ALK Rearrangement in a Large Series of Consecutive Non–Small Cell Lung Cancers

Comparison Between a New Immunohistochemical Approach and Fluorescence In Situ Hybridization for the Screening of Patients Eligible for Crizotinib Treatment

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• ***Context.***—**Echinoderm microtubule associated protein-like 4–anaplastic lymphoma receptor tyrosine kinase (*EML4-ALK*) translocation has been described in a subset of patients with non–small cell lung cancer (NSCLC) and has been shown to have oncogenic activity. Fluorescence in situ hybridization (FISH) is used to detect ALK-positive NSCLC, but it is expensive, time-consuming, and difficult for routine application.**

Objective.—**To evaluate the potential role of immunohistochemistry (IHC) as a screening tool to identify candidate cases for FISH analysis and for ALK inhibitor therapy in NSCLC.**

Design.—**We performed FISH and IHC for *ALK* and mutational analysis for epidermal growth factor receptor (*EGFR*) and *KRAS* in 523 NSCLC specimens. We conducted IHC analysis with the monoclonal antibody D5F3 (Ventana Medical Systems, Tucson, Arizona) and a highly sensitive detection system. We also performed a MassARRAY-based analysis (Sequenom, San Diego, California) in a small subset of 11 samples to detect *EML4-ALK* rearrangement.**

Results.—**Of the 523 NSCLC specimens, 20 (3.8%) were positive for *ALK* rearrangement by FISH analysis. *EGFR* and *KRAS* mutations were identified in 70 (13.4%) and 124 (23.7%) of the 523 tumor samples, respectively. *ALK* rearrangement and *EGFR* and *KRAS* mutations were mutually exclusive. Of 523 tumor samples analyzed, 18 (3.4%) were *ALK*$^+$ by IHC, 18 samples (3.4%) had concordant IHC and FISH results, and 2 *ALK*$^+$ cases (0.3%) by FISH failed to show ALK protein expression. In the 2 discrepant cases, we did not detect any mass peaks for the *EML4-ALK* variants by MassARRAY.**

Conclusions.—**Our results show that IHC may be a useful technique for selecting NSCLC cases to undergo *ALK* FISH analysis.**

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Lung cancer is the most frequent cause of cancer-related death worldwide, despite improvements in detection methods and treatments.[1,2] Although cytotoxic chemotherapy remains the mainstay of treatment for most patients with advanced non–small cell lung cancer (NSCLC),[3] the identification of specific genetic lesions, which drive the proliferation of cancer cells, has led to the development of new targeted therapies in a subset of patients.[4,5]

The echinoderm microtubule associated proteinlike 4–anaplastic lymphoma receptor tyrosine kinase (*EML4-ALK*) fusion in NSCLC was first described in 2007.[5,6] This is a novel fusion gene generated because of an inversion in chromosome 2, which juxtaposes the 5′-end of *EML4* with the 3′-kinase domain of *ALK*, thus permitting constitutive activation of *ALK* and inducing oncogenic cell transformation, as observed both in vivo and in vitro.[7] Thirteen variants of the *EML4-ALK* fusion have been described, according to the break point on *EML4* (from exon 2 to exon 20).[8] Furthermore, in addition to *EML4*, the TRK-fused gene (*TFG*)[6] and the kinesin family member 5B (*KIF5B*)[9,10] have been described to be fused to *ALK* in rare cases.

The reported incidence of *EML4-ALK* fusions in NSCLC is approximately 3% to 6.7%[11–13] but may be higher in a patient population selected based on specific clinicopathologic characteristics.[14,15] In Western populations, patients with *EML4-ALK* fusions are more commonly male, young, and nonsmokers.[14–16] Moreover, *EML4-ALK*$^+$ carcinoma is

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characterized by adenocarcinoma with a distinct histologic appearance.[16,17]

ALK rearrangement and other well-known oncogenic mutations, including epidermal growth factor receptor (*EGFR*) mutation and *KRAS* mutation, are mutually exclusive.[12] *EML4-ALK* is also associated with anti-EGFR tyrosine kinase inhibitor resistance.[14]

Recently, crizotinib, a small-molecule dual inhibitor of c-MET and ALK receptor tyrosine kinase, showed a significant clinical benefit in patients with *EML4-ALK*$^+$ NSCLC in a clinical trial.[18,19] Thus, the accurate identification of cases with *ALK*-rearrangement is essential for the selection of appropriate therapy. However, assessing *ALK* rearrangement in lung tumors remains a diagnostic challenge.[8]

Several methods, including reverse transcription-polymerase chain reaction (RT-PCR), fluorescence in situ hybridization (FISH), and immunohistochemistry (IHC), are currently used to identify *ALK* translocations in cases of NSCLC.

Although RT-PCR is theoretically the most sensitive assay for the detection of mutant transcripts, there is published evidence suggesting a propensity for false-negative results because of the variability in the *EML4-ALK* fusion structure and the existence of other *ALK* fusion partner. Moreover, high-quality RNA is difficult to obtain from routine, formalin-fixed, paraffin-embedded pathology material.[20]

Although FISH analysis, using an *ALK* break-apart probe, has been identified as the best method for selecting patients in crizotinib trials,[18–21] FISH is not readily available in routine pathology practice for screening every patient with NSCLC.

Contrastingly, IHC is relatively inexpensive, fast, and may be performed routinely in most laboratories; thus, it may serve as a practical tool for clinicians.

In the present study, we performed *ALK* FISH in 523 randomly selected NSCLC specimens derived from our clinical workflow that included concurrent mutational analysis of *EGFR* and *KRAS*. In the same cohort, we compared the test concordance of *ALK* FISH and IHC by evaluating the role of ALK IHC as a screening tool to identify candidates for *ALK* inhibitor therapy in advanced NSCLC. Furthermore, we also performed a MassARRAY-based method (Sequenom, San Diego, California) in a small subset of 11 patients to detect and characterize *EML4-ALK* variants.

MATERIALS AND METHODS

Patients and Tumor Specimens

Five hundred twenty-two consecutive patients with NSCLC, who presented at our clinic between February 2007 and January 2013, were included in the study. In one case, the patient presented a metachronous second primary lung sarcomatoid carcinoma after a resection of a lung adenocarcinoma and both samples were included in the study. The specimens included 344 whole-tissue sections (65.8%), 149 preoperative small biopsies (28.5%), and 30 cell blocks (5.7%) from pleural effusions. Samples were sent to the Unit of Pathological Anatomy in the Department of Surgical, Medical, Molecular Pathology, and Critical Area at the University of Pisa (Pisa, Italy) from the Unit of Thoracic Surgery and the Endoscopic Section of the Pneumology Unit and from external laboratories of pathologic anatomy in the Tuscany, Italy, area.

