Worse Disease-Free Survival in Never-Smokers with *ALK*+ Lung Adenocarcinoma

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Introduction: The *EML4*–anaplastic lymphoma kinase (*ALK*) translocation is a recognized oncogenic driver in non-small cell lung cancer. We investigated immunohistochemistry (IHC) screening with fluorescence in situ hybridization (FISH) confirmation for *ALK* detection and estimated the prevalence of *ALK* positivity in our patient cohort of never-smokers, together with differences in clinical outcomes and prognostic factors for patients with *ALK*-positive and *ALK*-negative tumors.

Methods: We designed a three-phase study (training, validation, and testing) in 300 never-smokers with lung adenocarcinoma from the observational Mayo Clinic Lung Cancer Cohort. Tumor samples were tested using IHC and FISH, and concordance between the methods was assessed. Clinical outcomes were assessed via 5-year progression- or recurrence-free survival from diagnosis. Prognostic factors for *ALK*-positive tumors and metastases were also investigated.

Results: *ALK*-positive patients were significantly ($p < 0.05$) younger and had higher grade tumors than *ALK*-negative patients. *ALK* positivity was 12.2% by IHC and confirmed at 8.2% of tumors by FISH, with complete concordance between IHC 3+/0 and FISH+/− assessments, respectively. Five-year risk of progression or recurrence was doubled for patients with *ALK*-positive compared with *ALK*-negative tumors; *ALK*-positive tumors also appeared to be associated with a higher risk of brain and liver metastases.

Conclusions: Our findings suggest that *ALK* positivity is associated with a significantly poor outcome in nonsmoking-related adenocarcinoma and that *ALK*-positive tumors may be associated with an increased risk of brain and liver metastases compared with *ALK*-negative disease. Consequently, an unmet medical need exists in *ALK*-positive lung cancer patients, and effective *ALK*-specific therapies are needed.

Key Words: *EML4*–anaplastic lymphoma kinase, Non-small cell lung cancer, Immunohistochemistry, Fluorescence in situ hybridization, Progression- and recurrence-free survival.

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The echinoderm microtubule-associated protein-like 4 (*EML4*)–anaplastic lymphoma kinase (*ALK*) translocation was discovered in non-small cell lung cancer (NSCLC) in 2007.[1] The *EML4–ALK* translocation can result in constitutive *ALK* kinase activity and represents an oncogenic addiction pathway in lung cancer.[2] Other *ALK* fusion variants have also been reported, with uncertain oncokinase functions in NSCLC.[3–5] Several studies have shown *ALK* gene rearrangement (*ALK* positivity) to correlate with never or light/former smoking status, younger age, adenocarcinoma histology, and to rarely coincide with *EGFR* or *KRAS* mutation.[6–11] Retrospective studies have reported prevalence estimates for *ALK* positivity ranging from 1.6[7] to 13%,[8] the variability resulting from factors including the methodology used to detect *ALK* gene rearrangement (e.g., polymerase chain reaction with limited probe sets) and patient or tissue selection criteria. Therefore, several questions remain to be answered, including the true prevalence of *ALK* positivity in unselected patient populations and whether prevalence varies by disease stage, geography, or ethnicity. Moreover, the natural history and clinical outcome of *ALK*-positive versus *ALK*-negative patients needs to be fully defined.

In an effort to find a cost-effective method for selecting *ALK*-positive NSCLC patients who may benefit from *ALK* inhibitor therapies, we studied the role of scored immunohistochemistry (IHC) as a screening test. Given the high concordance between IHC score and *ALK* status by fluorescence in situ hybridization (FISH), we proposed an algorithm for

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The protocol for this study was reviewed and approved by the Mayo Foundation Institutional Review Board and Biospecimens Committee.

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FISH confirmation.[12] In this study, we have further validated and tested the algorithm and performed a controlled comparison of clinical outcomes for *ALK*-positive versus *ALK*-negative adenocarcinoma patients. Our four specific objectives were to (1) assess whether IHC can be a practical tool for *ALK* screening in adenocarcinoma with a confirmatory FISH test; (2) estimate the prevalence of *ALK* positivity in our enriched study cohort by either or both IHC and FISH tests; (3) describe clinical characteristics of cases by IHC score, FISH status, and combined IHC and FISH results; and (4) most importantly, evaluate clinical outcomes of *ALK*-positive versus *ALK*-negative cases, while controlling for clinically important prognostic factors, in a naturally followed observational patient cohort.

