occurs with chromosomal rearrangements that partner the 3' coding sequences for the intracellular signaling domain (the kinase domain) with 5' promoter elements and coding sequences of other genes. This aberrant protein then drives many oncogenic processes. An inversion within chromosome 2p, resulting in the formation of a fusion gene product comprising portions of the echinoderm microtubule associated protein-like 4 (EML4) gene and the ALK gene, was discovered in non-small cell lung carcinoma (NSCLC) cell lines and archived human specimens [3]. Studies indicated that EML4-ALK inversion events included at least nine fusion variants, all containing the same portion of the ALK C-terminal kinase domain, rendering them catalytically active [4-8]. Consistent with this, EML4-ALK expression in lung alveolar epithelial cells in transgenic mice was a potent oncogenic factor [9]. The fusion typically renders ALK in a cytoplasmic localization.

ALK is now recognized as a key oncogenic driver in NSCLC, and although EML4 is the predominant fusion partner, other fusion partner genes have been identified [10-12]. The incidence of ALK gene rearrangements in NSCLC is reported to range from 2-7% [3-8]. ALK gene rearrangements tended to be present in lung adenocarcinoma specimens compared with squamous or small cell histology [3-8]. There is also evidence that ALK gene rearrangements tend to correlate with patients who are of "never or light" smoking status, although this may not be a statistically significant cofactor [3, 4, 7, 9]. Importantly, ALK gene rearrangements are rarely coincident with EGFR, HER2, or KRAS mutations, demonstrating that ALK positivity is a distinct disease subtype [9]. ALK positivity is defined as ALK rearrangements present in at least on tumor cell. The current estimate of ALK prevalence in NSCLC may be underestimated due to limited cohort sizes in the studies, and different testing methods used to detect ALK.

Pfizer has developed a small molecular kinase inhibitor, XALKORI® (crizotinib), which inhibits the ALK protein. Thus, determination of ALK status in NSCLC patients is critical for directing patient care. XALKORI® was approved in China in 2013, and the original clinical trials used the Abbott/Vysis FISH (fluorescent in situ hybridization) ALK Break-Apart assay to determine ALK status. However, ALK FISH is a manual assay that requires specialized microscopes to evaluate the results. The staining patterns can present technical challenges in evaluating the results, leading to potential false negatives [10]. Recent studies report that immunohistochemistry (IHC) is sensitive and specific for determination of ALK status, and is considered as a viable alternative to ALK FISH, although concordance rates between the two assays have varied [10-14]. Since most pathologists are familiar with IHC, it is also preferred in many hospitals. Case studies were also showing that ALK IHC positive, FISH negative patients do respond clinically to treatment with XALKORI® [15]. Clearly, definitive studies comparing ALK IHC with FISH assays are needed. However, many of the previously published studies that have attempted to do this have used different anti-ALK clones (D5F3 and 5A4), different detection systems, and varying scoring methods that remain unproven in their validity. The VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody, used with the OptiView DAB IHC Detection Kit and OptiView Amplification Kit, is a fully automated IHC assay with high through-put compared with FISH. In the present study, we compared the VENTANA ALK IHC assay with Vysis ALK FISH assay in a large cohort of Chinese lung cancer patients.

Materials and methods

Sample selection

A total of 1998 NSCLC cases were selected according to pathological diagnosis and H&E staining result from three provincial hospitals in China, including: Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Site 1570 cases), Peking University Cancer Hospital & Institute (Site 2878 cases), and Fudan University Shanghai Cancer Center (Site 3550 cases). Because of the low prevalence of ALK positive cases in the NSCLC patient population, we also included other tumor types and some normal lung samples as well. A summary of sample information are provided in **Table 1**. Note that some specimens were not included in the final sampling number because of lack of sufficient tumor content or tissue content for testing (e.g., Vysis FISH requires at least 50 tumor cells be present on the matched H&E), or because of failures to stain with one or both assays, or because of a failure to have sufficient tumor during the creation of tissue microarrays (TMAs). Finally, a total number of 832 cases of NSCLC, 91 cases of SCLC, 90 normal lung specimens and 161 other tumor specimens (1174 samples in total) were tested with both Vysis FISH ALK Break-Apart Assay and VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody assay.

This study was carried out in accordance with the guidelines of Declaration of Helsinki, and was approved by the ethical committee for clinical research at Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Approval No. 12-101/635, Date: Sep 27, 2012), Peking University Cancer Hospital & Institute (Approval No. 2012101602, Date: Oct 16, 2012), and Fudan University Shanghai Cancer Center (Approval No. 1211116-2, Date: Nov 26, 2012). Written informed consent was obtained from all participants.