All tumor samples were formalin-fixed, paraffin-embedded for microscopic examination. Histologic diagnoses were reviewed by two pathologists (G.A. and G.F.), according to the criteria of the 2004 World Health Organization classification of lung tumors[22] and the European Respiratory Society/American Thoracic Society/International Association for the Study of Lung Cancer multidisciplinary classification of lung adenocarcinoma.[23,24] Any disagreements concerning histologic diagnoses were mutually agreed on after a critical discussion. For surgical samples, the surgical-pathologic staging was performed according to the International Union Against Cancer TNM classification.[25]

For all of the samples, mutational analyses were required by clinicians after informed consent of the patients. The present research received the institutional review board approval.

For some patients, the response to oral crizotinib therapy (250 mg twice daily) and clinical outcomes were recorded. Tumor response was evaluated according to response evaluation criteria in solid tumors (RECIST).[26]

ALK Fluorescence In Situ Hybridization

The FISH analyses was performed on 4- to 6-μm-thick paraffin sections of tumor tissues using a commercially available break-apart probe specific to the *ALK* locus (Vysis LSI ALK dual color, break apart rearrangement probe, Abbott Molecular, Abbott Park, Illinois), according to the manufacturer's instructions for cases of NSCLC. All patients were analyzed at progression of the disease. The probe, which hybridizes to the band 2p23 on either side of the *ALK* gene breakpoint, was used to detect any rearrangement involving the *ALK* gene. Before hybridization, paraffin sections were deparaffinized in xylene (3 times, 10 minutes each), dehydrated with 5-minute washes in 100% ethanol (2 times) and 5-minute washes in 96% ethanol (2 times), and air-dried at room temperature. Tissue sections were then transferred to a pretreatment solution at 80°C for 15 minutes, followed by a 3-minute wash in purified water, and incubated in a protease solution for 10 minutes at 37°C to digest proteins. After a brief wash in purified water, the slides were sequentially dehydrated in 70%, 85%, and 100% alcohol and air-dried at room temperature. Tissue sections were placed in a Hybrite (Abbott) for 3 minutes at 73°C to denature the DNA, and probe hybridization was carried out overnight at 37°C. Tissue sections were washed in 0.1% NP40/2× saline-sodium citrate buffer at 76°C for 4 minutes and then washed in 0.1% NP40/2× saline-sodium citrate at room temperature for 1 minute. Slides were mounted with 1.5 μg/mL 4′,6-diamidino-2-phenylindole. Tumor samples were scored by 3 independent investigators (G.A., A.P., and G.F.), who were blinded to the clinicopathologic characteristics of the patients and to the immunohistochemical and molecular results.

According to the scoring methodology proposed by Kwak et al,[18] the test was considered positive if 15% or more of the tumor cells had separate 5′ (green) and 3′ (red) probe signals or had isolated 3′ signals. Overlapping red and green signals (yellowish) indicated cells in which *ALK* was not rearranged.

ALK Immunohistochemistry

A representative tissue block from each lesion was selected. Tissue sections (4-μm thick) were deparaffinized in xylene and rehydrated using a graded series of ethanol solutions, and then subjected to immunohistochemical staining with a rabbit monoclonal primary anti-ALK antibody (clone D5F3, ready to use) using the OptiView DAB IHC detection kit and the OptiView amplification kit (Ventana). Immunostaining was performed as a fully automated assay using BenchMark XT automated slide stainers (Ventana).

The negative controls were carried out by omitting the primary antibodies. In all cases, the immunohistochemical evaluation was performed independently by 2 pathologists (G.A. and G.F.) who were blinded to the clinicopathologic characteristics and molecular data of the patients.

Positive immunostaining for ALK was clearly visible as strong, granular, cytoplasmic staining in tumor cells. Tumor samples were considered positive when any percentage of the tumor cells showed strong, granular, cytoplasmic staining.

Analysis of *EGFR* and *KRAS* Mutations

Genomic DNA was isolated from tissue sections using a standard method. Paraffin was removed by xylene extraction, and the sample was subsequently lysed with proteinase K. DNA

extraction was then performed using a spin column procedure (QIAamp tissue kit, Qiagen, Monza, Italy).

Mutational profiling of *EGFR* (exons 18–21) was performed as previously reported.[27] Briefly, the eluted DNA was used as a template in a standard 20-μL PCR reaction mixture. The sizes of the PCR products for *EGFR* exons 18, 19, 20, and 21 were 207, 194, 247, and 235 base pairs (bp), respectively. Because the primers had similar melting temperatures, the same PCR conditions were used to simultaneously amplify the 4 exons (in separate reaction tubes). The conditions used to amplify the *EGFR* exons were as follows: initial denaturation at 94°C (7 minutes), 35 cycles of denaturation at 94°C (60 seconds), annealing at 58°C (60 seconds), synthesis at 72°C (60 seconds), and a final extension for 7 minutes. As a negative control, the DNA template was omitted from the reaction. The amplification products were separated onto 2% agarose gels and visualized by ethidium bromide staining. For the detection of mutations, PCR products were purified using a QIAquick PCR purification kit (Qiagen) and were sequenced using a cyclic sequencing kit (ALFexpress II, Amersham Biosciences, Glattbrugg, Switzerland) following the manufacturers' recommendations.

Pyrosequencing assays were performed for sequence analysis of *KRAS* (codons 12 and 13) as previously described.[28] Briefly, template DNA (6-ng genomic DNA or external PCR products) was amplified using a HotStarTaq Plus DNA polymerase kit (Qiagen), a standard protocol (0.2 mM of each primer, 160 mM deoxyribonucleotide triphosphates, 2 U of enzyme), and cycling conditions. Reverse PCR primers were biotinylated for subsequent pyrosequencing analysis. Pyrosequencing reactions were carried out using a PSQ HS96A instrument, PSQ HS96A single nucleotide polymorphism reagents, and pyrosequencing single nucleotide polymorphism analysis software (formerly Biotage AB, now PyroMark Q96MD, Qiagen). Raw data signals from pyrosequencing were normalized using known wild-type signals. Significant mutation calling was delineated from experimental noise by statistical analysis of the raw data using a *t* test ($P < .05$).

MassARRAY Method for the Detection of *EML4-ALK*

Formalin-fixed, paraffin-embedded tissue sections, each with a thickness of 5 μm, were deparaffinized with xylene. For every sample, a minimum of 2 sections with a surface area of 16 mm^2 each and a maximum of 2 sections with a surface area of approximately 280 mm^2 each were processed.