MATERIALS AND METHODS

Study Design, Sampling, and Data Collection

To evaluate the *ALK* test concordance and estimate *ALK* positivity prevalence, we designed a three-phase study in an *ALK*-positive-enriched patient population: approximately 100 patients were included in each phase as a training ($n = 100$), validation ($n = 99$), and testing set ($n = 101$), respectively (Supplemental Figure 1).

All patients were never-smokers, defined as having smoked zero to 99 cigarettes during their lifetime, and were selected from the Mayo Clinic Lung Cancer Cohort, an observational follow-up study.[13,14] The sample enrichment strategy included only never-smokers with adenocarcinoma diagnosed between 1997 and 2008 (none treated with crizotinib), with surgically resected samples, preserved as formalin-fixed paraffin-embedded tissue. These samples were from surgical treatments (i.e., lobectomy, pneumonectomy, and sleeve resection) or biopsies (i.e., wedge, pleural, and/or thoracotomy-based). In total, 300 cases were eligible for inclusion in the analyses. All eligible cases had banked tissue samples from surgical resections consisting of wedge resection or more extensive surgeries; available slides from each case were reviewed by two pathologists (E.S.Y. and M.C.A.) to verify the diagnosis and to select the representative block containing the most architecturally intact tumor cells.

Follow-up is accomplished through detailed medical record review and patient questionnaires at within 6 months postdiagnosis and then annually. For deceased patients, the follow-up questionnaire is sent to the next-of-kin. Additional details regarding patient identification and follow-up have been described previously.[15,16] Information abstracted from the medical record for each patient included age, gender, race, smoking status, history of tobacco exposure, other medical conditions, symptoms at presentation, and Eastern Cooperative Oncology Group performance status. For the initial diagnosis of primary NSCLC, date of diagnosis, clinical stage, histopathological type and grade of tumor, type of surgical resection, and the use of adjuvant therapy were recorded. The evidence of progression and/or recurrent disease was abstracted from Mayo Clinic medical records or other reliable sources. Additional sources of data collection included lung cancer follow-up questionnaires, tumor registry questionnaires routinely sent to the patient from Mayo Clinic, and correspondence letters or copied medical records received from outside clinicians. When the presence or absence of progression and/or recurrent disease was documented at another institution, data were included only when the information was specific and considered reliable.[15] To standardize the quality of follow-up information across all patients, follow-up information completed as of December 31, 2010, was included in the analysis.

IHC and FISH for *ALK* Rearrangement

Immunohistochemistry

IHC using *ALK1* monoclonal antibody (Dako, Carpinteria, CA) was performed as described previously by Yi et al.[12] An IHC score was assigned to each case according to the following criteria with at least 10% of tumor cells showing the designated staining pattern: 3+, intense, granular cytoplasmic staining; 2+, moderate, smooth cytoplasmic staining; 1+, faint cytoplasmic staining; and 0, no staining. IHC scoring was performed without the knowledge of FISH results.

FISH for ALK Rearrangement

Interphase molecular cytogenetic studies using a commercially available *ALK* probe (Vysis, Des Plaines, IL) were performed as described previously by Yi et al.[12] FISH for *ALK* locus rearrangement was considered positive if 15% or more of at least 100 cells counted showed splitting of the florescent probes flanking the *ALK* locus. All FISH interpretation was performed without the knowledge of IHC results for *ALK*.

Study End Points

Survival was assessed as 5-year progression-free or recurrence-free survival (PFS/RFS) from diagnosis (events occurring after 5 years were censored and cases died before recurrence/progression counted as events). To control for confounding effects of known predictors for lung cancer progression or recurrence, the following variables were matched or adjusted for age at diagnosis, sex, lung cancer stage (I, II, III, and IV), and mode of treatment (surgery only, surgery and chemo/radiation, other/none/ unknown).