Stained specimens were blindly evaluated by pathologists from their respective hospitals according to manufacturer instructions. One pathologist from Site 1 evaluated the cases in the TMA from Site 1, two pathologists evaluated the cases from Site 2, and two pathologists evaluated the cases from Site 3. Note that duplicate cores from each unique case were included in the TMAs. If either of the cores was deemed to be positive for ALK, that case was considered to be positive. If two pathologists reading the TMAs disagreed, the result from the second reader was used to report the result (due to seniority of the 2nd reader). For information only, discordant cases were also tested with a 3rd assay, an IHC assay using the clone anti-ALK (D5F3) antibody from Cell Signaling Technologies Ltd.

Immunohistochemistry

Staining for ALK protein with VENTANA anti-ALK (D5F3) Rabbit monoclonal primary antibody was performed on BenchMark XT fully-automated staining instrument (Ventana Medical Systems, Inc.), together with Optiview DAB IHC Detection Kit and Optiview Amplification Kit. Online deparaffinization and antigen retrieval were done according to manufacturer's instructions. VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody was incubated for 16 minutes at 37°C. ALK detection was performed via Optiview DAB IHC Detection Kit (an indirect, biotin-free system) and Optiview Amplification Kit which includes HQ hapten conjugate for signal amplification (Ventana Medical Systems, Inc.). A matched TMA slide was stained with Rabbit Monoclonal Negative Control Ig Antibody in parallel as a control for interpretation. As a system level control, VENTANA ALK 2-in-1 control slides were stained with VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody and Rabbit Monoclonal Negative Control Ig Antibody. Each control slide contains two different cell lines representing positive and negative ALK expression (H2228 & CALU-3 respectively).

IHC interpretation

Staining results with VENTANA anti-ALK (D5F3) rabbit monoclonal antibody assay were evaluated according to manufacturer's interpretation guide. Strong granular cytoplasmic staining in tumor cells (any percentage of positive tumor cells) was considered ALK positive. Absence of strong granular cytoplasm staining signal in tumor cells indicated ALK negative status. Known staining artifacts for the IHC assay were excluded, including mild punctate cytoplasmic stain in alveolar macrophage cytoplasm, staining in nerve initiating cells (nerve and ganglion cells), epithelial staining granules and lymphocytic infiltrating cells. Some background staining was observed in normal NSCLC mucosa (including Mucin) and necrotic tumor areas. These staining patterns were excluded from clinical evaluation.

FISH for ALK rearrangement

FISH analysis was performed on the study cases using Vysis LSI ALK Dual color, Break Apart Rearrangement Probe (Vysis/Abbott, Abbott Park, IL) according to the manufacturer's instructions. In brief, 4-μm-thick sections were pretreated and followed by dual probe hybridization using the LSI ALK dual-color probe. The slides were then washed and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Results were then observed under fluorescence microscope.

FISH interpretation

Results were evaluated according to manufacturer's instruction. At least one pair of orange/green signals' spacing that was greater than the diameter of two signals; or only one orange signal, but no corresponding green signal were positive. In those cases, there may be fused and/or separate signals altogether. These cases with the above staining patterns were interpreted as ALK positive. Cases with orange and green signals adjacent to each other, or become fused (at this time if observe under the orange/green V2 filter, it expresses as a superposition of yellow signal); or if the orange and green signals' spacing is less than two signals in diameter, they are also considered to form a fusion signal; or if there was only one green signal but no corresponding orange signal, were interpreted as negative.

Data analysis

The statistical methods used to compare the IHC and FISH data are described in following scheme. IHC data were compared against the FISH data for positive or negative ALK status to

obtain percent positive, negative and overall agreement rates.

	FISH results	
IHC results	Positive	Negative
Positive	a	b
Negative	c	d

Positive percent agreement = a/(a+c) × 100%

Negative percent agreement = d/(b+d) × 100%

Overall percent agreement = (a+d)/(a+b+c+d) × 100%

Results

A total of 1212 specimens were originally chosen for the study from three different provincial hospitals in China including: Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Site 1), Peking University Cancer Hospital & Institute (Site 2), and Fudan University Shanghai Cancer Center (Site 3). With 38 rejected specimens, 1174 total specimens were tested with both FISH and IHC. The patient cohort included cases of NSCLC, SCLC, normal lung specimens and other tumor types. More details about sample information are provided in the Methods section and **Table 1**.

The FISH and IHC data were firstly compared within each site. Results were summarized in **Table 2**. Among total 395 valid specimens evaluated in Site 1, ALK IHC (+) was determined in 63 cases and FISH (+) was determined in 62 cases. One case that was IHC (+) was negative for ALK by FISH. All 332 ALK IHC (-) were negative for ALK by FISH. Compared with FISH, the IHC achieved a positive percent agreement rate of 100%, negative percent agreement rate of 99.70%, and overall agreement rate of 99.75%. When considering only the 277 NSCLS tumors, one case was identified as ALK+ by IHC, but ALK- by FISH. All ALK IHC (-) cases were negative for ALK confirmed by FISH, resulting in 100% positive percent agreement rate, 99.53% negative percent agreement rate and 99.64% overall agreement rate. Notably, for 27 SCLC tumors, 30 normal lung samples and 61 other tumors, all cases were consistently identified as ALK- by both IHC and FISH tests, resulting in 100% negative percent agreement rate and overall agreement rate.

