Parallel FISH and Immunohistochemical Studies of ALK Status in 3244 Non–Small-Cell Lung Cancers Reveal Major Discordances

Florian Cabillic, PharMD, PhD,†‡ Audrey Gros, PharMD, PhD,§ Frédéric Dugay, PharMD,*‡‖ Hugues Begueret, MD, PhD,¶ Laura Mesturoux, MD,§ Dan Cristian Chiforeanu, MD,# Leila Dufrenot, MD,¶ Vincent Jauffret,‡ Dominique Dachary, MD,§ Romain Corre, MD,** Alexandra Lespagnol, PhD,†† Gwendoline Soler, MD, PhD,§ Julien Dagher,*‡‖ Véronique Catros, PharMD, PhD,*†‡ Michèle Le Calve, PharMD,*‡ Jean-Philippe Merlio, MD, PhD,§ and Marc-Antoine Belaud-Rotureau, PharMD, PhD*‡‖*

Introduction: Anaplastic lymphoma kinase (*ALK*) rearrangements occur in 1% to 7% of non–small-cell lung cancers (NSCLCs). Crizotinib, an ALK inhibitor, has been demonstrated to provide dramatic clinical benefits in ALK-positive advanced-stage NSCLC. Fluorescent in situ hybridization (FISH) has been established in clinical trials as the standard procedure method for detecting *ALK* rearrangements. Although the detection of ALK by immunohistochemistry (IHC) has been proposed for the screening of patients, large-scale studies are warranted to validate such a hierarchical approach.

Methods: In this article, we report the largest series thus far of parallel FISH and IHC ALK testing in 3244 consecutive NSCLC cases analyzed at two independent French centers.

Results: FISH-positive and/or IHC-positive results were demonstrated in 150 of 3244 cases (4.6%). An imbalanced sex ratio was detected, with women exhibiting a 2.2-fold relative risk for an alteration. Strikingly, only 80 of 150 specimens were classified as ALK positive by both techniques. The specimens with discordant FISH/IHC analyses were FISH-positive/IHC-negative (36), FISH-negative/IHC-positive (19), or FISH-noncontributive/IHC-positive (15). Thus, a single FISH or IHC analysis performed alone would have failed to detect approximately one-fourth of the ALK-positive cases with similar findings in our two centers.

Conclusions: This study highlights the feasibility of systematic NSCLC testing by both FISH and IHC in routine practice. Many preanalytical factors may account for the apparent discrepancies between both methods, suggesting that hierarchical screening may underscore ALK-positive cases. This significant level of discrepancy supports the need of combined testing to optimize the detection of ALK-inhibitor–eligible patients given that some patients with discordant testing were found to respond to crizotinib.

Key Words: Non–small-cell lung cancer, Anaplastic lymphoma kinase, Fluorescent in situ hybridization, Immunohistochemistry, Biomarker.

(*J Thorac Oncol***.** 2014;9: 295–306)

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase encoded by a gene located on the chromosome arm 2p. *ALK* was so named when it was discovered to be translocated [t(2;5)(p23;q35)] in anaplastic large-cell lymphoma, a subset of non-Hodgkin's lymphoma. Recently, a fusion protein with transforming activity was described in non–-small-cell lung cancer (NSCLC); this fusion was formed by a small inversion in the region of the chromosome 2 [Inv (2)(p21;p23)] that joins the *echinoderm microtubule–associated protein-like 4* (*EML4*) and *ALK* genes.[1,2] The *ALK* chromosomal breakpoint commonly lies between exons 19 and 20 but is variable on the *EML4* side; more than 21 EML4-ALK variants have been identified.[3] In addition to *EML4*, other translocation partners have been identified in NSCLC, notably *kinesin family member 5B*, *TRK-fused gene*, and *kinesin light chain 1*, leading to activation of signaling pathway and both experimental and clinical responses to ALK inhibitors.[4] Crizotinib is a potent and selective ATP-competitive inhibitor of the MNNG HOS transforming gene (MET) and ALK tyrosine kinases which has been patented by Pfizer. In the phase I/II trials enrolling NSCLC patients with documented *ALK*

*Université de Rennes 1, Faculté de Médecine, Rennes, France; †Inserm, UMR991, Liver Metabolisms and Cancer, Rennes, France; ‡Service de Cytogénétique et Biologie Cellulaire, CHU de Rennes, Rennes, France; §Service de Biologie des Tumeurs, CHU de Bordeaux, Bordeaux, France; ‖UMR CNRS 6290, IFR 140, Rennes, France; ¶Service de Pathologie, CHU de Bordeaux, Pessac, France; #Service d'Anatomie et Cytologie Pathologiques, CHU de Rennes, Rennes, France; **Service de Pneumologie, CHU de Rennes, Rennes, France; and ††Service de Génétique Moléculaire et de Génomique Médicale, CHU de Rennes, Rennes, France.

Disclosure: The authors declare no conflict of interest.

The last two authors have to be considered as co-last authors.

Address for correspondence: Florian Cabillic, PharMD, PhD, Laboratoire de Cytogénétique et Biologie Cellulaire, Inserm UMR991, Université de Rennes 1, F35043 Rennes, France. E-mail: florian.cabillic@chu-rennes.fr

ISSN: 1556-0864/14/0903-0295

rearrangements, the objective response rates were impressive (60%). Most responses were achieved during the first 8 to 9 weeks of treatment, and the duration of the response was approximately 45 to 50 weeks.[5] Consequently, crizotinib (Xalkori, Pfizer) obtained by an accelerated Food and Drug Administration approval in 2011 and, more recently, the marketing authorization of the European Medicines Agency. In addition, recent data from phase I/II trials with second-generation ALK tyrosine kinase inhibitors (LDK378, AP26113) revealed promising response rates in patients who had relapsed from crizotinib.[6,7] These results highlight the ethical need to perform exhaustive and efficient screening of NSCLC patients to diagnose the patients who are most likely to benefit from this new therapy. According to the majority of the series, the *ALK* locus rearrangement was observed in 1% to 7% of NSCLCs, without Kirsten rat sarcoma (*KRAS*)- or epidermal growth factor receptor (*EGFR*)-associated mutations.[8–12] Moreover, *ALK*-rearranged NSCLCs are thought to exhibit unique clinicopathological features, such as young age, a negative or light history of smoking, advanced clinical stage, and solid histology with signet ring cells.[13–15] The selection of patients on the basis of adenocarcinoma histology, the absence of *EGFR/KRAS* and *ERBB2* mutation, less than a 20 pack/year history of smoking, and poor or moderate cell differentiation has been shown to increase the rate of detection up to 29.6% and has been proposed as an algorithm to select patients for testing.[16] However, other studies demonstrated that selecting patients on a clinical and morphological basis is not sufficient to identify NSCLC patients with *ALK* rearrangements.[14,17]

Currently, the only approved companion test for the detection of ALK positivity is fluorescent in situ hybridization (FISH) using break-apart (BA) probes (Food and Drug Administration new drug application: 202570). Such testing has been validated in clinical trials to select patients who respond to crizotinib. For some authors, FISH may not be suitable for large-scale screening because of its running costs, the need for trained observers, and adapted equipment. Reverse-transcription polymerase chain reaction (RT-PCR) or immunohistochemistry (IHC) are thought to represent alternative methods. However, the large number of currently known and not yet unraveled *ALK* translocation partners makes the identification of all variants by molecular techniques such as RT-PCR prohibitively difficult.[18] With respect to IHC, new antibodies for the detection of the chimeric ALK protein have been validated along with FISH and RT-PCR.[14,17,19–23] IHC was found to be a reliable screening tool, but standardization of its interpretation has not yet been established.[24]

In this article, we have compiled the unselected data of two independent groups who performed daily parallel analyses of *ALK* rearrangements by FISH and ALK protein detection by IHC. This study documents a large-scale testing of ALK in 3244 unselected NSCLC patients. Concordance of the data obtained by the two groups highlights the feasibility of *ALK* FISH testing in routine practice using automated systems and reveals a significant level of discrepancy between the FISH and IHC results.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the institutional ethics committees of the Rennes and Bordeaux university hospitals, which waived the need for informed consent because of the observational nature of the study. The study was performed using routine FISH and IHC testing. Collection of the data and further analyses, such as statistical testing, were performed anonymously.

Patient Cohort

A total of 3244 NSCLC patients were consecutively referred to the Rennes and Bordeaux pathology and cytogenetics departments for the evaluation of ALK status. In Rennes, the cohort consisted of 1843 cases of NSCLCs, including 1289 male and 554 female patients. This cohort was histologically classified as 1393 adenocarcinoma, 294 squamous cell carcinoma (SCC), eight adenosquamous cell carcinoma, and 148 not otherwise specified (NOS) cases. In Bordeaux, the recruitment focused on samples with adenocarcinoma histology. Nine patients with SCC were also studied because they occurred in young patients or in light smokers. The cohort consisted of 1401 patients (842 male and 559 female patients) with tumors histologically classified as 1203 adenocarcinoma, nine SCC, six adenosquamous cell carcinoma, and 183 NOS (Tables 1 and 2). The histological classification was on the basis of hematoxylin-eosin staining and phenotypical markers (thyroid transcription factor-1, P63, and cytokeratins 5/6/7).