Total RNA was extracted with RNeasy formalin-fixed, paraffin-embedded kits (Qiagen), following the September 2011 protocol version, and eluted in 18 μL or 30 μL, depending on the amount of starting material.

Seven microliters of RNA was subjected to reverse transcription with a PrimeScript first-strand complementary DNA (cDNA) synthesis kit (Takara Bio, Ōtsu, Shiga, Japan) using 2 μL of random 6-mer and performing the reverse transcription reaction at 42°C for 60 minutes.

To verify a suitable quality and quantity of the isolated RNA, the cDNA was amplified with primers spanning an exon-exon junction of the *ABL* gene, producing a 123-bp amplicon. The reaction was performed on the Rotor Gene 6000 (formerly Corbett, now Qiagen) using the ABL Mix (Diatech Pharmacogenetics, Jesi, Ancona in the Marche, Italy) in combination with Premix Ex Taq (Perfect Real Time, Takara). The expected PCR product was detected by the dual-labeled fluorescent probe contained in the ABL Mix in all samples except one.

Two microliters of 1:2-diluted cDNA was then tested with the MassARRAY iPLEX *EML4-ALK* panel (Sequenom, San Diego, California).[29] The MassARRAY iPLEX system involves 3 main steps: PCR, single-base primer extension (SBE), and separation of the products on a matrix-loaded silicon chip by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The PCR and SBE reactions were performed in a thermal cycler (Labcycler, SensoQuest, Göttingen, Germany), whereas the extension products were analyzed using the matrix-assisted laser desorption ionization time-of-flight MassARRAY Analyzer 4 (Sequenom), according to the iPLEX Pro application guide (Sequenom), and using all reagents and consumables contained in the SQ TYPING 960 kit (Diatech). For small biopsies, it was necessary to analyze a double-extension product. Data analysis was performed with MassARRAY Typer software version 4.0.53 (Sequenom).

The MassARRAY iPLEX *EML4-ALK* panel, which consists of 4 multiplexes, is able to detect *EML4-ALK* variants v1 (E13;A20), v2 (E20;A20), v3a (E6a;A20), v3b (E6b;A20), v4 (E14;ins11del49A20), v4′ (E15del19;del20A20), v5a (E2;A20), v5b (E2;ins117A20), v5′ (E18;A20), v6 (E13;ins69A20), and v7 (E14;del12A20), as well as *ALK* point mutations C1156Y and L1196M, which confer resistance to ALK tyrosine kinase inhibitor.

The PCR primer pairs were designed so that a forward primer targeted *ALK* exon 19, a reverse primer targeted *ALK* exon 20, a forward primer targeted the *EML4* exon upstream of the fusion site, and a reverse primer targeted the *EML4* exon downstream of the fusion site. The SBE primers were designed to target the translocation junction both on the *EML4* side (forward) and on the *ALK* side (reverse), so each variant could be detected by 2 assays. Depending on the substrate, the SBEs bind to either a translocated exon or to the wild-type exon of the unextended primer targeted transcript.

The estimated limit of detection for forward assays is 10%, but reverse SBE primers are expected to be more sensitive when the number of amplifiable cDNA template copies is at least 3000/multiplex. Moreover, the MassARRAY iPLEX *EML4-ALK* panel contains an internal quality control assay able to discriminate cDNA from genomic DNA (gDNA): a forward PCR primer targeting the *EML4* exon 1 and a couple of reverse PCR primers targeting exon 2 and the intron between exon 1 and exon 2 in the *EML4* gene. The discrimination between cDNA and gDNA is achieved from the base extended in the *EML4* exon 1 to exon 2 junction. In other words, further differentiation of the *EML4-ALK* transcript variants (versus the respective wild-type transcript or gDNA contamination) is dependent on the actual nucleotide extended.

EML4-ALK Reverse Transcription PCR

Total RNA was extracted with RNeasy formalin-fixed, paraffin-embedded kits. The RNA (500 ng) from each sample was reverse-transcribed with RevertAid first-strand cDNA synthesis kit (Thermo Scientific Life Science Research, Waltham, Massachusetts) using random hexamer primers to produce cDNA from all RNAs.

For detection of *EML4-ALK* fusion cDNAs, we carried out 3 independent PCR analyses. The first PCR was performed using a set of primers to identify variant 1: The forward primer is located at exon 13 of *EML4* (5′-GAG ACT CAG GTG GAG TCA TGC-3′), whereas the reverse primer is located at exon 20 of *ALK* (5′-GGT CAT GAT GGT CGA GGT G-3′), resulting in a 170-bp PCR product.[30]

To search for others variants of rearrangement, we used primers that targeted fusion variant 1 (247 bp). These primers could also detect variant 2, which would yield a considerably longer amplicon (approximately 1 kilobase [kb]): The forward primer Fusion-RT-S, located in exon 13 of *EML4*, is 5′-GTG CAG TGT TTA GCA TTC TTG GGG-3′, whereas the reverse primer Fusion-RT-AS, in exon 20 of *ALK* is 5′-TCT TGC CAG CAA AGC ACT AGT TGG-3′.[5]

To analyze the shorter variant of *EML4-ALK* transcript (155/188-bp), variant 3, the *ALK* Fusion-RT-AS primer was combined with a forward primer located in exon 6 of *EML4*: EML4-ex6F 5′-GCA TAA AGA TGT CAT CAT CAA CCA AG-3′.

The PCR primers glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-S 5′-ACC ACA GTC CAT GCC ATC AC-3′ and GAPDH-AS 5′-TCC ACC ACC CTG TTG CTG TA-3′ for GAPDH cDNA (452 bp) were used as control for cDNA integrity.

The PCR products were assessed and visualized by 1,5% agarose gel electrophoresis, and were performed in triplicate. Each 25-μL reaction contained 100 ng cDNA, 12.5 μL of Taq PCR Master Mix (Qiagen), and 0.5 μL of each primer (20 μM).