Statistical Analyses

Descriptive statistics were summarized and compared for a number of factors, including age at diagnosis, sex, ethnicity (U.S. white versus other), grade of tumor differentiation (well differentiated, moderately differentiated, poorly or undifferentiated), stage, and treatment modality. Preprogression treatment included all treatment received before primary progression, recurrence, or development of second primary tumors. Both univariate and multivariate survival analyses were conducted by *ALK* status using IHC, FISH, and both combined IHC and FISH test scores comparing patients with *ALK*-positive tumors with patients with *ALK*-negative tumors. Five levels of analyses were performed: (1) comparison of patients' characteristics by *ALK* status using Kruskal–Wallis tests (continuous variables) and χ^2 tests or Fisher's exact tests (categorical variables); (2) IHC and FISH tests concordance of *ALK* status analyzed by χ^2 tests for homoge-

neity; (3) the prevalence of *ALK*-positive cases estimated by IHC test only, by FISH test only, and by both tests; (4) PFS/RFS by *ALK* status using adjusted (unmatched) and matched (a nested case–case) Cox proportional hazard models in which the hazard ratio or relative risk of the end point was estimated; and (5) comparison of detailed events of progression and recurrence between *ALK*-positive and *ALK*-negative cases. The "full model" used all covariates (age, sex, stage, and preprogression treatment) and the "select model" employed a stepwise model selection procedure to pick the significant covariates. A Cox proportional hazards model was used to estimate the partial effects of stage; specifically for each individual, two survival curves were estimated for each value of stage, with individual covariates remaining fixed. The adjusted survival curves are then calculated as the within-stage mean estimated survival of all study subjects.

RESULTS

Descriptive Analysis

Patient characteristics by FISH status, IHC score, and both IHC score and FISH status combined are shown in Table 1. In total, 300 patients had IHC scores, 216 had FISH results, and 300 had either of the two tests. All cases with IHC1+, 2+, or 3+ have been tested for FISH, and the 84 cases without FISH were IHC score 0. Across the three groups, a statistically significant difference between *ALK*-positive and *ALK*-negative cases was observed for age at diagnosis, tumor cell differentiation (grade), and treatment modality. In addition, stage was significantly different between FISH-positive and FISH-negative groups. Patients with *ALK*-positive tumors were younger and had more aggressive histologic grade

TABLE 1. Patient Characteristics by IHC Score, FISH Status, and IHC Score/FISH Status Combined

	Characteristics of 300 Patients with IHC Scores				Characteristics of 216 Patients by FISH Status		Characteristics of 300 Patients by IHC Score/FISH Status (FISH+/IHC3+/2+ vs. FISH−/IHC0/1+)	
	IHC3+ (n = 18)	IHC2+ (n = 14)	IHC1+ (n = 65)	IHC0 (n = 203)	Positive (n = 22)	Negative (n = 194)	Positive (n = 34)	Negative (n = 266)
Age at diagnosis, yr								
Median	59.0	56.5	70.0	70.0	62.5	70.0	58.0	70.0
Range	36.0–77.0	40.0–84.0	41.0–91.0	17.0–89.0	36.0–77.0	32.0–91.0	36.0–84.0	17.0–91.0
Gender, *n* (%)								
Female	11 (61.1)	13 (92.9)	46 (70.8)	155 (76.4)	14 (63.6)	147 (75.8)	26 (76.5)	199 (74.8)
Male	7 (38.9)	1 (7.1)	19 (29.2)	48 (23.6)	8 (36.4)	47 (24.2)	8 (23.5)	67 (25.2)
Race, *n* (%)								
Caucasian	18 (100.0)	11 (78.6)	58 (89.2)	176 (86.7)	21 (95.5)	172 (88.7)	31 (91.2)	232 (87.2)
Other	0 (0.0)	3 (21.4)	7 (10.8)	27 (13.3)	1 (4.5)	22 (11.3)	3 (8.8)	34 (12.8)
Grade of differentiation, *n* (%)								
Well differentiated	4 (22.2)	5 (35.7)	27 (41.5)	125 (61.6)	5 (22.7)	105 (54.1)	10 (29.4)	151 (56.8)
Moderately differentiated	10 (55.6)	8 (57.1)	31 (47.7)	62 (30.5)	12 (54.5)	75 (38.7)	19 (55.9)	92 (34.6)
Poorly/undifferentiated	4 (22.2)	1 (7.1)	7 (10.8)	16 (7.9)	5 (22.7)	14 (7.2)	5 (14.7)	23 (8.6)
Stage, *n* (%)								
I	6 (33.3)	7 (50.0)	32 (49.2)	130 (64.0)	7 (31.8)	122 (62.9)	14 (41.2)	161 (60.5)
II	2 (11.1)	2 (14.3)	2 (3.1)	10 (4.9)	2 (9.1)	7 (3.6)	4 (11.8)	12 (4.5)
III	5 (27.8)	4 (28.6)	16 (24.6)	37 (18.2)	7 (31.8)	37 (19.1)	10 (29.4)	52 (19.5)
IV	5 (27.8)	1 (7.1)	15 (23.1)	26 (12.8)	6 (27.3)	28 (14.4)	6 (17.6)	41 (15.4)
Treatment modality, *n* (%)								
Only surgery	5 (27.8)	9 (64.3)	37 (56.9)	136 (67.0)	7 (31.8)	122 (62.9)	15 (44.1)	172 (64.7)
Surgery and chemo/radiation	9 (50.0)	5 (35.7)	15 (23.1)	44 (21.7)	11 (50.0)	49 (25·3)	15 (44.1)	58 (21.8)
Other/none/unknown	4 (22.2)	0 (0.0)	13 (20.0)	23 (11.3)	4 (18.2)	23 (11.9)	4 (11.8)	36 (13.5)
Preprogression treatment, *n* (%)								
Only surgery	8 (44.4)	11 (78.6)	40 (61.5)	147 (72.4)	10 (45.4)	137 (70.6)	20 (58.8)	186 (69.9)
Surgery and chemo/radiation	5 (27.8)	3 (21.4)	12 (18.5)	32 (15.8)	7 (31.8)	34 (17.5)	9 (26.5)	43 (16.2)
Other/none/unknown	5 (27.8)	0 (0.0)	13 (20.0)	24 (11.8)	5 (22.7)	23 (11.9)	5 (14.7)	37 (13.9)
Median PFS/RFS	2.05	3.33	3.89	4.40	2.18	4.48	2.66	4.27