Fluorescent In Situ Hybridization

Specimens for biopsy originating from different peripheral sites were extemporaneously prepared in the central molecular analysis platform and analyzed within 1 week. Interphase FISH analysis was performed on 4-μm sections of formalin- or alcohol–formol–acetic-acid–fixed, paraffin-embedded tumor

TABLE 1. Characteristics of the Patients

	Total Cases	ALK-Positive Cases
Number	3244	150
Age	65 ± 11 (24–95)	63 ± 13 (28–95)
Sex		
Male	2131	69
Female	1113	81
Histology		
ADK	2596	131
SSC	303	3
ADSK	14	2
NOS	331	14
Mutations		
EGFR	311	8
KRAS	681	14

Age: mean ± SD (min–max).
ADK, adenocarcinoma; ADSK, adenosquamous cell carcinoma, SCC, squamous cell carcinoma; NOS, not otherwise specified; *ALK*, anaplastic lymphoma kinase; *EGFR*, epidermal growth factor receptor; *KRAS*, Kirsten rat sarcoma.

TABLE 2. Characteristics of the Patients with ALK Alterations

Patient	ALK FISH	FISH Pattern	Positive Nuclei (%)	Score IHC	Age (yr)	Sex	Histology	Tumor Site	Surgical Procedure	Tumor Cell (%)	*EGFR*/*KRAS* Mutation	Therapy	Crizotinib Response
Rennes University Hospital													
1	POS	IRS	76	2/3+	72	F	ADK	P	OS	>50	No	Chemo	
2	POS	IRS	54	2/3+	69	M	ADK	P	B	25–50	No	Crizo	Yes
3	POS	BA	16	2/3+	66	M	ADK	P	B	>50	No	Surg	
4	POS	IRS	30	2/3+	54	M	ADK	P	B	10–25	No	Crizo	Yes[a]
5	POS	BA	27	2/3+	74	F	ADK	P	B	>50	No	Crizo	Yes
6	POS	BA	17	2/3+	49	M	ADSK	P	B	10–25	No	Crizo	Yes[a]
7	POS	BA	24	2/3+	49	F	ADK	M	L	>50	No	Crizo	Yes
8	POS	BA	16	2/3+	60	F	ADK	P	B	10–25	No	Chemo	
9	POS	IRS	26	2/3+	87	M	ADK	P	B	>50	No	Died	
10	POS	BA	45	2/3+	49	F	ADK	M	B	25–50	No	Crizo	Yes
11	POS	BA	25	2/3+	64	F	ADK	P	B	25–50	No	Surg	
12	POS	BA	50	2/3+	63	F	ADK	M	OS	>50	No	Crizo	Yes
13	POS	BA	55	2/3+	67	F	ADK	P	TP	>50	No	Crizo	Yes
14	POS	BA	50	2/3+	58	F	ADK	P	B	>50	No	Crizo	Yes
15	POS	BA	60	2/3+	70	F	ADK	P	B	>50	No	Chemo	
16	POS	BA	20	2/3+	59	F	ADK	P	B	>50	No	Chemo	
17	POS	BA	65	2/3+	35	F	ADK	M	OS	>50	No	Crizo	Yes
18	POS	BA	40	2/3+	60	F	ADK	M	OS	>50	No	Crizo	Yes
19	POS	BA	65	2/3+	79	M	ADK	M	B	10–25	No	Died	
20	POS	BA	57	2/3+	77	F	ADK	M	B	>50	No	Crizo	Yes
21	POS	BA	18	2/3+	78	F	ADK	M	L	10–25	No	Crizo	Yes
22	POS	BA	60	2/3+	78	F	ADK	P	B	>50	No	Crizo	Yes
23	POS	IRS	50	2/3+	74	F	ADK	M	B	>50	ND	Died	
24	POS	BA	50	2/3+	76	M	ADK	P	OS	25–50	No	Crizo	Yes
25	POS	IRS	30	2/3+	76	F	ADK	M	L	25–50	No	Chemo	
26	POS	BA	55	2/3+	67	F	ADK	P	OS	>50	No	Chemo	
27	POS	IRS	60	2/3+	78	M	ADK	P	B	>50	No	Crizo	Yes
28	POS	IRS	80	2/3+	74	F	ADK	P	B	>50	No	Chemo	
29	POS	BA	50	2/3+	49	M	ADK	P	B	10–25	No	Chemo	
30	POS	BA	55	2/3+	31	F	ADK	P	OS	25–50	No	Chemo	
31	POS	BA	40	2/3+	41	M	ADK	P	B	>50	No	Crizo	Yes
32	POS	IRS	70	2/3+	65	M	ADK	ND	B	25–50	No	Radioth	
33	POS	BA	80	1+	93	F	NOS	M	L	>50	No	Died	
34	POS	BA	40	0	73	F	ADK	P	OS	>50	No	Surg	
35	POS	BA	18	0	58	M	ADK	M	B	>50	No	Died	
36	POS	BA	50	0	60	M	ADK	P	B	>50	No	None	
37	POS	BA	20	0	72	F	NOS	P	B	10–25	No	Died	
38	POS	BA	18	0	55	M	ADK	M	OS	>50	No	Chemo	
39	POS	BA	63	0	61	M	ADK	P	B	>50	No	Chemo	
40	POS	IRS	60	0	72	M	SCC	P	B	25–50	No	Chemo	
41	POS	IRS	60	0	65	M	ADK	P	OS	>50	L858R + T790M	Crizo	Yes
42	POS	IRS	60	0	63	F	ADK	M	B	>50	No	Crizo	Yes
43	POS	IRS	60	0	80	F	NOS	P	B	>50	No	None	
44	POS	IRS	30	0	58	M	ADK	P	B	>50	KRAS	Chemo	
45	POS	IRS	45	0	67	M	ADK	P	OS	>50	KRAS	Chemo	
46	POS	BA	60	0	66	M	ADK	M	B	>50	No	Crizo	Yes
47	NEG	/	/	2/3+	45	F	ADK	M	OS	>50	No	Crizo	Yes
48[c]	NEG	/	/	2/3+	66	F	ADK	P	OS	>50	No	Chemo	

(*Continued*)

TABLE 2. (*Continued*)