Table 1. Clinicopathologic, Immunohistochemical, and Molecular Features of Patients

Clinicopathologic Characteristics	Cases, No. (%)
Age, y, n = 522	
Range, 25–87	
Median, 67	
Sex, n = 522	
M	335/522 (64.2)
F	187/522 (35.8)
Histology, n = 523	
Adenocarcinoma	406/523 (77.6)
Squamous cell carcinoma	60/523 (11.5)
Non–small cell carcinoma, not otherwise specified	30/523 (5.7)
Large cell carcinoma	14/523 (2.7)
Large cell neuroendocrine carcinoma	5/523 (1.0)
Adenosquamous carcinoma	4/523 (0.8)
Sarcomatoid carcinoma	4/523 (0.8)
Tumor size, n = 205	
T1 (T1a + T1b)	36/205 (17.6)
T2 (T2a+T2b)	102/205 (49.8)
T3	49/205 (23.9)
T4	18/205 (8.8)
Lymph-node metastases, n = 205	
N0	104/205 (50.7)
N1	46/205 (22.4)
N2	55/205 (26.9)
ALK immunohistochemistry, n = 523	
Positive	18/523 (3.4)
Negative	505/523 (96.6)
ALK fluorescence in situ hybridization, n = 523	
Positive	20/523 (3.8)
Negative	503/523 (96.2)
EGFR mutational status, n = 523	
Wild-type	453/523 (86.6)
Mutated	70/523 (13.4)
KRAS mutational status, n = 523	
Wild type	399/523 (76.3)
Mutated	124/523 (23.7)

Abbreviations: ALK, anaplastic lymphoma kinase; *EGFR*, epidermal growth factor receptor.

Statistical Analysis

Statistical analyses were conducted using the statistical software IBM SPSS 19.0.1 (IBM, Armonk, New York). We used the Cohen κ test to analyze the agreement level between different diagnostic tests. A χ^2 test was used to analyze the associations between the different variables. The a priori level of significance was set at a P value $<.05$.

RESULTS

Clinicopathologic Characteristics

Five hundred twenty-two patients were analyzed in this study, including 335 males (64.2%) and 187 females (35.8%), with ages at diagnosis ranging from 25 to 87 years (mean, 66 years; median, 67 years). The cohort consisted of 523 samples, with 406 samples (77.6%) of adenocarcinoma (ADC); 60 samples (11.5%) of squamous cell carcinoma; 30 samples (5.7%) of NSCLC, not-otherwise specified; 14 samples (2.7%) of large cell carcinoma; 5 samples (1%) of large cell neuroendocrine carcinoma; 4 samples (0.8%) of adenosquamous carcinoma; and 4 samples (0.8%) of sarcomatoid carcinoma.

Postsurgical staging was available for 205 tumor samples. According to tumor-status, there were 21 T1a (10.2%), 15 T1b (7.3%), 87 T2a (42.4%), 15 T2b (7.3%), 49 T3 (23.9%), and 18 T4 (8.8%). Forty-six cases were classified as N1 (22.4%), and 55 were classified as N2 (26.8%), whereas 104 (50.7%) did not show any metastatic nodal involvement at the time of diagnosis. The clinicopathologic characteristics of the patients are summarized in Table 1.

ALK Fluorescence In Situ Hybridization

ALK rearrangement assessed using FISH was identified in 20 out of 523 tumor samples (3.8%), including both the metachronous sarcomatoid carcinoma and adenocarcinoma that were diagnosed in the same patient (Table 1). The *ALK* $FISH^+$ NSCLC cases showed 2 main patterns using the LSI ALK dual-color break-apart probes: a balanced rearrangement with a break-apart (split) pattern, and an unbalanced rearrangement with an isolated (single) red signal (Figure 1, A). The mean percentage of positive nuclei was 34.7% in the $FISH^+$ NSCLC cases. At least 100 cells were analyzed in each case. The *ALK* $FISH^-$ cases showed 2 fusion signals or close proximity of the red and green signals (Figure 1, B).

ALK Immunohistochemistry

Of 523 tumor samples 18 (3.4%) were ALK^+ by IHC (Table 1). All of the IHC^+ tumor samples showed diffuse and strong, granular, cytoplasmic staining (Figure 1, C). Of 523 samples, 505 (96.6%) showed negative immunohistochemical staining (Figure 1, D).

We observed extremely weak, background staining, which was visible as light cytoplasmic stippling in alveolar macrophages, cells of neural origin (nerve and ganglion cells), lymphocytic cells, normal mucosa in NSCLC (including mucin), and necrotic tumor areas. That result did not impair the evaluation of tumor cell immunostaining.

Comparison Between *ALK* Fluorescence In Situ Hybridization and Immunohistochemistry

Eighteen IHC^+ tumor samples showed *ALK* rearrangement by FISH. In 2 of the 505 IHC^- cases (0.4%; Figure 2, A and B), an *ALK* rearrangement was detected by FISH (Figure 2, C and D). All other cases (503 of 523; 96.2%) were negative by both the IHC and FISH tests (Table 2). We analyzed the agreement level between the IHC and FISH results by performing a Cohen κ test. We found almost perfect agreement between the 2 tests (Cohen κ, 0.945; confidence interval, 0.87–1; P value $< .001$).

Detection of *EML4-ALK* Using a MassARRAY-Based Method and Comparison With FISH and IHC Results

We performed an assay based on the MassARRAY platform for the detection of *EML4-ALK* in a subset of 11 samples, including the 2 discrepant cases ($FISH^+/IHC^-$).

We detected the presence of *EML4-ALK* variants known to be ALK^+ by FISH and IHC analysis: variant 1 in 2 samples (Figure 3, A), variant 2 in 1 sample (Figure 3, B), and variant 5′ (Figure 3, C) in another sample. No mass peaks for *EML4-ALK* variants were detected by the MassARRAY assay in the remaining specimens that were ALK^- by both FISH and IHC analysis, including the 2 cases with discrepant results (Table 3).

