A Novel, Highly Sensitive ALK Antibody 1A4 Facilitates Effective Screening for ALK Rearrangements in Lung Adenocarcinomas by Standard Immunohistochemistry

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Introduction: Successful treatment of lung cancer patients with crizotinib depends on the accurate diagnosis of anaplastic lymphoma receptor tyrosine kinase (ALK) gene rearrangements. The approved fluorescence in-situ hybridization test is complex and difficult to use in daily diagnostic practice. Immunohistochemical assays—rapid and perfectly adapted for routine pathology practice—have been proposed as alternatives. We evaluated the novel high affinity ALK 1A4 antibody for routine diagnostics in formalin fixed, paraffin-embedded tumor specimens.

Methods: Detection of ALK protein expression was investigated by comparing the new 1A4 antibody and the established D5F3 antibody/Ventana system in 218 lung cancer specimens with known *ALK* status preanalyzed by quantitative reverse transcription-polymerase chain reaction and fluorescence in-situ hybridization (20 *ALK*-positive cases, 198 *ALK*-negative cases).

Results: The accuracy of both immunohistochemical assays for the detection of *ALK* rearrangements was high. Using a conventional staining procedure without signal enhancement, the 1A4 antibody assay identified all 20 *ALK*-rearranged tumors (100% sensitivity) and correctly characterized 196 of 198 negative cases (99.1% specificity). The D5F3/Ventana assay detected 19 *ALK*-rearranged tumors and typed 217 of 218 tumors correctly (95% sensitivity, 99.5 % specificity).

Conclusions: The novel 1A4 antibody represents a promising candidate for screening lung tumors for the presence of *ALK* rearrangements.

Key Words: Lung adenocarcinoma, *ALK*, Immunohistochemistry, Antibody 1A4, Routine diagnostics.

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The identification of aberrantly activated tyrosine kinases in subsets of non–small-cell lung cancer (NSCLC) together with the development of specific kinase inhibitors has provided a major breakthrough in lung cancer therapy. Genomic rearrangements of the anaplastic lymphoma receptor tyrosine kinase (*ALK*) gene result in the formation of fusion proteins containing the ALK kinase domain.[1,2] As a consequence, downstream signaling pathways implicated in cell growth and proliferation are constitutively activated. In lung adenocarcinoma, signaling of the activated tyrosine kinase can effectively be inhibited by the ALK inhibitor crizotinib.[3,4] Patients with *ALK*-rearranged tumors who benefit from treatment, however, merely represent 3%–6 % of adenocarcinoma patients,[5] and reliable preselection by clinical or histopathological criteria hitherto is not possible. Given the high incidence of lung carcinoma, rapid screening for the identification of those few NSCLC patients with *ALK* rearrangements is desirable. Thus, the development of robust and reliable but also rapid and cost–effective laboratory tests is of importance. Currently, fluorescence in-situ hybridization (FISH) is the only approved diagnostic test to detect *ALK* rearrangement which, however, requires specialized technical equipment, expertise and is a low-throughput approach. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis reliably identifies *ALK* rearrangements by detecting unbalanced *ALK* transcript expression,[6] but is not readily available as a routine method. Immunohistochemistry (IHC) is relatively inexpensive, rapid, and perfectly adapted for routine pathology practice, therefore representing a promising candidate for a screening test. However, reliable detection of the low ALK protein expression in *ALK*-rearranged NSCLC turned out to be difficult. Depending on the affinity of the antibody, the sensitivity of the detection system, the scoring system, and the experience of the scorer ALK immunostaining yields variable results. Substantial improvement was achieved by employing high affinity antibodies such as D5F3 and 5A4 in combination with sensitive detection methods.[7–9]

The aim of this study was to evaluate the use of the novel ALK 1A4 antibody for routine diagnostics. The antibody was stained in a large cohort of adenocarcinoma specimens enriched for *ALK*-rearranged cases to determine the reliability of the assay to detect *ALK* rearrangements in comparison with the D5F3/Ventana system, qRT-PCR, and FISH.

MATERIALS AND METHODS

Specimen Collection

Two-hundred and eighteen lung adenocarcinoma specimens with known *ALK* status (20 *ALK*-positive, 198 *ALK*-negative) determined by qRT-PCR as described earlier[6] were

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studied. Break-apart FISH confirmed the result in all 20 ALK-positive and 55 ALK-negative cases. Triplicate 0.6 mm cores from formalin-fixed paraffin-embedded tumor samples had been inserted into tissue microarrays (TMA). TMAs were used for all IHC analyses, and cases with ambiguous results were restained using full tissue sections. The study was approved by the ethics committee of the Eberhard-Karls-University Tübingen (Germany).