Patient	ALK FISH	FISH Pattern	Positive Nuclei (%)	Score IHC	Age (yr)	Sex	Histology	Tumor Site	Surgical Procedure	Tumor Cell (%)	*EGFR*/*KRAS* Mutation	Therapy	Crizotinib Response
49	NEG	/	/	2/3+	62	M	ADK	M	L	>50	KRAS	Chemo	
50	NEG	/	/	2/3+	67	F	ADK	M	B	>50	No	Chemo	
51	NC	/	/	2/3+	52	F	ADK	M	B	25–50	NC	Crizo	Yes
52	NC	/	/	2/3+	95	F	ADK	M	L	25–50	No	None	
53	NC	/	/	2/3+	65	F	ADK	P	B	10–25	No	Crizo	Yes[b]
54	NC	/	/	2/3+	82	F	ADK	P	B	25–50	No	Lost	
55	NC	/	/	2/3+	61	M	ADK	P	B	25–50	NC	Chemo	
56	NC	/	/	2/3+	68	F	ADK	P	OS	>50	NC	Surg	
57	NC	/	/	2/3+	45	M	ADK	P	B	<10	NC	Chemo	
58	NC	/	/	2/3+	77	F	ADK	M	B	<10	Del19	Died	
59	NEG	/	/	1+	52	M	ADK	P	OS	25–50	Del19	Surg	
60	NEG	/	/	1+	51	M	ADK	P	B	>50	No	Surg	
61	NEG	/	/	1+	65	F	ADK	P	TP	25–50	Del19	Surg	
62	NEG	/	/	1+	65	M	ADK	P	OS	25–50	L858R	Gefitinib	
63	NEG	/	/	1+	63	F	ADK	M	OS	>50	No	Chemo	
64	NEG	/	/	1+	69	M	ADK	P	B	25–50	KRAS	Died	
65	NEG	/	/	1+	70	F	ADK	P	OS	>50	KRAS	Chemo	
66	NC	/	/	1+	56	M	ADK	P	B	25–50	KRAS	Chemo	
67	NC	/	/	1+	77	M	ADK	P	B	>50	No	Died	
68	NC	/	/	1+	80	F	ADK	P	B	>50	NC	Chemo	
Bordeaux University Hospital													
69	POS	BA	15	2/3+	67	F	ADK	P	B	>50	No	Crizo	Yes[a]
70	POS	BA	20	2/3+	80	F	ADK	P	OS	>50	No	Chemo	
71	POS	BA	20	2/3+	64	F	ADK	M	OS	>50	No	Lost	
72	POS	IRS	80	2/3+	62	F	ADK	P	OS	>50	No	Crizo	Yes
73	POS	IRS	15	2/3+	52	M	ADK	P	B	25–50	No	Crizo	Yes[a]
74	POS	BA	35	2/3+	82	M	ADK	P	L	>50	No	Chemo	
75	POS	IRS	30	2/3+	38	M	ADK	P	B	10–25	No	Crizo	Yes
76	POS	IRS	40	2/3+	56	F	ADK	P	OS	>50	No	Chemo	
77	POS	BA	60	2/3+	68	M	ADK	P	B	>50	No	Died	
78	POS	BA	40	2/3+	35	F	ADK	P	OS	>50	No	Crizo	Yes[a]
79	POS	BA	60	2/3+	58	F	ADK	P	OS	>50	No	Lost	
80	POS	BA	55	2/3+	80	M	ADK	M	B	10–25	NC/No	Lost	
81	POS	IRS	30	2/3+	44	F	ADK	P	B	>50	No	Radioth	
82	POS	BA	16	2/3+	28	M	NOS	P	B	>50	No	Crizo	Yes[a]
83	POS	IRS	50	2/3+	41	F	ADK	P	OS	25–50	No/NC	Lost	
84	POS	IRS	90	2/3+	65	F	ADK	P	OS	>50	No	Lost	
85	POS	IRS	50	2/3+	70	F	ADK	M	OS	>50	ND	Crizo	Yes
86	POS	BA	60	2/3+	28	M	ADK	P	B	10–25	ND	Crizo	Yes
87	POS	IRS	60	2/3+	67	F	ADK	M	OS	25–50	No	Radioth	
88	POS	IRS	50	2/3+	84	F	ADK	P	B	10–25	No	Chemo	
89	POS	IRS	70	2/3+	72	F	ADK	P	B	25–50	No	Crizo	Yes
90	POS	IRS	50	2/3+	78	M	ADK	P	OS	25–50	No	Crizo	Yes
91	POS	BA	80	2/3+	41	M	ADK	P	OS	>50	No	Crizo	Yes
92	POS	BA	40	2/3+	71	M	ADK	P	B	25–50	No	Chemo	
93	POS	BA	90	2/3+	58	F	ADK	P	OS	>50	No	Lost	
94	POS	IRS	90	2/3+	57	F	ADK	P	OS	>50	No	Surg	
95	POS	BA	40	2/3+	40	M	ADK	P	OS	>50	No	Crizo	Unknown
96	POS	BA	90	2/3+	67	M	ADK	P	B	25–50	ND	Lost	

(*Continued*)

TABLE 2. (*Continued*)

Patient	ALK FISH	FISH Pattern	Positive Nuclei (%)	Score IHC	Age (yr)	Sex	Histology	Tumor Site	Surgical Procedure	Tumor Cell (%)	*EGFR*/*KRAS* Mutation	Therapy	Crizotinib Response
97	POS	BA	35	2/3+	72	M	ADK	M	B	>50	No	Crizo	Not evaluated
98	POS	BA	30	2/3+	86	M	ADK	P	B	>50	No	Crizo	Stabilization
99	POS	BA	20	2/3+	48	M	ADK	M	B	ND	No	Surg	
100	POS	BA	40	2/3+	59	M	ADK	P	B	>50	No	Lost	
101	POS	IRS	40	2/3+	51	F	NOS	P	B	>50	No	Died	
102	POS	BA	20	2/3+	65	M	NOS	P	B	25–50	No	Erlotinib	
103	POS	BA	80	2/3+	51	F	ADK	P	B	25–50	No	Chemo	
104	POS	BA	60	2/3+	79	F	ADSK	P	B	25–50	No	Crizo	Yes[b]
105	POS	BA	60	2/3+	55	M	ADK	M	OS	>50	No	Chemo	
106	POS	BA	30	2/3+	51	M	ADK	P	L	25–50	No	Died	
107	POS	BA	50	2/3+	39	M	NOS	M	B	25–50	No	Lost	
108	POS	BA	50	2/3+	54	M	ADK	M	B	>50	No	Lost	
109	POS	BA	50	2/3+	70	F	ADK	M	B	>50	No	Chemo	
110	POS	IRS	65	2/3+	56	F	ADK	P	B	>50	No	Crizo	Yes
111	POS	IRS	50	2/3+	66	F	ADK	P	OS	10–25	No	Lost	
112	POS	BA	15	2/3+	59	F	ADK	ND	B	25–50	No	Chemo	
113	POS	BA	40	2/3+	44	M	ADK	M	B	>50	No	Lost	
114	POS	BA	50	1+	82	F	ADK	M	B	>50	No	Crizo	Yes
115	POS	BA	15	1+	82	F	ADK	P	B	25–50	No	Died	
116	POS	BA	15	0	70	M	ADK	P	B	>50	KRAS	Chemo	
117	POS	BA	15	0	65	F	ADK	P	OS	>50	KRAS	Chemo	
118	POS	IRS	70	0	69	M	ADK	P	OS	>50	No	Surg	
119	POS	IRS	70	0	56	M	NOS	P	OS	>50	No	Surg	
120	POS	BA	15	0	61	F	ADK	M	OS	>50	KRAS	Chemo	
121	POS	BA	60	0	53	M	ADK	P	B	25–50	No	Lost	
122	POS	IRS	20	0	62	M	ADK	P	B	25–50	L858R	Gefitinib	
123	POS	IRS	15	0	63	F	ADK	M	OS	>50	KRAS	Radioth	
124	POS	IRS	40	0	72	M	ADK	P	OS	>50	No	Surg	
125	POS	BA	20	0	64	M	ADK	P	OS	10–25	KRAS	Crizo	Stabilization[a]
126	POS	IRS	15	0	46	F	ADK	M	B	25–50	No	Died	
127	POS	BA	15	0	52	F	ADK	P	OS	>50	No	Surg	
128	POS	BA	15	0	73	F	NOS	P	B	>50	L858R	Gefitinib	
129	POS	BA	15	0	63	M	ADK	P	OS	>50	No	Crizo	Yes
130	POS	BA	15	0	59	M	NOS	M	B	>50	No	Chemo	
131	POS	BA	25	0	46	F	ADK	P	B	25–50	No	Died	
132	POS	BA	15	0	71	M	SCC	P	B	>50	No	Crizo	No
133	POS	BA	15	0	83	M	ADK	P	B	10–25	No	Chemo	
134	POS	BA	30	0	40	F	NOS	P	OS	>50	No	Surg	
135	POS	IRS	15	0	42	M	ADK	M	B	>50	No	Died	
136	POS	BA	20	0	66	F	NOS	P	B	>50	No	Chemo	
137	POS	IRS	15	0	50	F	ADK	P	TP	>50	KRAS	Erlotinib	
138	POS	BA	20	0	54	M	ADK	M	B	>50	No	Not yet treated	
139	NEG	/	/	2/3+	48	M	NOS	P	B	25–50	No	Chemo	
140	NEG	/	/	2/3+	67	F	ADK	P	OS	>50	No	Chemo	
141	NEG	/	/	2/3+	76	M	SCC	M	OS	>50	No	Chemo	
142	NEG	/	/	2/3+	68	M	ADK	P	OS	>50	L858R	Surg	
143	NEG	/	/	2/3+	77	M	ADK	M	B	>50	No	Chemo	
144	NEG	/	/	2/3+	60	F	NOS	P	B	25–50	KRAS	Died	
145	NEG	/	/	2/3+	63	F	ADK	M	OS	>50	No	Chemo	

(*Continued*)

TABLE 2. (*Continued*)

Patient	ALK FISH	FISH Pattern	Positive Nuclei (%)	Score IHC	Age (yr)	Sex	Histology	Tumor Site	Surgical Procedure	Tumor Cell (%)	*EGFR*/*KRAS* Mutation	Therapy	Crizotinib Response
146	NEG	/	/	1+	68	F	ADK	P	B	>50	No	Crizo	Yes
147	NC	/	/	2/3+	65	M	ADK	P	L	>50	No	Chemo	
148	NC	/	/	2/3+	70	F	ADK	P	B	10–25	No	Crizo	Toxicity
149	NC	/	/	2/3+	61	F	ADK	P	B	25–50	No	Crizo	Yes[b]
150	NC	/	/	1+	66	F	ADK	P	L	25–50	KRAS	Surg	