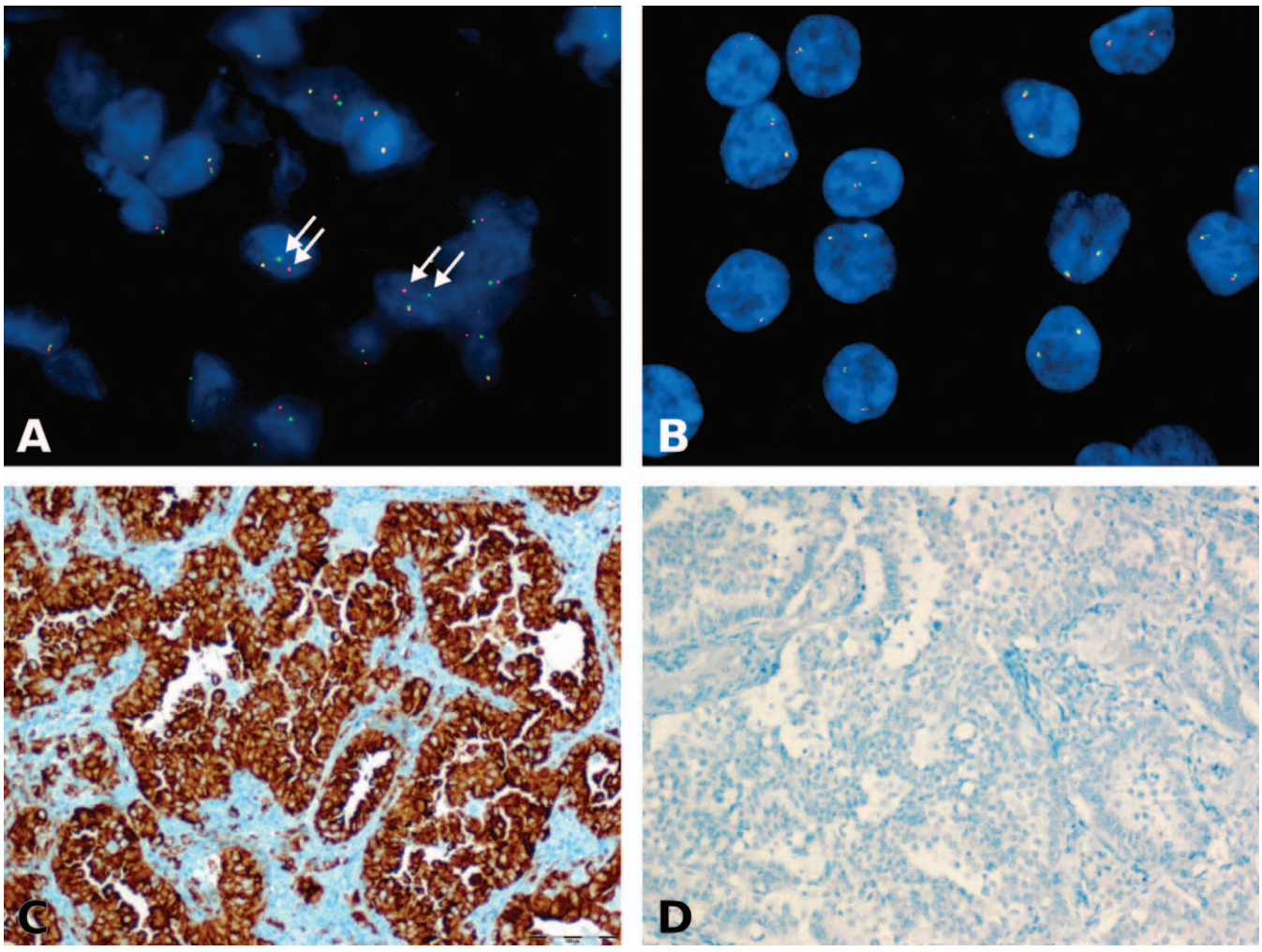

Figure 1. *A and B, Fluorescence in situ hybridization (FISH) analysis in non–small cell lung cancer (NSCLC) using a break-apart probe. A, FISH-positive case demonstrating cells with typical anaplastic lymphoma kinase (*ALK*) translocation with one normal (paired) signal and 2 pairs of separated signals (arrows). B, A FISH-negative case showing fusion or very close apposition of the probes adjacent to the 3′ and 5′ ends of the gene. C and D, Immunohistochemistry for ALK in NSCLC using the D5F3 antibody. C, Positive immunohistochemical staining of the ALK protein showing strong granular cytoplasmic expression in NSCLC. D, An example of ALK$^-$ immunohistochemical staining in a case of NSCLC (original magnifications ×1000 [A and B] and ×100 [C and D]).*

In addition, we observed a point mutation (C1156Y) of the *ALK* gene in a FISH, IHC, and MassARRAY assay-negative case (Figure 3, D; Table 3).

EML4-ALK Detection by RT-PCR in the 2 Cases With Discrepant IHC and FISH Results

Using RT-PCR for *EML4-ALK* fusion messenger RNA (mRNA), we investigated the presence of the *EML4-ALK* variants 1, 2, and 3 in the 2 cases with discrepant IHC and FISH results. The RT-PCR analysis was successful in all samples; however, the 2 cases were negative for the analyzed variants.

Relationship Between *ALK* FISH Rearrangement, Clinicopathologic Characteristics and Mutational Status

Correlations between *ALK* rearrangement, patient clinicopathologic characteristics, and mutational status are summarized in Tables 4 and 5.

Among the 19 patients who were positive for *ALK* by FISH, there were 11 males (58%) and 8 females (42%), with ages at diagnosis ranging from 37 to 77 years (mean, 57.5 years; median, 62.5 years). One patient presented with a metachronous sarcomatoid carcinoma and adenocarcinoma, both of which had *ALK* rearrangement. Patients who were *ALK*$^+$ by FISH were younger than those patients who were *ALK*$^-$ by FISH (range, 25–87 years; mean, 66 years; median, 67 years) ($P < .001$) (Table 4). We defined early onset lung cancers by classifying patients as either younger or older than 63 years (median age).

Nine of 19 patients (47%) who were *ALK*$^+$ by FISH underwent crizotinib treatment, including one of the 2 cases with discrepant IHC and FISH results. Eight of the 9 patients (89%) achieved partial response. The patient with discrepant IHC and FISH results was a 66-year-old man with a significant smoking history, a large right middle lobe mass, and hilar and mediastinal lymphadenopathy at the time of presentation. This specimen was a bronchial biopsy showing a NSCLC, not-otherwise specified, with neuroendocrine differentiation that indicated *ALK* translocation. Based on those data, the patient was treated with crizotinib. Surprisingly, after 2 months, a computed tomography scan of the chest and brain showed progression of the disease.