Among total 409 valid specimens evaluated in Site 2, ALK IHC (+) result was determined in 46 cases and FISH (+) was found in 41 cases. Therefore, five cases were ALK IHC (+) and FISH (-). All 363 ALK IHC (-) cases were also negative for ALK as confirmed by FISH. Compared with FISH, the IHC achieved a positive percent agreement rate of 100%, negative percent agreement rate of 98.64%, and overall agreement rate of 98.78%. When considering only the 294 NSCLS tumors, 41 cases were identified as ALK+ by both IHC and FISH, and 253 cases were identified as ALK- by both IHC and FISH, giving 100% positive percent agreement rate and 100% negative percent agreement rate. For 35 SCLC tumors, FISH (-) was found for all cases, but five cases were identified as ALK+ by IHC. For 30 normal lung samples and 50 other tumors, all cases were consistently identified as ALK negative by both IHC and FISH, resulting in 100% negative percent agreement rate and overall agreement rate.

Among total 370 valid specimens evaluated in Site 3, ALK IHC (+) results were determined in 41 cases and FISH (+) were determined in 40 of those cases. Two cases of IHC (+) were FISH (-), while one case of FISH (+) was found to be IHC (-). Compared with FISH, the IHC achieved a positive percent agreement rate of 97.5%, negative percent agreement rate of 99.39%, and overall agreement rate of 99.19%. When considering only the 261 NSCLS tumors, 38 cases were identified as ALK+ by both IHC and FISH. One out of 223 IHC (-) cases was found to be ALK + by FISH. For 29 SCLC tumors, FISH (-) was found for all cases, but two cases were identified as ALK+ by IHC. For 30 normal lung samples, all cases were consistently identified as ALK- by both IHC and FISH. For 50 other tumors, one case of ALK+ and 49 cases of ALK- were consistently identified by both IHC and FISH, resulting in 100% positive percent agreement rate, negative percent agreement rate and overall agreement rate.

Finally, we combined all the samples collected from three sites for comparison. Among the total 1174 valid specimens for testing, IHC (+) was found in 150 cases and FISH (+) was found in 143 cases. Eight cases of IHC (+) were FISH (-), while one case of FISH (+) was found to be IHC (-). Compared with FISH, the IHC had a positive percent agreement rate of 99.3%, negative

Table 1. Summary of specimens obtained from three provincial hospitals in China

Participating site	Sample Size (test method)		Cases included in the IHC/FISH comparison study						
			NSCLC			SCLC	Normal Lung	Other tumor	Total
			Screen (+) for ALK	Screen (-) for ALK	Previously determined as FISH (+)				
Site 1: Cancer Hospital of Chinese Academy of Medical Sciences	570 (IHC)	Included cases	20	229	43	30	30	74	426
		Valid case*		277		27	30	61	395
Site 2: Peking University Cancer Hospital & Institute	878 (IHC)	Included cases	41	253	/	35	30	50	409
		Valid case*		294		35	30	50	409
Site 3: Fudan University Shanghai Cancer Center	550 (FISH or PCR)	Included cases	/	225	40	29	30	53	377
		Valid case*		261		29	30	50	370
Total	1998	Included cases	61	707	83	94	90	177	1212
		Valid cases*		832		91	90	161	1174 Immunohistochemistry as a screening tool for ALK rearrangement in NSCLC: evaluation of five different ALK antibody clones and ALK FISH

*Valid cases-cases had sufficient tumor content and valid results of both FISH and IHC analyses in the comparison study.

Table 2. Concordance between FISH and IHC results on the 1174 cases obtained from three provincial hospitals in China

Participating site			FISH (+)	FISH (-)	Positive agreement (%)	Negative agreement (%)	Overall agreement (%)
Site 1: Cancer Hospital of Chinese Academy of Medical Sciences	Overall	IHC (+)	62	1	100%	99.70%	99.75%
		IHC (-)	0	332			
	NSCLC	IHC (+)	62	1	100%	99.53%	99.64%
		IHC (-)	0	214			
	(adenocarcinoma)	IHC (+)	61	0	100%	100%	100%
		IHC (-)	0	186			
	(squamous)	IHC (+)	1	1	100%	96.55%	96.66%
		IHC (-)	0	28			
	SCLC	IHC (+)	0	0	NA	100%	100%
		IHC (-)	0	27			
	Normal	IHC (+)	0	0	NA	100%	100%
		IHC (-)	0	30			
	Other tumors	IHC (+)	0	0	NA	100%	100%
		IHC (-)	0	61			