[a]Relapse after initial response.
[b]Treatment was stopped because of toxicity.
[c]Of note, a sample from a distant metastatic site has been analyzed blindly several months later and revealed same discrepancy. FISH/IHC discordant results are highlighted in grey.
BA, break-apart profile; IRS, isolated red signal profile; M, male; F, female; ADK, adenocarcinoma; ADSK, adenosquamous cell carcinoma; SCC, squamous cell carcinoma; NOS, not otherwise specified; P, primitive tumor; M, metastasis; OS, operative specimen; B, biopsy; TP, transparietal punction; L, liquid; NC, unsuccessful analyse; ND, undetermined or missing data; L858R, arginine for leucine substitution at amino acid 858 in EGFR; T790M, methionine for threonine substitution at amino acid 790 in EGFR; del19, exon 19 deletion in *EGFR*; Surg, patient treated only by surgery; chemo, chemotherapy; radioth, radiotherapy; crizo, crizotinib; died, patient died soon after being diagnosed before a treatment could be started; lost, lost to follow-up; ALK, anaplastic lymphoma kinase; FISH, fluorescent in situ hybridization; IHC, immunohistochemistry; EGFR, epidermal growth factor receptor; KRAS, Kirsten rat sarcoma; POS, positive; NEG, negative.

tissues. The Abbott BA probe (Vysis ALK Dual-Color; Abbott, Rungis, France) and the Dako split probe (Dako, Glostrup, Denmark) were assessed in the Rennes and Bordeaux cohorts, respectively. Both Vysis and Dako probes had been previously validated to provide equivalent results with the use of 100 NSCLC samples (our unpublished results).

Because tumor cells could be unequally distributed within the sample (focal infiltrations), an adjacent hematoxylin-eosin–stained section was used to delimit the area of interest and to determine the percentage of tumor cells. The protocols differ slightly between the two centers. In Rennes, the slides were deparaffinized with xylene using a VP2000 processor (Abbott, Wiesbaden, Germany). The tissue was then digested with pepsin (Dako) for 8 minutes. The target DNA and probe were codenatured for 3 minutes at 73°C by using a programmable system (Thermobrite; Abbott), and probe hybridization was performed overnight in a humidified atmosphere at 37°C. In Bordeaux, FISH analysis was conducted with the histology FISH accessory kit (Dako). Slides were deparaffinized with toluene. The tissue was then digested with pepsin (Sigma-Aldrich, St. Louis, MO) for 10 minutes. The target DNA and probe were codenatured for 5 minutes at 82°C by using a programmable system (Dako), and probe hybridization was performed overnight in a humidified atmosphere at 45°C.

Slides were analyzed with a fluorescence microscope (Axioskop2, Axio Imager Z2 or Axioplan [Zeiss, Göttingen, Germany]; BX61 [Olympus, Rungis, France]) and Isis imaging software (Metasystems, Altlussheim, Germany). The entire hybridized surface was screened using a double band-pass filter with an ×63 objective to detect areas with abnormal patterns and to focus the scoring. FISH scoring was performed under both real-time conditions at the microscope and with the use of z-stack images. Specific recommendations for ALK-rearranged pattern determination were used.[25,26] At least 100 nonoverlapping tumor nuclei were examined. Nuclei were not scored if the signals were weak, diffuse, of only one color, or in areas with stretching of the signal and nuclei. The pattern was considered positive for cells exhibiting BA (signals separated by a gap larger than two diameters) or isolated red signals (IRSs, deletion of the 5' *ALK* region). Scoring was performed in areas or clusters with the most abnormal pattern and not as a mean of randomly selected tumor areas. FISH for *ALK* locus rearrangement was considered positive if 15% or more nuclei were positive. Cases with high levels of polysomy, defined by the presence of greater than six fusion signals in more than 15% of tumor cells, were also registered. After establishing the case report by two independent observers, we also took into consideration the immunohistochemical results. All FISH slides with borderline positivity (15%) or discordant data between FISH and IHC were then reviewed by two additional experienced observers without the knowledge of which pattern had been reported and the IHC data. A final consensus decision was then determined on the basis of the scoring of at least three observers.

Immunohistochemistry

IHC was assayed in both centers on 4-µm formalin- or alcohol–formol–acetic-acid–fixed, paraffin-embedded tumor tissues by using a primary monoclonal ALK antibody (mAb) from Abcam (clone 5A4; Abcam, Cambridge, United Kingdom). In Rennes, IHC analysis was performed using a Ventana automated immunostainer (Ventana Medical Systems, Illkirch Graffenstaden, France); the slides were dried at 60°C for 1 hour, deparaffinized using EZ Prep at 75°C for 4 minutes, and incubated with the primary mAb at a dilution of 1:50 for 1 hour at 37°C. Detection was performed using a multimer-technology system with the UltraView Universal DAB detection kit with which the pathologists have a vast experience. Note that Ventana markets another detection kit, the OptiView system, which was not used in Rennes. In Bordeaux, IHC assays were performed by using a Bond-maX automated immunostainer (Leica Microsystems, Inc., Buffalo Grove, IL); the slides were dried at 60°C for 30 minutes, deparaffinized using Bond Dewax Solution (Leica Microsystems) at 72°C for 3 minutes, and incubated with the primary mAb at a dilution of 1:50 for 15 minutes at room temperature. Detection was performed using the Bond Polymer Refine Detection system (Leica Microsystems).

A positive external control consisting of a slide of a previously FISH-validated *ALK*-rearranged and IHC-positive sample was included in all tests. Semi-quantitative assessment was performed by one observer (HB, LD, or DC) by estimating the staining intensity and percentage of tumor cells with positive cytoplasmic staining. Samples were then placed into four categories with negative, faint, and/or doubtful heterogeneous staining (1+) and moderate to intense homogeneous staining (2 to 3+); noncontributive IHC results were also recorded when the number of tumor cells was to found to be too low because of sample exhaustion or artifacts (necrosis, etc.).

Statistical Analysis

The Mann–Whitney rank-sum test and χ^2 test were performed with Sigma-Stat software (Systat Software, San Jose, CA). A p value of 0.05 was considered statistically significant.

RESULTS

Characteristics of ALK-Positive Patients Evidenced Either by FISH or by IHC

A total of 150 patients (4.6%) were found to be ALK positive by FISH and/or IHC (Tables 1 and 2). Among those identified as positive, 68 patients (3.7%) were in Rennes and 82 patients (5.8%) were in Bordeaux. The mean age of the ALK-positive patients was not significantly different from that of the entire cohort ($p = 0.055$; Table 1). The data revealed an imbalanced sex ratio ($p < 0.0001$; Table 1), with a relative risk of 2.2-fold for female patients to exhibit an ALK alteration. Female patients represented 57% (39 of 68) and 51% (42 of 82) of the ALK-positive patients, whereas they accounted for 30% (554 of 1843) and 40% (559 of 1401) of the patients in Rennes and Bordeaux, respectively. The vast majority of the tumors screened in the study were adenocarcinomas, for which the rate of ALK positivity reached 5% (Tables 1 and 2). Of note, the rate of detection of ALK positivity for NOS NSCLC was 4.2% (14 of 331). By contrast, only three of 303 SCCs (1%) were classified as positive. This may partly account for the lower detection rate in the Rennes cohort, where 294 SCC cases were included, in contrast to the Bordeaux cohort. Moreover, among the ALK-positive cases, 22 of 150 cases were found in association with *KRAS* (14) or *EGFR* (8) mutations (Tables 1 and 2).

FISH Analysis

FISH analysis identified 116 *ALK* gene rearrangements, accounting for 3.6% of the patients (Table 2). The percentages of FISH-positive cases were 2.5 and 5 in Rennes and Bordeaux, respectively. Illustrations of positive BA and IRS patterns are depicted in Figure 1, as are samples with polysomy. The percentage of positive nuclei exhibiting either a BA or IRS pattern was highly variable (Fig. 2). The positive cases more frequently exhibited the BA pattern (66%). Moreover, the BA pattern was characterized by a lower percentage of positive nuclei compared with that observed in the IRS pattern ($p = 0.021$) (Fig. 2).