Immunohistochemistry

IHC was accomplished on 3-µm TMA or formalin-fixed paraffin-embedded tissue sections applying the monoclonal anti-ALK antibodies 1A4 (Origene, Rockville, MD) and D5F3 (Ventana Medical Systems, Tucson, AZ). The D5F3 assay was performed using the Ventana Benchmark XT System, prediluted D5F3, the Optiview DAB detection kit and the Optiview Amplification kit according to the manufacturer's instructions. The 1A4 assay was performed using the Leica BOND MAX System (Menarini Diagnostics, Berlin, Germany) with conventional DAB staining (no amplification). After heat-induced epitope retrieval at pH 6.0, 1A4 (1:50) was incubated for 30 minutes, followed by washing and detection according to the manufacturer's protocol. IHC was evaluated in a blinded fashion. For 1A4, ALK expression was assessed by estimating the intensity of cytoplasmic staining: 0/negative, no staining; 1+, faint; 2+, moderate; 3+, strong staining intensity in at least 10% tumor cells. D5F3/Ventana staining was classified as ALK-positive if there was any strong, granular, cytoplasmic staining; faint cytoplasmatic staining was regarded as negative according to the manufacturer's instructions.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Unbalanced *ALK* transcript expression indicative of ALK gene rearrangement was measured by qRT-PCR amplification of the *ALK* 5′ and 3′ portions as previously described.[6] Expression values were normalized to *PGK1* and calculated relative to the average value in cell line RH30, which was arbitrarily defined as 1. The cut-off value for relevant expression was 0.3.

Fluorescence In-Situ Hybridization

FISH was performed as described applying a break-apart probe specific for the *ALK* locus (ZytoLight SPEC ALK/EML4 TriCheck Probe; Zytomed Systems, Berlin, Germany).[6]

RESULTS

Two-hundred and eighteen NSCLC specimens with known *ALK* status as determined by qRT-PCR and FISH (20 *ALK*-positive, 198 *ALK*-negative cases) were analyzed for ALK protein expression using two different immunohistochemical assays. The D5F3/Ventana assay produced more intense cytoplasmic signals, but higher background staining; the 1A4 antibody assay gave weaker cytoplasmic staining but less background. Concordance between IHC and qRT-PCR/FISH data and representative immunohistochemical stainings are shown in Tables 1, 2, and Figure 1.

The 1A4 antibody assay identified all 20 *ALK*-rearranged tumors (100% sensitivity) and predicted 196 of 198 negative cases correctly (99.1% specificity; Table 1). In detail, 1A4 stained 17 cases with moderate to strong and five tumors with weak intensity (Table 2, Fig. 1). *ALK* rearrangement was confirmed by both qRT-PCR and FISH in all tumors with moderate to strong protein expression and three of five cases revealing weak staining intensity. Although weak staining was only observed in *ALK*-positive cases with low to moderate transcript expression (#21: 0.15, #16: 0.62; #11: 0.95), the staining intensity did not exactly correlate with transcript expression. Two cases (#118, #119) judged as 1+ were negative by FISH and failed to show unbalanced *ALK* transcript expression; they were therefore considered to be false-positive by IHC. No staining signal was observed in the remainder of 196 cases without *ALK* rearrangement.

The D5F3/Ventana assay identified 19/20 *ALK*-rearranged tumors and typed 217 of 218 tumors correctly (95% sensitivity, 99.5 % specificity; Table 1). All 19 ALK positive and *ALK*-rearranged cases showed a strong, granular cytoplasmic reactivity that did not correlate with the amount of *ALK* fusion transcripts (Table 2, Fig. 1). In three tumors, a weaker immunoreactivity with atypical or focal staining of small cytoplasmic spots (#21, #366) or membranous and cytoplasmic staining (#330) was seen (Fig. 1*B*). According to the manufacturer's interpretation guidelines, these three cases were classified as negative. Although #330 and #366 were confirmed as *ALK*-negative, #21 had an *ALK* translocation detected by FISH, resulting in a weak fusion transcript

TABLE 1. Summary of Immunohistochemical Staining Results for 218 Adenocarcinoma Cases

ALK Status qRT-PCR/FISH	*n*	IHC Using ALK Antibody 1A4			IHC Using ALK Antibody D5F3		
		Positive[b]	Critical[c]	Negative	Positive[b]	Critical[d]	Negative
Positive[a]	20	17	3	0	19	1	0
Negative	198	0	2	196	0	2	196
Total	218	17	5	196	19	3	196