Site 2: Peking University Cancer Hospital & Institute	Overall	IHC (+)	41	5	100%	98.64%	98.78%
		IHC (-)	0	363			
	NSCLC	IHC (+)	41	0	100%	100%	100%
		IHC (-)	0	253			
	(adenocarcinoma)	IHC (+)	41	0	100%	100%	100%
		IHC (-)	0	221			
	(squamous)	IHC (+)	0	0	NA	100%	100%
		IHC (-)	0	31			
	(adenosquamous)	IHC (+)	0	0	NA	100%	100%
		IHC (-)	0	1			
	SCLC	IHC (+)	0	5	NA	85.71%	85.71%
		IHC (-)	0	30			
	Normal	IHC (+)	0	0	NA	100%	100%
		IHC (-)	0	30			
	Other tumors	IHC (+)	0	0	NA	100%	100%
		IHC (-)	0	50			
Site 3: Fudan University Shanghai Cancer Center	Overall	IHC (+)	39	2	97.50%	99.39%	99.19%
		IHC (-)	1	328			
	NSCLC	IHC (+)	38	0	97.44%	100%	99.62%
		IHC (-)	1	222			
	(adenocarcinoma)	IHC (+)	34	0	100%	100%	100%
		IHC (-)	0	200			
	(squamous)	IHC (+)	2	0	100%	100%	100%
		IHC (-)	0	20			
	(adenosquamous)	IHC (+)	2	0	66.67%	100%	80%
		IHC (-)	1	2			
	SCLC	IHC (+)	0	2	NA	93.10%	93.10%
		IHC (-)	0	27			
	Normal	IHC (+)	0	0	NA	100%	100%
		IHC (-)	0	30			
	Other tumors	IHC (+)	1	0	100%	100%	100%
		IHC (-)	0	49			

Combination of three sites	Overall	IHC (+)	142	8	99.30%	99.22%	99.23%
		IHC (-)	1	1023			
	NSCLC	IHC (+)	141	1	99.30%	99.86%	99.76%
		IHC (-)	1	689			
	(adenocarcinoma)	IHC (+)	136	0	100%	100%	100%
		IHC (-)	0	607			
	(squamous)	IHC (+)	3	1	100%	98.75%	98.80%
		IHC (-)	0	79			
	(adenosquamous)	IHC (+)	2	0	66.67%	100%	83.33%
		IHC (-)	1	3			
	SCLC	IHC (+)	0	7	NA	92.31%	92.31%
		IHC (-)	0	84			
	Normal	IHC (+)	0	0	NA	100%	100%
		IHC (-)	0	90			
	Other tumors	IHC (+)	1	0	100%	100%	100%
		IHC (-)	0	160			