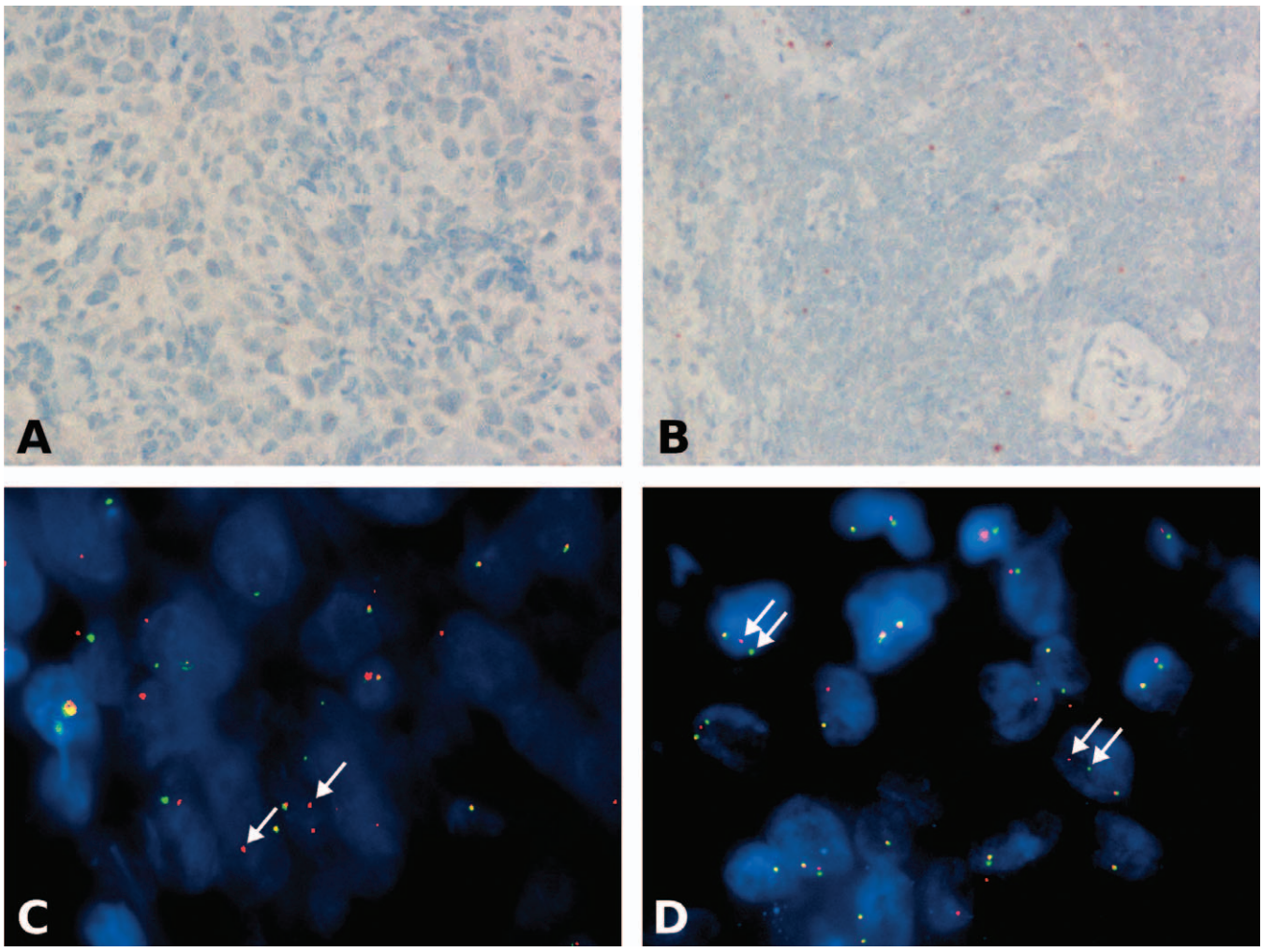

Figure 2. *A and B, Anaplastic lymphoma kinase (ALK)–negative immunohistochemical staining of the 2 cases with discrepant immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) results. C and D, The FISH analysis in the 2 cases with discrepancy between FISH and IHC demonstrates the presence of* ALK *translocation (arrows) (original magnifications ×200 [A and B] and ×1000 [C and D]).*

The other patient with discordant IHC and FISH results died before the start of treatment. At the time of analysis, the median follow-up of the 9 patients treated with crizotinib was 22 months (range, 4–45 months) among the living patients; at that time, 4 patients (44%) had died. The median overall survival was 22 months, and progression-free survival was 18 months.

By histologic classification, most patients with a positive *ALK* by FISH (n = 20) exhibited ADC (n = 17; 85%), 1 (5%) had sarcomatoid carcinoma, 1 (5%) had adenosquamous carcinoma, and 1 (5%) had NSCLC, not-otherwise specified, with neuroendocrine differentiation. No squamous histotypes were detected in our cases.

Moreover, by ADC histology in patients positive for *ALK* by FISH (n = 17), we identified the predominant histologic pattern in surgical specimens according to the revised classification of lung adenocarcinoma provided by the European Respiratory Society/American Thoracic Society/International Association for the Study of Lung Cancer.[23,24] Briefly, a predominant solid pattern was observed in 9 cases (53%), whereas 5 cases (29%) presented with a predominant acinar pattern, and the 3 other cases (18%) presented with a predominant papillary pattern. Signet ring features were observed in 6 of 17 patients (35%) with *ALK*$^+$ findings. The distribution of FISH-positive cases according to *t* status and nodal involvement in surgically resected samples is reported in Table 4.

All of the molecular analyses for *EGFR* and *KRAS* mutation and for *ALK* rearrangement were performed on the same paraffin block on serial sections. All alterations were mutually exclusive.

Of the 523 analyzed specimens, *EGFR* mutations were identified in 70 cases (13.4%) (Table 1) with the following distribution: 39 (55.7%) in exon 19, 2 (2.9%) in exon 18, 3 (4.3%) in exon 20, and 26 (37.1%) in exon 21. Exon 19 showed classic in-frame deletions involving 5 amino acids

Table 2. Comparison Between Immunohistochemistry (IHC) and Fluorescence In Situ Hybridization (FISH) Data[a]

Cases, N = 523	FISH-Positive Cases, No., n = 20	FISH-Negative, No., n = 503
IHC positive, n = 18	18	0
IHC negative, n = 505	2	503

[a] Cohen κ, 0.945; confidence interval, 0.87–1; $P < .001$.