Shaded variables are statistically different ($p < 0.05$) among the comparison groups.

Not all patients had surgically resected lung cancer. Not all resected tissue came from curative surgery. Some tissue samples were from wedge, pleural, or thoracotomy-based biopsies.

"Unknown" treatments were for patients who received treatment outside our institution and the data are still being verified. "Other" treatments included TAC of pleura and removal of solitary brain metastasis.

FISH, fluorescence in-situ hybridization; IHC, immunohistochemistry; PFS/RFS, progression- or recurrence-free survival.

and more advanced disease stage than those with *ALK*-negative tumors.

Concordance Analysis

There were 225 samples tested by both assays from 221 distinct patients (four patients each had two samples tested). Concordance rates between IHC scores and FISH status are presented in Supplemental Table 1. Complete concordance (100%) was observed between IHC3+ scores and FISH-positive status and between IHC0 scores and FISH-negative status. Relatively high concordance was seen between IHC1+ scores and FISH-negative status (96.9% of IHC1+ was FISH negative); while reasonable concordance was noted between IHC2+ scores and FISH-negative status (85.7% of IHC2+ was FISH negative).

ALK Positivity Prevalence Estimation

The prevalence of *ALK* tumors was estimated using five methods (Supplemental Table 2): (1) using IHC as a screening test, the prevalence of *ALK* positivity was 10.7% for IHC3+/2+, 6.0% for IHC3+, and 4.7% for IHC2+; (2) by FISH, the prevalence was 8.2%; (3) using IHC as a screening test and FISH as a confirmatory "gold standard" test, the prevalence was 8.2% for FISH-positive or IHC3+; (4) the prevalence was 12.2% for FISH-positive or IHC3+ or IHC2+ in the training and validation sets; and (5) counting all FISH-positive and all IHC3+/2+ cases in the entire prevalence cohort, the prevalence was 11.3%.

Survival Analysis

PFS/RFS was evaluated using two comparative analyses. In the unmatched analysis, three models (unadjusted, full, and select) considered samples as *ALK*-positive using three alternative definitions: IHC3+, FISH+, or both FISH and IHC3+. The select model adjusted for tumor stage only. As shown in Table 2, results were consistent across all models: the risk of experiencing lung cancer progression or recurrence within 5 years after diagnosis was greater than two-fold in *ALK*-positive cases compared with *ALK*-negative cases. Stage-adjusted survival curves for the three *ALK*-positive definitions are shown in Figure 1.