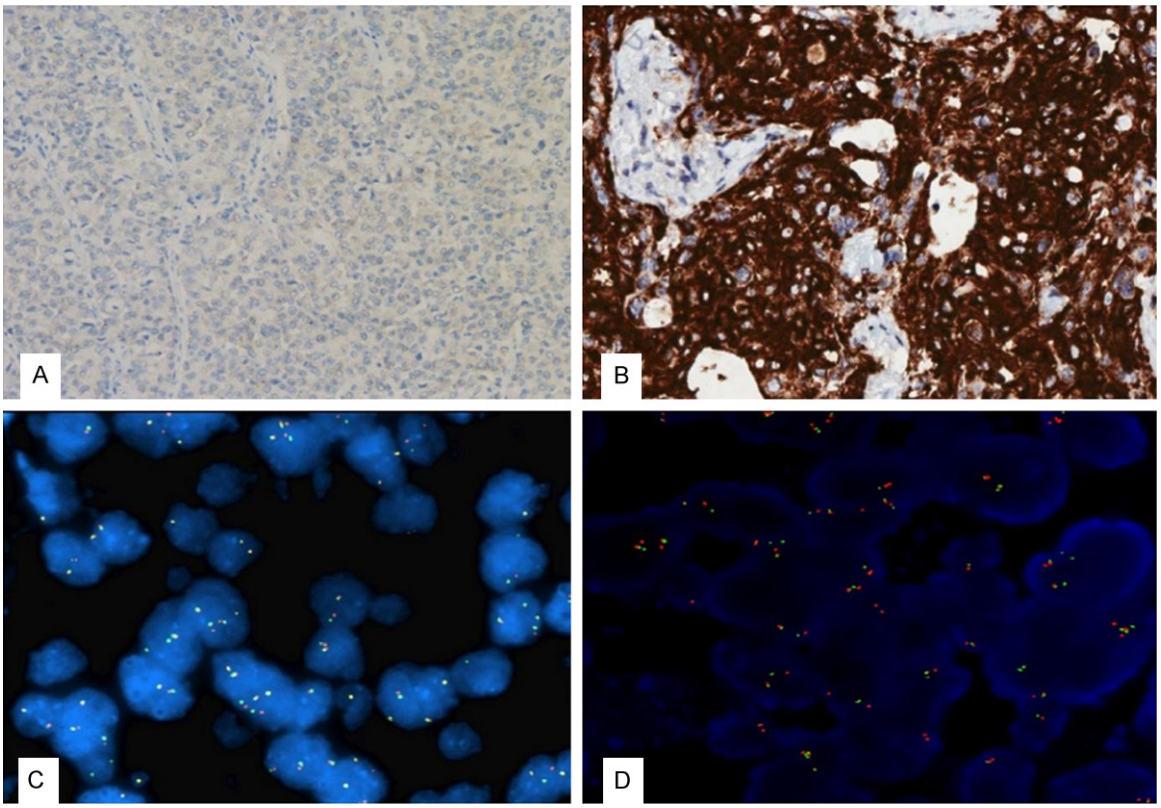

Figure 1. Example images of positive, negnative immonuhistochemistry staining and FISH results. One ALK IHC-negative case and one ALK IHC-positive case are shown in upper panel (A, B. Original magnifications × 200) and their corresponding FISH images in the lower panel (C, D. Original magnifications × 400).

Table 3. Staining analysis of disconcordant cases between FISH and IHC assays

Testing ID	FISH assay	IHC assay	Pathological Diagnosis
ALK-12-7	-	+	NSCLC (squamous cell carcinoma)
G73352	-	+	SCLC
G73473	-	+	SCLC
G75147	-	+	SCLC
G73678	-	+	SCLC
G74413	-	+	SCLC
90-D5-6	-	+	SCLC
90-C7-8	-	+	SCLC
89-D3-4	+	-	NSCLC (mixed squamous cell carcinoma and adenocarcinoma)

percent agreement rate of 99.22%, and overall agreement rate of 99.23%. When considering only the 832 NSCLS tumors, 141 cases were identified as ALK+ by both IHC and FISH. One case of IHC (+) was found to be ALK- by FISH. Another case of FISH (+) was identified as ALK- by IHC. Compared with FISH, the IHC had a positive percent agreement rate of 99.3%, negative percent agreement rate of 99.86%, and overall agreement rate of 99.76%. For 91 SCLC tumors, FISH (-) was found for all cases, but seven cases were identified as ALK+ by IHC, resulting in 92.31% negative percent agreement rate and overall agreement rate. For 90 normal lung samples, all cases were consistently identified as ALK- by both IHC and FISH. For 161 other tumors, one case of ALK+ and 160 cases of ALK- were consistently identified by both IHC and FISH, resulting in 100% positive percent agreement rate, negative percent

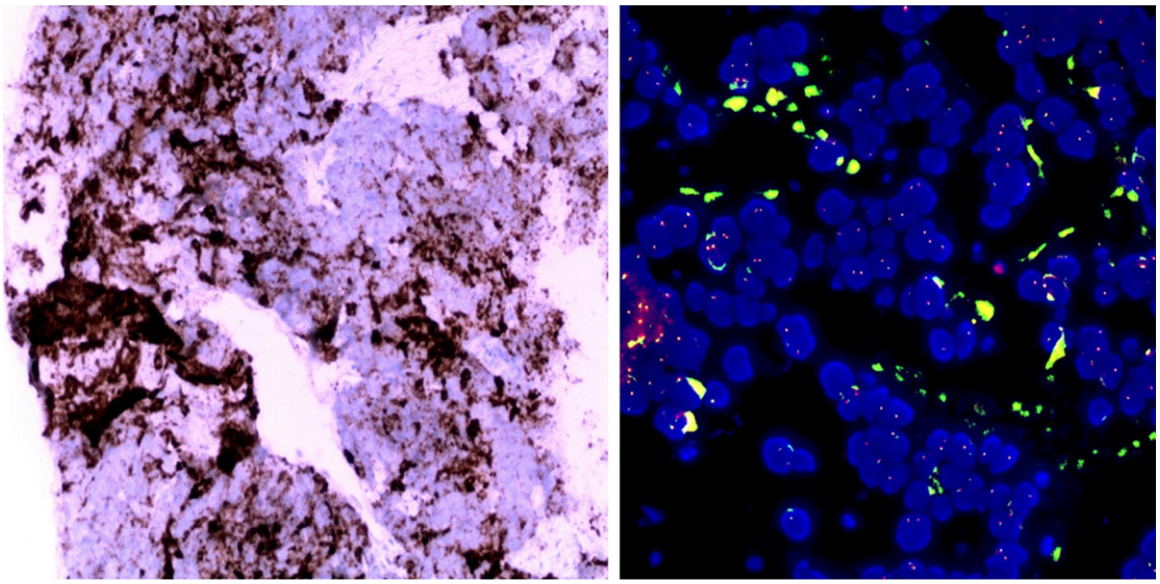

Figure 2. Example images of inconcordant SCLC cases. One ALK IHC-positive and FISH-negative SCLC case with the corresponding IHC (left panel, magnification × 200) and FISH images (right panel, managification × 400).

agreement rate and overall agreement rate. **Figure 1** shows representative IHC & FISH images for both ALK positive and negative cases.