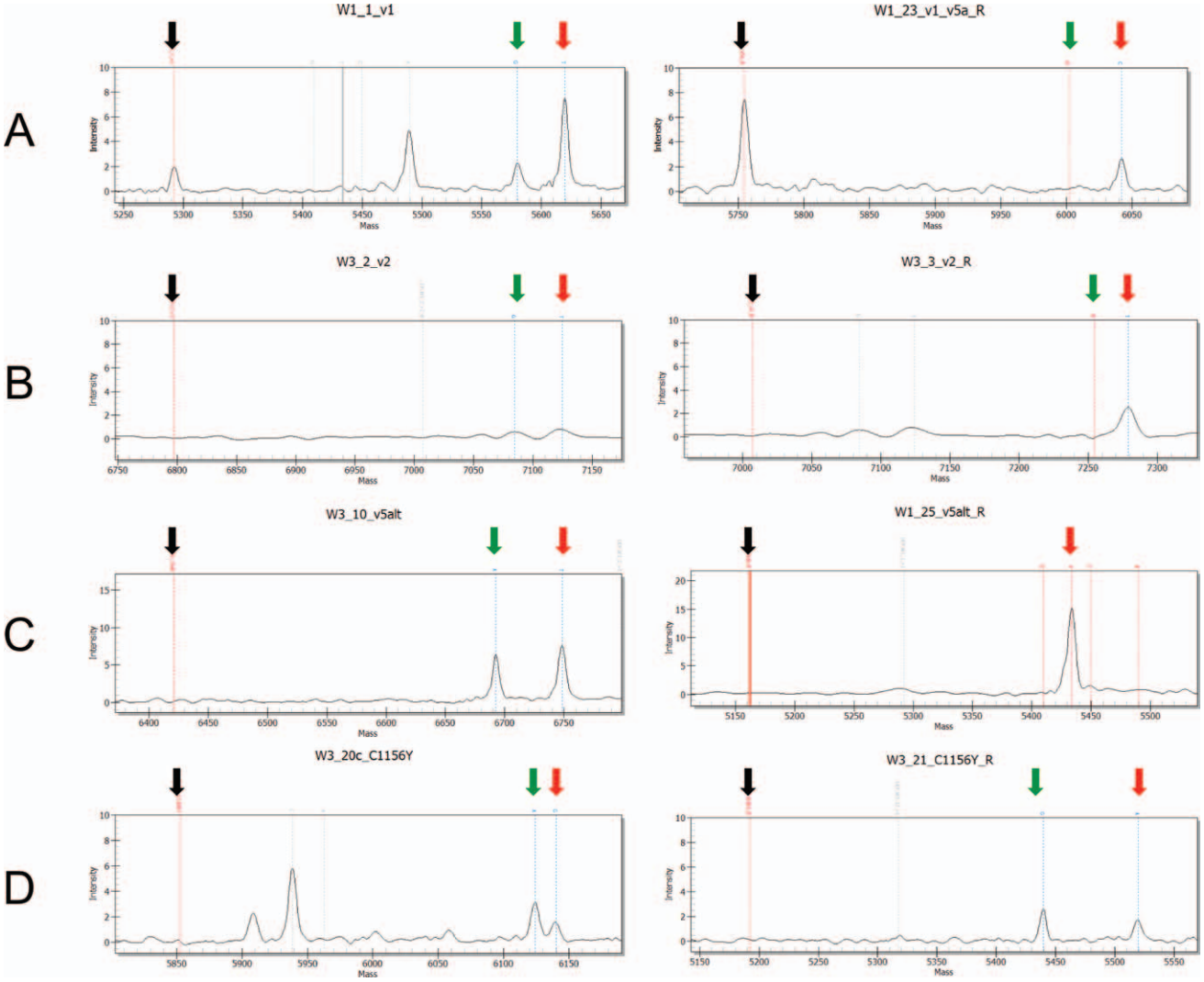

Figure 3. *Lanes A through D, Representative spectra for anaplastic lymphoma kinase (*ALK*) variants with forward assay results on the left and reverse assay results on the right. Lanes A through C, Representative spectra for echinoderm microtubule associated proteinlike 4–anaplastic lymphoma kinase (*EML4-ALK*) variants 1, 2, and 5′ respectively. Lane D, Representative spectra for* ALK *C1156Y mutation. The black arrow shows the unextended primer, whereas the green and the red arrows correspond to the wild-type and the mutant allele, respectively. The v5alt_R assay doesn't have any peak correspondent to the wild-type allele, because when* ALK *is not rearranged, the relative single-base primer extension primer is not extended. The nonhighlighted peaks correspond to extended and/or unextended primers of other assays that are part of the same multiplex.*

encoded by codons 746 through 750 (ELREA) and the amino acids encoded by codons 747 to 759. Mutations in exon 21 were missense substitutions resulting in a leucine to arginine change at codon 858 (L858R). The mutations in the other exons were all single-nucleotide substitutions.

KRAS mutations were detected in 124 of the 523 analyzed samples (23.7%) (Table 1). Almost all of those mutations (118 of 124; 95.2%) involved codon 12, whereas 6 mutations (4.8%) involved codon 13.

COMMENT

ALK activation has been previously identified as a driver of oncogenic alteration in a subset of patients with NSCLC.[5,6] Recently, following impressive results in clinical trials, crizotinib, a small molecule that inhibits the tyrosine kinase activity of *ALK*, was approved for the treatment of patients with advanced *ALK*$^{+}$ NSCLC, as assessed by FISH.[18,19]

In the present study, we examined a cohort of 523 NSCLC samples derived from our molecular clinical workflow that

Table 3. Correlation Among Immunohistochemistry (IHC), Fluorescence In Situ Hybridization (FISH), and MassARRAY Data

Case No., n = 11	FISH Results	IHC Results	MassARRAY Results
1	Positive	Positive	*EML4-ALK* variant 1
2	Positive	Positive	*EML4-ALK* variant 1
3	Positive	Positive	*EML4-ALK* variant 5′
4	Positive	Positive	*EML4-ALK* variant 2
5	Positive	Negative	WT
6	Positive	Negative	WT
7	Negative	Negative	WT
8	Negative	Negative	WT
9	Negative	Negative	WT
10	Negative	Negative	WT
11	Negative	Negative	C1156Y

Abbreviation: WT, wild type.

Table 4. Patient Characteristics by *ALK* Status[a]					
Clinicopathologic Characteristics	**Cases, No. (%)**	***ALK*⁺ by FISH, No. (%)**	***ALK*⁻ by FISH, No. (%)**	**ALK⁺ by IHC, No. (%)**	**ALK⁻ by IHC, (%)**
Age, y, n = 522					
≤63	282 (54.0)	18* (94.7)	264 (52.5)	17* (100)	265 (52.5)
>63	240 (46.0)	1 (5.3)	239 (47.5)	0 (0)	240 (47.5)
Sex, n = 522					
M	335 (64.2)	11 (57.9)	324 (64.4)	9 (52.9)	326 (64.5)
F	187 (35.8)	8 (42.1)	179 (35.6)	8 (47.1)	179 (35.4)
Histology, n = 523					
ADC	406 (77.5)	17 (85.0)	389 (77.3)	16 (88.8)	390 (77.2)
SC	4 (0.8)	1 (5.0)	3 (0.6)	1 (5.6)	3 (0.6)
ASC	4 (0.8)	1 (5.0)	3 (0.6)	1 (5.6)	3 (0.6)
NSCLC-NOS	30 (5.7)	1 (5.0)	29 (5.8)	0 (0)	30 (5.9)
Others	79 (15.2)	0 (0)	79 (15.7)	0 (0)	79 (15.6)
Tumor size, n = 205					
T1a	21 (10.2)	1 (14.3)	20 (10.1)	1 (14.3)	20 (10.1)
T1b	15 (7.3)	0 (0)	15 (7.6)	0 (0)	15 (7.6)
T2a	87 (42.4)	2 (28.6)	85 (42.9)	2 (28.6)	85 (42.9)
T2b	15 (7.3)	0 (0)	15 (7.6)	0 (0)	15 (7.6)
T3	49 (23.9)	3 (42.9)	46 (23.2)	3 (42.8)	46 (23.2)
T4	18 (8.9)	1 (14.3)	17 (8.6)	1 (14.3)	17 (8.6)
Lymph-node metastases, n = 205					
N0	104 (50.7)	2 (28.6)	102 (51.5)	2 (28.6)	102 (51.5)
N1	46 (22.4)	1 (14.3)	45 (22.7)	1 (14.3)	45 (22.7)
N2	55 (26.8)	4 (57.1)	51 (25.8)	4 (57.1)	51 (25.8)

Abbreviations: ADC, adenocarcinoma; ALK, anaplastic lymphoma kinase; ASC, adenosquamous carcinoma; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NSCLC-NOS, non–small cell lung cancer, not-otherwise specified; SC, sarcomatoid carcinoma.