IHC Analysis

IHC analysis identified 114 ALK-positive samples (3.5%) (Tables 1 and 2). Overexpression of ALK protein

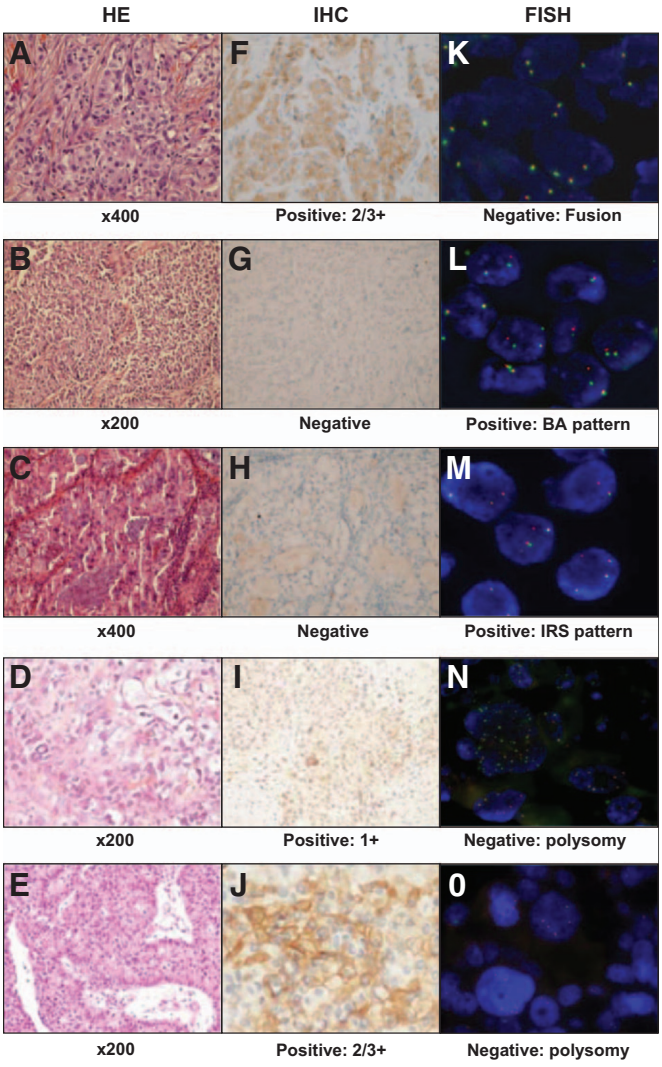

FIGURE 1. Illustration of discordant cases of *ALK* gene rearrangement and ALK protein overexpression. HE staining (*A–E*), IHC staining (*F–J*), and FISH patterns (*K–O*) of 4-μm–fixed, paraffin-embedded tissue sections of non–small-cell lung cancer. FISH analysis was performed with separating probes flanking the breakpoint of the *ALK* gene. IHC analysis was performed with an anti-ALK mouse monoclonal antibody (clone 5A4; Abcam). *A*, *F*, and *K*, A case exhibiting positive (2/3+) IHC staining (*F*) and a negative FISH result with adjacent orange/green or yellow fusion signals (*K*). *B*, *G*, and *L*, A case exhibiting negative IHC staining (*G*) and a rearranged-*ALK* gene with a BA pattern (*L*). *C*, *H*, and *M*, A case exhibiting negative IHC staining (*H*) and a rearranged-*ALK* gene with an IRS pattern (*M*). *D*, *I*, and *N*, A case exhibiting heterogeneity of ALK immunostaining with either low (1+) staining of most tumor cells or moderate staining (2+) of large polylobulated cells (*I*) and high polysomy with the presence of greater than six fusion signals (*N*). *E*, *J*, and *O*, A case exhibiting positive (2/3+) IHC staining (*J*) and high polysomy with the presence of greater than six fusion signals (*O*). IHC, immunohistochemistry; HE, hematoxylin-eosin; FISH, fluorescent in situ hybridization; BA, break-apart; IRS, isolated red signal; *ALK*, anaplastic lymphoma kinase.

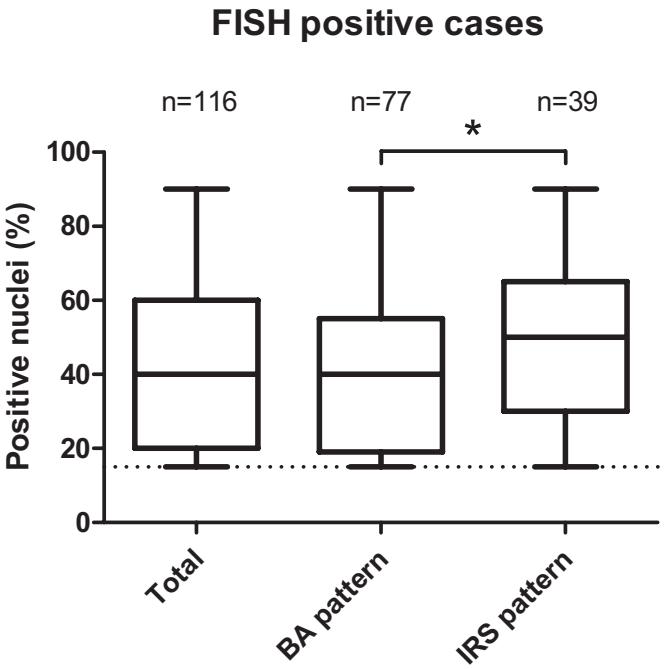

FIGURE 2. Percentage of positive nuclei in non–small-cell lung cancer harboring *ALK* rearrangements. Box-and-whisker plot of the percentage of positive nuclei in the FISH-positive samples. *ALK* locus rearrangement was considered positive if 15% or more nuclei exhibited BA or IRS patterns. The distribution is shown for all FISH-positive cases (n = 116), BA-positive cases (n = 77), and IRS-positive cases (n = 39). The line in the box represents the median, and the borders of the box represent the lower and upper quartiles of the data, respectively. The ends of the whiskers represent the minimum and maximum of all data. The threshold of 15% is depicted on the graph. FISH, fluorescent in situ hybridization; IRS, isolated red signal; BA, break-apart; *ALK*, anaplastic lymphoma kinase.

was detected in 3% and 4.2% of the cases in Rennes and Bordeaux, respectively. Moderate to intense homogeneous staining (score 2/3+) was observed in 99 cases although 15 samples were considered to exhibit faint and/or doubtful heterogeneous staining (score 1+). Illustrations of negative, faint/doubtful, and intense staining are presented in Figure 1.

Comparison between FISH and IHC Analyses

When compiling the results, a 4.6% (150 of 3244) rate of detection of ALK positivity was achieved using parallel combined FISH and IHC testing. Only 53% (80 of 150) of the samples classified as positive were in fact identified by both FISH and IHC analyses (Fig. 3*A*). Without considering the noncontributive FISH cases, the two analytic methods led to 55 discordant results (24 in Rennes and 31 in Bordeaux). In the FISH-positive/IHC-positive cases, IHC staining was mostly intense: 77 samples were scored 2/3+, whereas only three cases exhibited faint staining, suggesting an apparent correlation between both techniques for intensely stained IHC cases (Fig. 3*B*). However, there was no correlation between the percentage of tumor cells with *ALK* rearrangements and the intensity of IHC staining (Table 2).

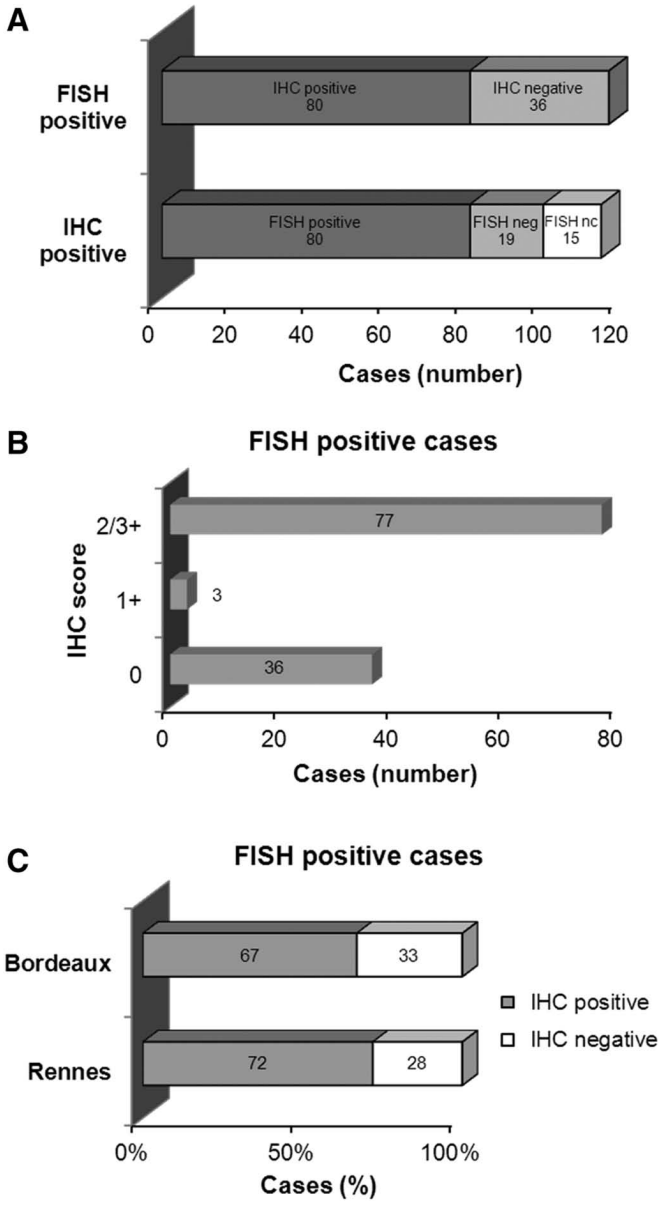

FIGURE 3. Comparative FISH and IHC analyses. *A*, Histograms of the repartition of the IHC results (positive or negative) in the 116 FISH-positive cases and the repartition of the FISH results (positive, negative, and noncontributive) in the 114 IHC-positive cases. *B*, Histograms of the repartition of the IHC score (0, 1+, 2/3+) in the 116 FISH-positive cases. *C*, Histograms of the IHC results in the 116 FISH-positive cases. Rennes: 46 FISH-positive cases; Bordeaux: 70 FISH-positive cases. The values on the bars are the percentage of IHC-positive and IHC-negative results. FISH, fluorescent in situ hybridization; IHC, immunohistochemistry.