For the nine samples with inconsistent results between FISH and IHC assay, another anti-ALK (D5F3) rabbit monoclonal antibody assay from Cell Signaling Technologies was used for further confirmation. Results were summarized in **Table 3**. For three out of nine cases, the results of VENTANA IHC assay were confirmed by the third IHC assay.

Discussion

The goal of this study was to evaluate the VENTANA anti-ALK IHC assay as a useful method for determining ALK status in NSCLC patients in clinical practice. A total of 1174 specimens were tested with both Vysis FISH assay and VENTANA anti-ALK IHC assay. With Vysis FISH as the relative "gold standard", our results showed that the VENTANA anti-ALK IHC assay reached a positive percent agreement of 99.30% (95% CI: 96.15%~99.88%), a negative percent agreement of 99.22% (95% CI: 98.48%~99.61%), and an overall agreement rate of 99.23% (95% CI: 98.55%~99.60%). Therefore, the VENTANA anti-ALK IHC assay was proven to be highly concordant with Vysis FISH assay using samples coming from three different provincial hospitals in China.

The specimens in this study contained common clinical specimen types, including NSCLC, SCLC, normal lung tissue specimens and other tumor specimens. Since ALK is relevant for NSCLC cases clinically, we analyzed the data specifically for NSCLC samples. Out of a total of 832 cases of NSCLC specimens, the positive percent agreement with FISH was 99.30%, negative percent agreement was 99.86%, and overall agreement rate was 99.76%. For the 90 cases of normal lung tissue specimens, the overall agreement rate reached 100%. For the 161 cases of other tumor samples, the overall agreement rate also reached 100%. Thus, the VENTANA IHC assay showed excellent clinical utility and its test results were very reliable.

There were a total of 9 specimens with discordant IHC and FISH results. One case was positive by FISH but negative by the VENTANA assay. The other 8 cases were positive by IHC but negative for FISH. Notably, the discrepant cases were mainly SCLC. Of the 91 cases of SCLC specimens, the negative percent agreement between FISH and IHC was 92.31%, while the overall total percent agreement was 99.22%. In fact, SCLC tumors may often originate from cells of neurocrine origin, like Kultchitsky cells in the bronchus where wild-type ALK can sometimes be detected [2]. **Figure 2** shows a discrepant case of SCLC with ALK IHC (+) & FISH (-) staining. Such wild-type

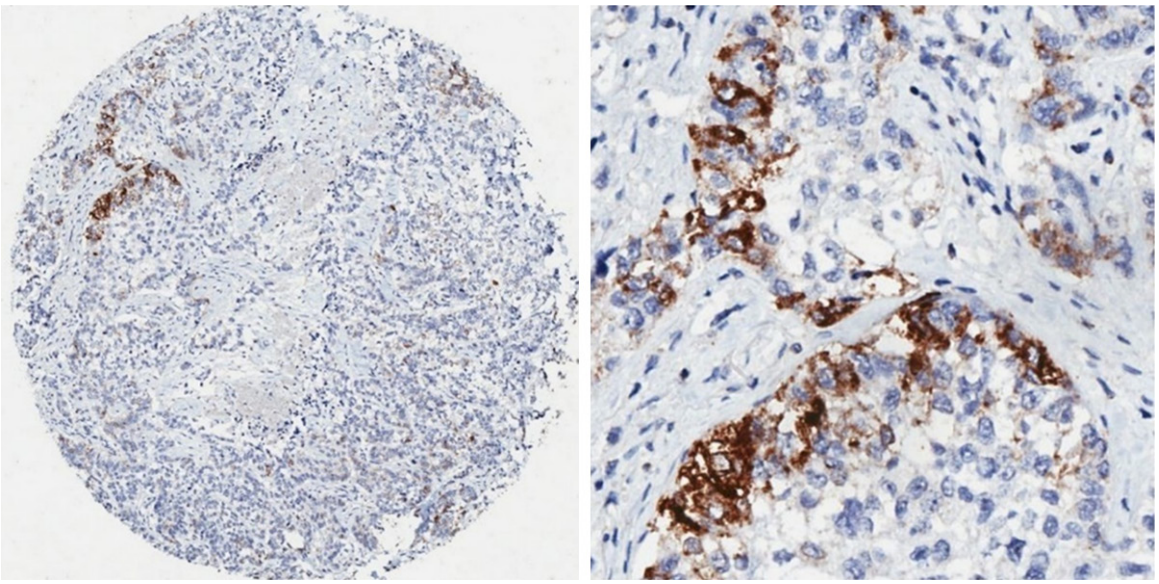

Figure 3. Example images of positive immunohistochemistry staining in squamous cell carcinoma from Site 1. One ALK IHC-positive case of suqamous cell carcinoma is shown in left panel (original magnification × 50) and right panel (original magnification × 200).