[a]Positive refers to *ALK* rearrangement indicated by unbalanced *ALK* transcript expression and separated green and orange signals or single orange signals upon FISH.
[b]Positive refers to moderate or strong IHC staining in at least 10% tumor cells.
[c]Faint IHC staining scored as 1+.
[d]Focal IHC staining (judged as negative according to the manufacturer's guidelines).
ALK, anaplastic large-cell lymphoma kinase; FISH, fluorescence in-situ hybridization; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; IHC, immunohistochemistry

TABLE 2. Molecular Data of Adenocarcinoma Cases Positive for ALK Immunostaining

	qRT-PCR[a]		FISH[b]	Immunohistochemistry	
Case No.	*ALK* 3′ Portion	*ALK* 5′ Portion	*ALK* Break-Apart Probe	ALK Antibody 1A4	ALK Antibody D5F3
62	3.84	0.01	Translocation with deletion	3+	Positive
14	0.72	0.02	Translocation with deletion	3+	Positive
9	1.03	0.00	Translocation	3+	Positive
3	5.94	0.02	Translocation	2+	Positive
2	6.23	0.03	Translocation	2+	Positive
10	1.01	0.16	Translocation	2+	Positive
8	1.30	0.01	Translocation	2+	Positive
17	0.55	0.00	Translocation with deletion	2+	Positive
7	1.58	0.01	Translocation with deletion	2+	Positive
19	0.41	0.01	Translocation	2+	Positive
6	1.84	0.00	Translocation with deletion	2+	Positive
133	1.96	0.00	Translocation	2+	Positive
137	0.79	0.02	Translocation	2+	Positive
964	0.93	0.00	Translocation	2+	Positive
967	1.61	0.02	Translocation	2+	Positive
965	0.57	0.01	Translocation	2+	Positive
11	0.95	0.19	Translocation with deletion	1+	Positive
16	0.62	0.11	Translocation	1+	Positive
140	0.25	0.00	Translocation	2+	Positive
21	0.15	0.01	Translocation	1+	Focal staining
118	0.04	0.01	Wild type	1+	Negative
119	0.01	0.02	Wild type	1+	Negative
330	0.03	0.04	Wild type	Negative	Focal staining
366	0.01	0.00	Wild type	Negative	Focal staining

[a]*ALK* transcript expression was measured by qRT-PCR amplification of the *ALK* 5′ and 3′ portion normalized to *PGK1* and calculated relative to the average value in cell line RH30, which was arbitrarily defined as 1. Cases #140 and #21 had a low, yet unbalanced expression.

[b]Translocation refers to *ALK* rearrangement indicated by separated green and orange signals upon FISH. Translocation with deletion refers to *ALK* translocation with deletion of the *ALK* 5′ portion indicated by single orange signals.

ALK, anaplastic large-cell lymphoma kinase; FISH, fluorescence in-situ hybridization; qRT-PCR, quantitative reverse transcription-polymerase chain reaction

(expression level 0.15) and protein expression (scored as 1+ using 1A4). Of 198 *ALK*-negative cases, 196 were undoubtedly scored as negative. Ten cases showed background staining of tumor cells and macrophages and were judged as negative according to the manufacturer's guidelines.

DISCUSSION

Immunohistochemical stainings of a panel of lung adenocarcinoma specimens using the novel ALK antibody 1A4 and the D5F3/Ventana system yielded highly concordant results. Both systems typed more than 99% tumor specimens correctly. Notably, the 1A4 assay detected all (100% sensitivity), and the D5F3/Ventana system all but one of the *ALK*-rearranged cases (95% sensitivity). Other studies report on a sensitivity of 83%–100% with D5F3/Ventana[9–11] and 95% with 5A4/Ventana[12] (both with signal amplification), and 100% with D5F3[8,7] and 83%–100% with 5A4[8,9,10,11,13] using signal enhancement. In most studies, a high correlation in predicting *ALK* rearrangements was reached using D5F3 or 5A4 in combination with highly sensitive detection methods. In contrast, 1A4 IHC was performed using a conventional staining procedure, without signal enhancement. The D5F3/Ventana assay produced more intense cytoplasmic signals than 1A4 but with higher background and focal staining. Likely, this is the consequence of a tyramide signal amplification step incorporated into the Ventana assay; it demands experienced and trained evaluation.

IHC detects *ALK* rearrangements independent of the fusion partner and represents a suitable screening approach. If applied in routine diagnostics of NSCLC, it is expected to drastically reduce molecular pathological efforts. In our study, discordant IHC results in comparison with qRT-PCR/FISH were rare (1A4: two cases; D5F3: three cases) and restricted to cases with faint or focal immunostaining. Therefore, only tumors with weak 1A4 staining (2.3% in our series) or focal D5F3 staining (1.4% in our series) needed to be reanalyzed using alternative assays as qRT-PCR or FISH. In other words, 98% NSCLC were reliably predicted in a fast and cost–effective manner using IHC alone.