In Rennes, 68 samples (3.7%) were diagnosed as ALK positive, but only 33 patients (1.8%) were positive for both *ALK* gene rearrangement and ALK protein overexpression (Table 2). FISH evidenced 13 *ALK* rearrangements without protein immunodetection. FISH-positive/IHC-negative cases accounted for 28% (13 of 46) of the patients diagnosed by FISH analysis

TABLE 3. Comparative FISH/IHC Analysis in 3244 NSCLC Patients

		FISH			
		Positive	**Negative**	**NC**	**Total**
IHC	Positive	80	19	15	114
	Negative	36	2579	435	3050
	NC	0	49	31	80
	Total	116	2647	481	3244

FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NC, noncontributive analysis or technical failure; NSCLC, non–small-cell lung cancer.

(Fig. 3*C*). These 13 patients did not harbor a specific *ALK* rearrangement pattern (7 BA and 6 IRS) (Table 2). Furthermore, 11 IHC-positive samples (4 samples with a 2/3+ score and 7 with a 1+ score) were classified as negative by FISH.

In Bordeaux, 82 samples (5.9%) exhibited either *ALK* gene rearrangements or ALK overexpression. Only 47 patients (1.4%) were positive by both techniques (Table 2). FISH revealed *ALK* rearrangements in 23 IHC-negative cases. Consistent with the results in Rennes, the FISH-positive/IHC-negative cases represented 33% (23 of 70) of the cases identified by FISH (Fig. 3*C*). In such cases, *ALK* rearrangement patterns were BA in 15 and IRS in eight cases, which did not differ from patients with a combined FISH/IHC positivity (31 and 16, respectively). In addition, eight IHC-positive samples (7 scored 2/3+ and 1 scored 1+) were FISH negative (Table 2). Three of these eight discordant FISH-negative/IHC-positive cases were also analyzed by using the Abbott ALK BA probes, which revealed similar FISH-negative results (data not shown). Furthermore, four cases were IHC positive, whereas the FISH analysis was not contributive because of hybridization failure or an insufficient number of tumor cells.

The performance of each test, if theoretically performed sequentially, was compared in the entire cohort. First, FISH analysis performed would have detected the *ALK* rearrangements in 116 of the 3244 patients (3.6%) although IHC analysis would have detected ALK expression in an additional set of 34 patients that were negative (n = 19) or noncontributive (n = 15) by FISH (Table 3). Conversely, if IHC had been the first technique used, 114 positive cases (3.5%) would have been detected, and FISH testing would have revealed ALK positivity in an additional set of 36 patients (Fig. 3*A* and Table 3). Preliminary data from 44 evaluable patients demonstrate a high response rate in the crizotinib-treated population. Most interestingly, some responses were also observed in both discordant FISH+/IHC− and FISH−/IHC+ patients (Table 2).

DISCUSSION

The discovery of *ALK* rearrangements in a subset of NSCLC[2] has rapidly led to the validation of the ALK inhibitor crizotinib in a phase III trial in which patients were enrolled on the basis of a positive BA FISH assay.[18] For the detection of ALK-positive cases, BA or IRS patterns and a positive threshold of 15% with scoring at a minimum of 60 nuclei were defined.[25,26] The evaluation of several primary antibodies (reviewed in the study by Weickhardt et al.[24]), including ALK1 (Dako), 5A4 (from different sources including Abcam and Novocastra, Newcastle Upon Tyne, United Kingdom), or D5F3 (Cell Signaling Technology, Danvers, MA),[20] has been performed for IHC using FISH as a standard procedure comparator but occasionally in conjunction with RT-PCR.[22] The difficulty in detecting the ALK antigenicity in NSCLC with *ALK* rearrangements led to the use of signal amplification systems such as tyramide amplification[13] or other enhanced detection systems such as the ultraView system (Roche Ventana, Illkirch Graffenstaden, France)[19,22] to maximize IHC sensitivity. Despite such improvements, a scoring system was also used in most series to evaluate staining intensity by eye. A 2/3+ score seemed to be correlated with FISH-positive results, and IHC 1+ cases were mainly found to be FISH negative,[19,23,27] except in the series by Park et al.,[28] in which five of six IHC 1+ cases were also FISH positive. This suggests that IHC interpretation is not well standardized, as signal intensity depends on the IHC amplification procedure or preanalytical steps. Indeed, although most IHC cases displayed homogeneous staining, although at various intensities, we also recorded some focal-positive cases or cases with heterogeneous staining intensity as positive. Whether this represents tumor heterogeneity or fixation artifacts was not determined but may indicate that ALK immunoreactivity in NSCLC is peculiarly versatile and sensitive to preanalytical steps. Despite the use of a parallel positive control, the absence of an internal positive structure also makes it impossible to determine true noncontributive specimens for IHC.

Several studies have supported the concept of screening patients with NSCLC by IHC and by using FISH only in positive cases or in patients with clinical and histological features of ALK-positive NSCLC.[19,27,29] Generally, the above-mentioned parallel studies have not reported FISH positivity in IHC-negative cases except in the recent study by Wallander et al.[22] However, in some studies, only IHC-positive cases were screened by FISH, which would not allow the detection of FISH-positive/IHC-negative cases.[30] In addition, true independence of FISH and IHC performance cannot be achieved when the same pathologists participate in both FISH and IHC interpretations and in studies with selected or nonconsecutive inclusions. Such information is generally lacking in most published studies aiming at a parallel evaluation of both techniques.[13,20,22,31] Some individual ALK-positive cases with discordance between FISH and IHC were reported; however, this was explained either by absence of ALK protein detection because of a lack of staining protocol sensitivity[20] or by failure in detecting *ALK* rearrangement with minimal separation of the 3' and 5' ALK probes, as in the context of EML4/ALK paracentric inversion.[26] Finally, most comparative studies between IHC and FISH have been performed on a limited number of cases with artificial concordance generated by the high rate of double-negative cases of IHC and FISH.

During the last year, we have collected anonymous data from more than 3200 NSCLC patients with advanced or metastatic diseases consecutively referred to our two university hospital departments for the detection of both *ALK* rearrangements and ALK overexpression by FISH and IHC analyses, respectively. No further selection was performed on these samples at the biological platform level. Interpretation of both techniques

was performed similarly in Rennes and Bordeaux, with IHC scored by trained pathologists (HB and DC). FISH was blindly analyzed by at least two cytogenetic readers who participated in the Polaris interlaboratory quality control program at the European level (in Bordeaux: JPM, AG, DD, LM, and GS; in Rennes: FC, MLC, MABR, and VJ). Note that the samples had been processed by many different pathology laboratories using various preanalytic protocols that may have impacted the findings. Our study demonstrates an imbalanced sex ratio with a relative risk of 2.2-fold for female patients to harbor an ALK alteration. Such a sex ratio influence was previously reported by Zhou et al.[32] but was not confirmed by other studies.[14,27] ALK alteration was not shown to be restricted to adenocarcinoma but was present in the NOS NSCLC subtype. As previously found by other groups,[32,33] ALK alteration was also detected in some SCC samples but only at a very low frequency. These data challenge the detection strategies in patients with advanced or metastatic NSCLC that suggest limiting ALK screening to adenocarcinoma,[16] but the data are consistent with recent guidelines from the National Cancer Comprehensive Network that recommend analyzing all adenocarcinoma and NOS NSCLC cases and limiting analyses for SCC to never smokers and small biopsy specimens (version-2 2013 available from: www.nccn.org). The rate of FISH *ALK*-positive cases was 3.5%, which is consistent with previous data reported in white patients.[19,33] A similar rate of positive cases (3.6%) was also observed by IHC, thus demonstrating the absence of an overestimation by each method. The parallel studies conducted in Rennes and Bordeaux provided similar results and differed substantially from those claiming a global concordance between FISH and IHC for the detection of ALK abnormalities and supporting the use of IHC as a screening technique.[19,34] Indeed, only 80 of the 150 patients identified with an ALK alteration were detected by both FISH and IHC methods, whereas the remaining 70 patients were detected by only one of the two techniques. Such a discrepancy was also mentioned in previous studies in which cases were positive by either IHC or FISH[22,33,35,36] or in studies using RT-PCR, IHC, and FISH, demonstrating the complementarities of the different techniques.[22]

Our data support the fact that IHC may not detect all cases with ALK rearrangements, consistent with the recent report by Rodig et al.,[13] who reported that approximately 20% of FISH-positive cases remained IHC negative. The use of the D5F3 antibody (Cell Signaling Technology) in 17 FISH-positive/IHC-negative samples of the Bordeaux series in a second IHC testing allowed the detection of ALK expression in only five cases, whereas 12 cases remained negative, also underlining the versatility of ALK immunodetection in *ALK*-rearranged NSCLC (data not shown).