ALK could be detectable by IHC but not FISH, and therefore using IHC in SCLC specimens should be interpreted with care to ensure that any strong staining present in tissue elements other than the tumor cells is not reported as clinically positive for ALK. ALK positivity for treatment with crizotinib should be reported for strong staining present only in the tumor cells.

Recently several groups have reported ALK translocation in lung squamous carcinoma. Boland *et al.* has screened 150 adenocarcinomas and 150 squamous cell carcinomas with ALK immunohistochemistry. They found that the prevalence of ALK rearrangements is about 2.7% in adenocarcinomas but less than 1% (1 in 150 samples) in squamous cell carcinomas. That squamous cell carcinoma case positive for ALK IHC was very poorly differentiated but showed intercellular bridges and areas of keratinization. It seems that the prevalence of ALK rearrangements in squamous cell carcinomas is rare [15]. A group from Italy has screened 40 lung pure squamous cell carcinomas with FISH and found out 2.5% (one of 40 cases) showed ALK translocation [15]. From our study, in a total of 83 squamous cell carcinoma cases, three of them showed consistent positivity between IHC & FISH. One of the cases showed IHC (+) and FISH (-) result. According to our experience, a peripheral staining pattern is usually observed in ALK (+) squamous cell carcinoma. **Figure 3** shows a representative ALK IHC stain in squamous cell carcinoma. Once more data is collected to indicate the clinical significance of ALK positivity in SCC to targeted therapy, a separate interpretation method specific to this subtype may need to be developed accordingly.

This study includes by far the largest lung sample cohort comparing results of VENTANA IHC assay and Vysis FISH in China. Large number of similar studies have led to recommendations on testing algorithms, including using ALK IHC as a screening tool, with confirmation by FISH [16]. Concordance between FISH and IHC have varied widely in these studies, mainly because those IHC assays had employed different antibody clones and detection platforms. Additionally, several different scoring systems had been used, mainly to capture intensity of staining and percentage of tumor cells. Here, we reported results using the only commercially available, validated and fully automated IHC assay, the VENTANA anti-D5F3 anti-ALK IHC assay. Use of this standardized IHC assay and its corresponding instructions for scoring was also shown in other studies to exhibit excellent concordance with FISH for determining ALK status [17]. The binary positive/negative scoring algorithm was not

evaluated here for inter-reader agreement, as it was beyond the scope of the study. It is reported to be over 95% by manufacturer labeling and 100% in this study. There is still careful attention required to detail in the staining results evaluation, as the amplified detection can lead to higher background than seen with typical IHC assays and there are some tissue elements that exhibit staining (macrophages, neuroendocrine cells). However, when used as directed, the assay exhibits high concordance with FISH and we recommend that it be used as the primary tool for ALK testing in NSCLC, without the need for confirmation by FISH. A recommendation of VENTANA IHC assay as one of the confirmatory tests in NSCLC in China was published in June 2013.

In summary, the VENTANA anti-ALK assay shows an excellent concordance with the FISH assay. The test results are reliable and have clinical validity. The IHC assay is attractive for use in clinical settings due to its automation and fast turn-around time (~4 hours for 14 specimens), ease of evaluation of the staining results (any strong cytoplasmic staining in tumor cells is positive), and as this study demonstrates, its strong correlation with FISH in terms of sensitivity and specificity.

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Disclosure of conflict of interest

None.

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References

[1] Kutok JL and Aster JC. Molecular biology of anaplastic lymphoma kinase-positive anaplastic large-cell lymphoma. J Clin Oncol 2002; 20: 3691-3702.

[2] Iwahara T, Fujimoto J, Wen D, Cupples R, Bucay N, Arakawa T, Mori S, Ratzkin B and Yamamoto T. Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. Oncogene 1997; 14: 439-449.

[3] Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, Fujiwara S, Watanabe H, Kurashina K, Hatanaka H, Bando M, Ohno S, Ishikawa Y, Aburatani H, Niki T, Sohara Y, Sugiyama Y and Mano H. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007; 448: 561-566.

[4] Inamura K, Takeuchi K, Togashi Y, Nomura K, Ninomiya H, Okui M, Satoh Y, Okumura S, Nakagawa K, Soda M, Choi YL, Niki T, Mano H and Ishikawa Y. EML4-ALK fusion is linked to histological characteristics in a subset of lung cancers. J Thorac Oncol 2008; 3: 13-17.