[a] Values with an asterisk (*) are statistically significant at $P < .001$.
Percentages are for column groupings.

had undergone *ALK*, *EGFR*, and *KRAS* analyses. In that cohort, *ALK*⁺ samples by FISH represent 3.8% (20 of 523) of the total, concordant with publications from other investigators, in a randomly selected population of patients with NSCLC.[11–13]

As previously reported, patients who were *ALK*⁺ were predominantly men, young, and never/light smokers with ADC histology.[14,16] In our study, young age was significantly associated with *ALK* rearrangement ($P < .001$), whereas significant differences were not observed between sexes according to histology. However, for the first time, to our knowledge, we observed the presence of an *ALK* translocation in a sarcomatoid variant of NSCLC, which arose as a metachronous lesion in a woman with a previous papillary ADC.[31] We also observed the presence of an *ALK* rearrangement in an adenosquamous carcinoma, as previously described by Kwak and Paik.[18,32] Moreover, *ALK* rearrangement was observed in a bronchial biopsy with poorly differentiated NSCLC with neuroendocrine differentiation, which was not otherwise classified because of the small size of the sample. Squamous histotypes did not show *ALK* rearrangement.

Interestingly, in our study, *ALK*-rearranged cases did not harbor *EGFR* or *KRAS* mutations, as reported by other authors.[5,12] These data confirm that patients with *ALK* rearrangement represent a predominantly unique subgroup of NSCLC.

In clinical practice, it is important to determine the presence of an *ALK* rearrangement in patients with late-staged NSCLC. Although FISH analysis has been approved as the gold standard for *ALK* rearrangement detection, the method is relatively expensive and time-consuming. Furthermore, the evaluation of positive FISH signals requires considerable skill because *ALK* rearrangement in NSCLC is intrachromosomal, causing relatively close break-apart probes.[33] Moreover, because *ALK* alteration is infrequently determined at the time of primary excision of the lesion, the FISH method would need to be employed on biopsy or archived NSCLC samples, which is particularly difficult. Therefore, FISH analysis may not be practical for screening every patient with NSCLC.

Alternately, IHC is easily performed routinely in most pathology laboratories, is less time-consuming, and can be successfully conducted using archival tissues and small biopsies.

Table 5. Correlation Among *EGFR* and *KRAS* Mutations and *ALK* Alterations			
***ALK* Status, n = 523**	***EGFR* Mutations, No. (%)**	***KRAS* Mutations, No. (%)**	***EGFR-KRAS* Wild Type, No. (%)**
No *ALK* rearrangement	70 (13.4)	124 (23.7)	309 (59.1)
ALK rearrangement	0	0	20 (3.8)
Total	**70** (13.4)	**124** (23.7)	**329** (62.9)

Percentages are taken from the total of 523 specimens.

To evaluate the role of *ALK* IHC as a screening tool in the identification of candidates for *ALK* inhibitor therapy in advanced NSCLC, we retrospectively compared the test concordance of *ALK* FISH and ALK IHC in the cohort of 523 NSCLC samples. We used a new, high-sensitivity, and high-specificity monoclonal antibody (D5F3) and a highly sensitive detection system (OptiView DAB IHC detection kit and OptiView amplification kit) to enhance the assay sensitivity. The advantage of this anti-ALK IHC assay is that the use of these kits enabled most NSCLC cases to easily be interpreted as "positive" or "negative," providing more objective evaluation of the results by pathologists.

Data from the literature showed that the detection threshold of the rearranged protein is principally determined by the affinity of the primary antibody and of the signal amplification system.[34] The 5A4 and D5F3 clones are known to be high-affinity antibodies. In particular, the rabbit monoclonal antibody D5F3 appears to be promising and shows good correlation with FISH analysis results.[35–38]

In contrast to the most recent studies, we used a binary scoring system (positive or negative for ALK status) for the evaluation of the results. We found that all ALK^+ patients by IHC were also positive for *ALK* rearrangement by FISH. However, in 2 FISH-positive cases, IHC failed to indicate the rearranged protein, and MassARRAY did not detect any *ALK* variant RNA-transcripts in those cases. In the 2 cases, we also performed RT-PCR for *EML4-ALK* fusion mRNA to investigate the presence of variants 1, 2, and 3 of *EML4-ALK*. In both cases, amplification was not evident. Moreover, one of those 2 patients, who underwent crizotinib treatment, did not show any response, whereas the other 8 patients so treated showed partial response. These findings support the hypothesis that the detected rearrangements by FISH were most likely not transcribed or translated.[39] In fact, one possible explanation for those results is that the translocation was present, but the rearranged ALK protein was either not expressed or was expressed at a level so low that it was undetectable. Alternately, an *ALK* fusion partner different from *EML4* (such as *TFG* or *KIF5B*) could explain the difference between the results obtained at the DNA level and those obtained at the mRNA/protein level. All 503 FISH-negative cases were found to be negative by IHC.

Interestingly, we observed a point mutation, C1156Y, in the *ALK* gene in one of the cases found to be negative by FISH, IHC, and MassARRAY assay. This point mutation was previously described in patients with ALK^+ NSCLC with acquired resistance to crizotinib.[40,41] To our knowledge, this is the first report in which the presence of a C1156Y mutation is described in a patient without *ALK* rearrangement and, for that reason, deserves further investigation.

In conclusion, our results showed that IHC analysis using the monoclonal antibody D5F3 and a highly sensitive detection system is a rapid, simple, and useful tool for the selection of cases to submit to *ALK* FISH analysis. After further confirmatory studies in large subsets of FISH-positive cases, IHC could represent the best method for selecting patients for ALK inhibitor therapy in NSCLC.

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