Several hypotheses could explain the absence or low level of ALK protein in FISH-positive cases. ALK protein expression in *ALK*-rearranged NSCLC was thought to be much lower than in *ALK*-rearranged anaplastic large-cell lymphoma.[20] According to the fusion partner gene, the different chimeric ALK proteins were shown to have different hetero- or homo-dimerization properties and stabilities (for a review, see the study by Bergalet et al.[37]). The *ALK* rearrangement could also lead to a nontranslated ALK protein if the fusion gene is neither transcribed nor translated because of RNA decay or errors such as a stop codon in the open reading frame. As observed here, FISH-positive *ALK*-rearranged cases with negativity for ALK immunodetection have also been reported by several groups.[22,33] In our study, many technical artifacts such as late fixation or overfixation may have impaired the appropriate detection of the ALK protein in FISH-positive cases, as we have included unselected consecutive samples originating from various pathology laboratories with heterogeneous fixation times of small biopsies and delays between removal and fixation of surgical lung samples by perfusion protocols.[38] Other artifacts, including the possible mixture with acidic fixatives still used in France, may also have different consequences on the ability of FISH and IHC to detect ALK-positive cases, accounting for a portion of our discordant cases. The variability in the preanalytical steps among pathology laboratories may partly explain why our two laboratories observed a high rate of discordant cases compared with single-institution studies.

However, several RT-PCR studies comparing IHC and FISH results have also reported different levels of ALK protein according to the type of *EML4-ALK* fusion gene. In particular, cases with EML4-ALK variant 1, as detected by RT-PCR, were hardly detectable by IHC with no or faint staining or by FISH, as the distance between the two ALK probes was less than two signal distances apart.[22] It should be underlined that several experienced readers have reviewed the FISH slides of our discordant IHC and FISH cases, as the experience of the reader was found to be a critical parameter. However, it was not determined whether such cases contained EML4-ALK variant 1 transcripts. In the study by Wallander et al.,[22] several IHC-positive/FISH-negative cases were also reported to remain negative for EML4-ALK transcripts by RT-PCR amplification. In this study, fusion transcripts with other translocation partners such as *kinesin family member 5B*, *TRK-fused gene*, and *kinesin light chain 1* were not searched, but these rearrangements would have been detected by BA FISH, as such translocations lead to a wider physical separation of the 5' and 3' ALK probes than in the EML4-ALK inversion.[39,40] Together with our 19 IHC-positive/FISH-negative cases, the data by Wallander et al.[22] also suggest the possibility of mechanisms other than *ALK* rearrangement leading to ALK expression in the discordant FISH-negative NSCLC cases. Accordingly, ALK amplification or point-activating mutations have been shown to be associated with ALK expression and response to ALK inhibitors in neuroblastoma, renal clear cell adenocarcinoma, or myofibroblastic tumors.[41–43] In addition, among our discordant IHC-positive and FISH-negative cases, two cases were found to exhibit high levels of *ALK* polysomy with clusters of equal to or more than six fusion signals without BA or IRS signals. Such cases were classified among *atypical* FISH-negative cases using the criteria for EGFR amplification in NSCLC, and most of them were IHC negative for ALK.[44,45] As a subset of these atypical negative cases may be immunostained for ALK, such patients could be eligible for ALK inhibitors. The abundant focal amplification of ALK-native signals has been previously reported.[24,26] Such a pattern was also observed in our concordant FISH-negative and IHC-negative cases but was however most frequently observed in scarce cells with large nuclei below a threshold of 15% of cells.

In France, the Institut National du Cancer has founded a program for the prospective detection of emerging biomarkers in cancer, particularly for ALK in NSCLC. To reduce costs, a hierarchical testing for *ALK*, *BRAF*, human epidermal growth factor receptor 2 (*HER2*), or phosphatidylinositol-3-kinase catalytic subunit (*PIK3CA*) mutations in *EGFR* wild-type and *KRAS* wild-type samples has been discussed on the basis of studies reporting the absence of concomitant mutations in NSCLC cases.[8–11] This criterion has also been included in an algorithm for screening ALK in clinically selected patients with NSCLC and an *EGFR* and *KRAS* wild-type status.[16] However, the absence of concomitant mutations is questionable, as these mutations have been reported in other studies.[32,46–48] In the present comprehensive study, we have not followed a hierarchical screening algorithm and have identified eight cases with EGFR-activating mutations and 14 cases with *KRAS* mutations among our 150 ALK-positive cases. Whether such ALK-positive cases with concomitant *EGFR* or *KRAS* mutations have a different prognosis or response to ALK inhibitors remains to be determined. However, our data do not support a hierarchical algorithm based on molecular criteria.

Data on crizotinib response in patients who have been diagnosed differently by FISH and IHC are still preliminary. Thus, until large-scale studies in patients under therapy with crizotinib determine which testing is the most relevant to predict responses to ALK inhibition, our data support the need to routinely perform both analyses because of the difficulty in detecting the chimeric ALK protein in NSCLC and the presence of false-negative cases for each method. The limitations of each technique appear in our report of consecutive samples issued from various pathology laboratories, underlining the fact that the lack of standardization of preanalytical parameters may affect ALK protein detection by IHC and account for noncontributive cases of the FISH technique. These results also highlight that performing FISH analysis routinely for all NSCLC patients may require an automated process at various stages of the analysis (dewaxing, hybridization, screening of the hybridized slides with a camera to identify tumor cells, and signal scoring). Such a process is also mandatory because the biomarkers involved in the rearrangement or amplification in NSCLC also include renal oncocytoma and sarcoma (ROS1) and rearranged during transfection (RET).[49] In addition, we recently demonstrated that up to six sequential FISH hybridizations could be performed on a single section, demonstrating that FISH is a practical tool for the study of several chromosomal aberrations in samples of limited size and amount.[50] Multiple FISH testing will likely be required until a robust next-generation sequencing approach proves applicable on a large scale in routine practice for chromosomal rearrangement detection.