[5] Choi YL, Takeuchi K, Soda M, Inamura K, Togashi Y, Hatano S, Enomoto M, Hamada T, Haruta H, Watanabe H, Kurashina K, Hatanaka H, Ueno T, Takada S, Yamashita Y, Sugiyama Y, Ishikawa Y and Mano H. Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. Cancer Res 2008; 68: 4971-4976.

[6] Koivunen JP, Mermel C, Zejnullahu K, Murphy C, Lifshits E, Holmes AJ, Choi HG, Kim J, Chiang D, Thomas R, Lee J, Richards WG, Sugarbaker DJ, Ducko C, Lindeman N, Marcoux JP, Engelman JA, Gray NS, Lee C, Meyerson M and Janne PA. EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. Clin Cancer Res 2008; 14: 4275-4283.

[7] Shinmura K, Kageyama S, Tao H, Bunai T, Suzuki M, Kamo T, Takamochi K, Suzuki K, Tanahashi M, Niwa H, Ogawa H and Sugimura H. EML4-ALK fusion transcripts, but no NPM-, TPM3-, CLTC-, ATIC-, or TFG-ALK fusion transcripts, in non-small cell lung carcinomas. Lung Cancer 2008; 61: 163-169.

[8] Takeuchi K, Choi YL, Soda M, Inamura K, Togashi Y, Hatano S, Enomoto M, Takada S, Yamashita Y, Satoh Y, Okumura S, Nakagawa K, Ishikawa Y and Mano H. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. Clin Cancer Res 2008; 14: 6618-6624.

[9] Shaw AT, Yeap BY, Mino-Kenudson M, Digumarthy SR, Costa DB, Heist RS, Solomon B, Stubbs H, Admane S, McDermott U, Settleman J, Kobayashi S, Mark EJ, Rodig SJ, Chirieac LR, Kwak EL, Lynch TJ and Iafrate AJ. Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. J Clin Oncol 2009; 27: 4247-4253.

[10] Galetta D, Rossi A, Pisconti S and Colucci G. The emerging role of ALK inhibitors in the treatment of advanced non-small cell lung cancer. Expert Opin Ther Targets 2012; 16 Suppl 2: S45-54.

[11] McLeer-Florin A, Moro-Sibilot D, Melis A, Salameire D, Lefebvre C, Ceccaldi F, de Fraipont F, Brambilla E and Lantuejoul S. Dual IHC and FISH testing for ALK gene rearrangement in lung adenocarcinomas in a routine practice: a French study. J Thorac Oncol 2012; 7: 348-354.

[12] Yi ES, Boland JM, Maleszewski JJ, Roden AC, Oliveira AM, Aubry MC, Erickson-Johnson MR, Caron BL, Li Y, Tang H, Stoddard S, Wampfler J, Kulig K and Yang P. Correlation of IHC and FISH for ALK gene rearrangement in non-small cell lung carcinoma: IHC score algorithm for FISH. J Thorac Oncol 2011; 6: 459-465.

[13] Jokoji R, Yamasaki T, Minami S, Komuta K, Sakamaki Y, Takeuchi K and Tsujimoto M. Combination of morphological feature analysis and immunohistochemistry is useful for screening of EML4-ALK-positive lung adenocarcinoma. J Clin Pathol 2010; 63: 1066-1070.

[14] Mino-Kenudson M, Chirieac LR, Law K, Hornick JL, Lindeman N, Mark EJ, Cohen DW, Johnson BE, Janne PA, Iafrate AJ and Rodig SJ. A novel, highly sensitive antibody allows for the routine detection of ALK-rearranged lung adenocarcinomas by standard immunohistochemistry. Clin Cancer Res 2010; 16: 1561-1571.

[15] Peled N, Palmer G, Hirsch FR, Wynes MW, Ilouze M, Varella-Garcia M, Soussan-Gutman L, Otto GA, Stephens PJ, Ross JS, Cronin MT, Lipson D and Miller VA. Next-generation sequencing identifies and immunohistochemistry confirms a novel crizotinib-sensitive ALK rearrangement in a patient with metastatic non-small-cell lung cancer. J Thorac Oncol 2012; 7: e14-16.

[16] Lindeman NI, Cagle PT, Beasley MB, Chitale DA, Dacic S, Giaccone G, Jenkins RB, Kwiatkowski DJ, Saldivar JS, Squire J, Thunnissen E and Ladanyi M. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. Arch Pathol Lab Med 2013; 137: 828-860.

[17] Hutarew G, Hauser-Kronberger C, Strasser F, Llenos IC and Dietze O. Immunohistochemistry as a screening tool for ALK rearrangement in NSCLC: evaluation of five different ALK antibody clones and ALK FISH. Histopathology 2014; 65: 398-407.