Not to overlook "druggable" tumors is the critical issue in ALK diagnostics. Therefore, up-regulated expression of *nonrearranged ALK* transcripts associated with detectable amounts of ALK protein is a critical finding that was observed in 1.1% of 523 NSCLC specimens.[6] Reinvestigation of those cases in this study was not possible due to exhaustion of tumor material. Expression of nonrearranged *ALK* transcripts may be relevant for ALK inhibitor therapy in NSCLC, because (1) ALK-inhibitor treatment of NSCLC cells and xenograft

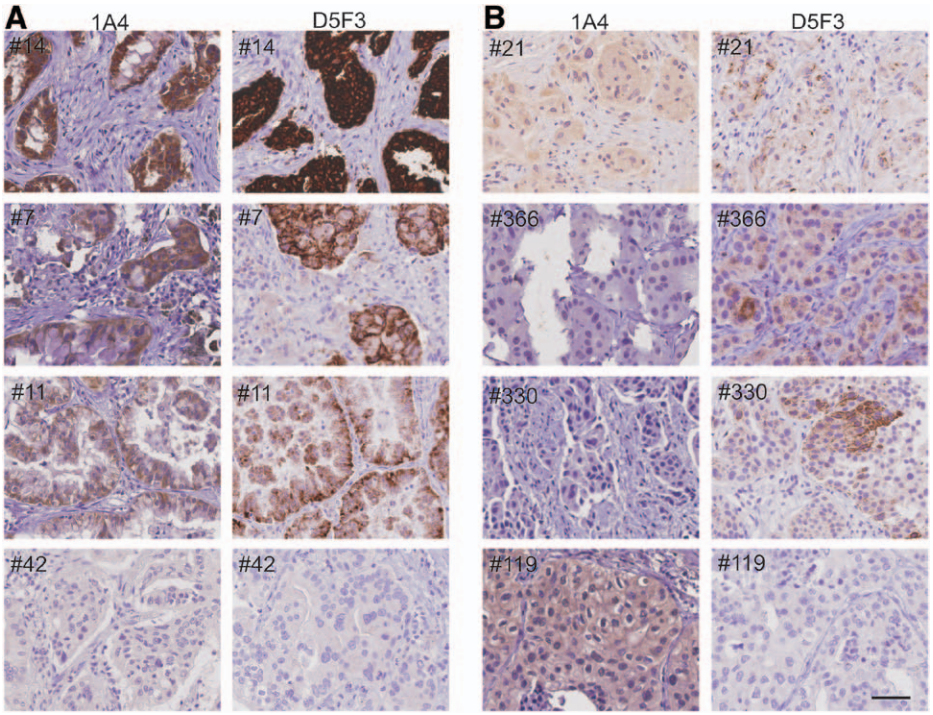

FIGURE 1. Examples of ALK staining in adenocarcinoma specimens using antibodies 1A4 and D5F3. *A,* concordant, *B* discordant results. *A,* Distinct cytoplasmic staining with differing intensity in three specimens with *ALK* translocations (#14, #7, #11). An *ALK*-negative case #42 is negative with both immunohistochemical assays. *B,* Case #21 with *ALK* translocation and weak fusion transcript expression shows weak cytoplasmic staining with 1A4 (1+) and focal cytoplasmic staining of small spots with D5F3 (negative according to the manufacturer's guidelines). Focal staining with D5F3 is also seen in two *ALK*-negative tumors (#366, #330), both negative with 1A4. Case #119 (*ALK*-negative according to qRT-PCR) is clearly negative with D5F3 but weakly positive with 1A4 (1+). *Scale bar* 50 μm.

tumors expressing wild-type *ALK* transcripts resulted in tumor regression and suppression of metastasis;[14] and (2) a reported IHC-positive, but FISH-negative patient was treated with crizotinib and showed a dramatic therapy response.[15] It should be mentioned, however, that a rare false-negative FISH result cannot reliably be excluded for this case, because the lack of rearrangement was not confirmed by other methods. IHC detects ALK protein overexpression in tumors with gene fusions and transcriptional up-regulation, but does not distinguish between the two. Therefore, until the question of whether tumors expressing nonrearranged ALK can be treated with crizotinib is answered, we propose a diagnostic screening algorithm using a sensitive IHC assay as a screening test, followed by further investigation of IHC-positive and questionable cases only. In more specific terms, this means that cases showing any *ALK* expression by IHC would be tested by qRT-PCR or FISH for confirmation of *ALK* positivity; cases with no *ALK* expression by IHC would be considered ALK-negative, and would not have to be tested further. Even if this algorithm is applied, routine diagnostic efforts are expected to be significantly reduced by prescreening of tumors with IHC.

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