REFERENCES

1. Rikova K, Guo A, Zeng Q, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell* 2007;131:1190–1203.
2. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming *EML4-ALK* fusion gene in non-small-cell lung cancer. *Nature* 2007;448:561–566.
3. Ou SH, Bartlett CH, Mino-Kenudson M, Cui J, Iafrate AJ. Crizotinib for the treatment of ALK-rearranged non-small cell lung cancer: a success story to usher in the second decade of molecular targeted therapy in oncology. *Oncologist* 2012;17:1351–1375.
4. Heuckmann JM, Balke-Want H, Malchers F, et al. Differential protein stability and ALK inhibitor sensitivity of EML4-ALK fusion variants. *Clin Cancer Res* 2012;18:4682–4690.
5. Camidge DR, Bang YJ, Kwak EL, et al. Activity and safety of crizotinib in patients with ALK-positive non-small-cell lung cancer: updated results from a phase 1 study. *Lancet Oncol* 2012;13:1011–1019.
6. Shaw TA, Mehra R, Kim DW, et al. Clinical activity of the ALK inhibitor LDK378 in advanced, ALK-positive NSCLC. *J Clin Oncology* 2013;31:Abstract 8010.
7. Camidge DR, Bazhenova L, Salgia R, et al. First-in-human dose-finding study of the ALK/EGFR inhibitor AP26113 in patients with advanced malignancies: updated results. *J Clin Oncology* 2013;31:Abstract 8031.
8. Boland JM, Erdogan S, Vasmatzis G, et al. Anaplastic lymphoma kinase immunoreactivity correlates with ALK gene rearrangement and transcriptional up-regulation in non-small cell lung carcinomas. *Hum Pathol* 2009;40:1152–1158.
9. Inamura K, Takeuchi K, Togashi Y, et al. EML4-ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset. *Mod Pathol* 2009;22:508–515.
10. Takahashi T, Sonobe M, Kobayashi M, et al. Clinicopathologic features of non-small-cell lung cancer with EML4-ALK fusion gene. *Ann Surg Oncol* 2010;17:889–897.
11. Zhang X, Zhang S, Yang X, et al. Fusion of EML4 and ALK is associated with development of lung adenocarcinomas lacking EGFR and KRAS mutations and is correlated with ALK expression. *Mol Cancer* 2010;9:188.
12. Gainor JF, Varghese AM, Ou SH, et al. ALK rearrangements are mutually exclusive with mutations in EGFR or KRAS: an analysis of 1,683 patients with non-small cell lung cancer. *Clin Cancer Res* 2013;19:4273–4281.
13. Rodig SJ, Mino-Kenudson M, Dacic S, et al. Unique clinicopathologic features characterize ALK-rearranged lung adenocarcinoma in the western population. *Clin Cancer Res* 2009;15:5216–5223.
14. Yoshida A, Tsuta K, Nakamura H, et al. Comprehensive histologic analysis of ALK-rearranged lung carcinomas. *Am J Surg Pathol* 2011;35:1226–1234.
15. Nishino M, Klepeis VE, Yeap BY, et al. Histologic and cytomorphologic features of ALK-rearranged lung adenocarcinomas. *Mod Pathol* 2012;25:1462–1472.
16. Kobayashi M, Sonobe M, Takahashi T, Yoshizawa A, Kikuchi R, Date H. Detection of ALK fusion in lung cancer using fluorescence in situ hybridization. *Asian Cardiovasc Thorac Ann* 2012;20:426–431.
17. Jokoji R, Yamasaki T, Minami S, et al. Combination of morphological feature analysis and immunohistochemistry is useful for screening of EML4-ALK-positive lung adenocarcinoma. *J Clin Pathol* 2010;63:1066–1070.
18. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 2010;363:1693–1703.
19. McLeer-Florin A, Moro-Sibilot D, Melis A, et al. 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.
20. Mino-Kenudson M, Chirieac LR, Law K, et al. 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.
21. Takeuchi K, Choi YL, Soda M, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. *Clin Cancer Res* 2008;14:6618–6624.
22. Wallander ML, Geiersbach KB, Tripp SR, Layfield LJ. Comparison of reverse transcription-polymerase chain reaction, immunohistochemistry, and fluorescence in situ hybridization methodologies for detection of echinoderm microtubule-associated proteinlike 4-anaplastic lymphoma kinase fusion-positive non-small cell lung carcinoma: implications for optimal clinical testing. *Arch Pathol Lab Med* 2012;136:796–803.
23. Yi ES, Boland JM, Maleszewski JJ, et al. 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.

24. Weickhardt AJ, Aisner DL, Franklin WA, Varella-Garcia M, Doebele RC, Camidge DR. Diagnostic assays for identification of anaplastic lymphoma kinase-positive non-small cell lung cancer. *Cancer* 2013;119:1467–1477.
25. Camidge DR, Kono SA, Flacco A, et al. Optimizing the detection of lung cancer patients harboring anaplastic lymphoma kinase (ALK) gene rearrangements potentially suitable for ALK inhibitor treatment. *Clin Cancer Res* 2010;16:5581–5590.
26. Camidge DR, Theodoro M, Maxson DA, et al. Correlations between the percentage of tumor cells showing an anaplastic lymphoma kinase (ALK) gene rearrangement, ALK signal copy number, and response to crizotinib therapy in ALK fluorescence in situ hybridization-positive nonsmall cell lung cancer. *Cancer* 2012;118:4486–4494.
27. Paik JH, Choe G, Kim H, et al. Screening of anaplastic lymphoma kinase rearrangement by immunohistochemistry in non-small cell lung cancer: correlation with fluorescence in situ hybridization. *J Thorac Oncol* 2011;6:466–472.
28. Park HS, Lee JK, Kim DW, et al. Immunohistochemical screening for anaplastic lymphoma kinase (ALK) rearrangement in advanced non-small cell lung cancer patients. *Lung Cancer* 2012;77:288–292.
29. Thunnissen E, Bubendorf L, Dietel M, et al. EML4-ALK testing in non-small cell carcinomas of the lung: a review with recommendations. *Virchows Arch* 2012;461:245–257.
30. Sakairi Y, Nakajima T, Yasufuku K, et al. EML4-ALK fusion gene assessment using metastatic lymph node samples obtained by endobronchial ultrasound-guided transbronchial needle aspiration. *Clin Cancer Res* 2010;16:4938–4945.
31. Kim H, Yoo SB, Choe JY, et al. Detection of ALK gene rearrangement in non-small cell lung cancer: a comparison of fluorescence in situ hybridization and chromogenic in situ hybridization with correlation of ALK protein expression. *J Thorac Oncol* 2011;6:1359–1366.
32. Zhou JX, Yang H, Deng Q, et al. Oncogenic driver mutations in patients with non-small-cell lung cancer at various clinical stages. *Ann Oncol* 2013;24:1319–1325.
33. Martelli MP, Sozzi G, Hernandez L, et al. EML4-ALK rearrangement in non-small cell lung cancer and non-tumor lung tissues. *Am J Pathol* 2009;174:661–670.
34. Blackhall FH, Peters S, Ker KM, et al. Prevalence and clinical outcomes for patients with ALK gene rearrangement in Europe. *Ann Oncol* 2012;23:ix73.
35. Conklin CM, Craddock KJ, Have C, Laskin J, Couture C, Ionescu DN. Immunohistochemistry is a reliable screening tool for identification of ALK rearrangement in non-small-cell lung carcinoma and is antibody dependent. *J Thorac Oncol* 2013;8:45–51.
36. Sholl LM, Weremowicz S, Gray SW, et al. Combined use of ALK immunohistochemistry and FISH for optimal detection of ALK-rearranged lung adenocarcinomas. *J Thorac Oncol* 2013;8:322–328.
37. Bergalet J, Fawal M, Lopez C, et al. HuR-mediated control of C/EBPbeta mRNA stability and translation in ALK-positive anaplastic large cell lymphomas. *Mol Cancer Res* 2011;9:485–496.
38. Ilie M, Hofman P. Pitfalls in lung cancer molecular pathology: how to limit them in routine practice? *Curr Med Chem* 2012;19:2638–2651.
39. Takeuchi K, Choi YL, Togashi Y, et al. KIF5B-ALK, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. *Clin Cancer Res* 2009;15:3143–3149.
40. Togashi Y, Soda M, Sakata S, et al. KLC1-ALK: a novel fusion in lung cancer identified using a formalin-fixed paraffin-embedded tissue only. *PLoS One* 2012;7:e31323.
41. Ogura T, Hiyama E, Kamei N, Kamimatsuse A, Ueda Y, Ogura K. Clinical feature of anaplastic lymphoma kinase-mutated neuroblastoma. *J Pediatr Surg* 2012;47:1789–1796.
42. Mano H. ALKoma: a cancer subtype with a shared target. *Cancer Discov* 2012;2:495–502.
43. Sukov WR, Hodge JC, Lohse CM, et al. ALK alterations in adult renal cell carcinoma: frequency, clinicopathologic features and outcome in a large series of consecutively treated patients. *Mod Pathol* 2012;25:1516–1525.
44. Hirsch FR, Varella-Garcia M, Dziadziuszko R, et al. Fluorescence in situ hybridization subgroup analysis of TRIBUTE, a phase III trial of erlotinib plus carboplatin and paclitaxel in non-small cell lung cancer. *Clin Cancer Res* 2008;14:6317–6323.
45. Varella-Garcia M, Diebold J, Eberhard DA, et al. EGFR fluorescence in situ hybridisation assay: guidelines for application to non-small-cell lung cancer. *J Clin Pathol* 2009;62:970–977.
46. Koivunen JP, Mermel C, Zejnullahu K, et al. EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. *Clin Cancer Res* 2008;14:4275–4283.
47. Tiseo M, Gelsomino F, Boggiani D, et al. EGFR and EML4-ALK gene mutations in NSCLC: a case report of erlotinib-resistant patient with both concomitant mutations. *Lung Cancer* 2011;71:241–243.
48. Barlesi F, Blons H, Beau-Faller M, et al. Biomarkers (BM) France: results of routine EGFR, HER2, KRAS, BRAF, PI3KCA mutations detection and EML4-ALK gene fusion assessment on the first 10,000 non-small cell lung cancer (NSCLC) patients (pts). *J Clin Oncology* 2013;31:Abstract 8000.
49. Takeuchi K, Soda M, Togashi Y, et al. RET, ROS1 and ALK fusions in lung cancer. *Nat Med* 2012;18:378–381.
50. Pham-Ledard A, Prochazkova-Carlotti M, Andrique L, et al. Multiple genetic alterations in primary cutaneous large B-cell lymphoma, leg type support a common lymphomagenesis with activated B-cell-like diffuse large B-cell lymphoma. *Mod Pathol* 2013 Sep 13 doi: 10.1038/modpathol.2013.156. [Epub ahead of print].