The matched analysis used three similar scenarios based on IHC/FISH tests and on the number of matches of *ALK*-negative cases to each *ALK*-positive case. Results were consistent, showing a greater than two-fold increased risk of progression and/or recurrence in patients with *ALK*-positive tumors (Supplemental Table 3).

Detailed Comparison of Progression and Recurrence Events between *ALK*-Positive and *ALK*-Negative Cases

Location of metastases at diagnosis by *ALK* status was available for 47 advanced-stage (local, regional, and remote metastasis) lung cancer cases and is presented in Supplemental Table 4 as descriptive information. The six patients with *ALK*-positive tumors appeared to have a greater frequency of chest cavity, brain, bone, and liver metastases than *ALK*-negative patients. However, no formal statistical test was performed due to small sample size.

TABLE 2. Analysis of 5-yr PFS/RFS by Cox Proportional Hazards Models

	HR (95% CI)	*p*
PFS/RFS, IHC3+ (n = 18) vs. IHC0/1+ (n = 264)		
Unadjusted	3.33 (1.79–6.17)	<0.0001
Full model[a]	2.14 (1.12–4.07)	0.0210
Age at diagnosis (per 1 yr)	0.99 (0.97–1.02)	0.5658
Sex (males)	1.47 (0.89–2.43)	0.1345
Stage (vs. I)		
II	1.40 (0.41–4.78)	0.5931
III	3.74 (1.97–7.09)	<.0001
IV	3.19 (1.48–6.82)	0.0029
Treatment (vs. only surgery)		
Surgery and chemotherapy or radiation	1.68 (0.89–3.18)	0.1101
Other/none/unknown	2.32 (1.09–4.91)	0.0287
Select model[b]	**2.37 (1.27–4.46)**	**0.0071**
Stage (vs. I)		
II	1.40 (0.42–4.46)	0.5862
III	5.26 (3.07–9.03)	<.0001
IV	5.04 (2.71–9.36)	<.0001
PFS/RFS, FISH+ (n = 22) vs. FISH− (n = 191)		
Unadjusted	3.10 (1.75–5.52)	0.0001
Full model[a]	1.95 (1.07–3.58)	0.0303
Age at diagnosis (per 1 yr)	1.00 (0.98–1.02)	0.7575
Sex (males)	1.36 (0.80–2.34)	0.2588
Stage (vs. I)		
II	0.99 (0.23–4.27)	0.9872
III	3.49 (1.78–6.83)	0.0003
IV	3.46 (1.43–8.36)	0.0058
Treatment (vs. only surgery)		
Surgery and chemotherapy or radiation	1.40 (0.72–2.71)	0.3183
Other/none/unknown	2.24 (0.91–5.53)	0.0800
Select model[b]	**2.11 (1.17–3.82)**	**0.0134**
Stage (vs. I)		
II	1.02 (0.24–4.36)	0.9741
III	4.48 (2.55–7.87)	<.0001
IV	5.59 (2.86–10.93)	<.0001
PFS/RFS, FISH+/IHC3+ (n = 22) vs. FISH−/IHC0/1+ (n = 274)		
Unadjusted	3.37 (1.92–5.90)	<0.0001
Full model[a]	2.00 (1.11–3.61)	0.0208
Age at diagnosis (per 1 yr)	0.99 (0.97–1.01)	0.3387
Sex (males)	1.34 (0.82–2.19)	0.2491
Stage (vs. I)		
II	1.23 (0.37–4.14)	0.7347
III	3.78 (2.04–7.00)	<.0001
IV	3.45 (1.65–7.19)	0.0010
Treatment (vs. only surgery)		
Surgery and chemotherapy or radiation	1.51 (0.82–2.79)	0.1881
Other/none/unknown	2.15 (1.03–4.46)	0.0408
Select model[b]	**2.31 (1.31–4.10)**	**0.0040**
Stage (vs. I)		
II	1.22 (0.37–4.04)	0.7421
III	5.03 (2.99–8.46)	<.0001
IV	5.24 (2.88–9.53)	<.0001

[a] Adjusted for age at diagnosis, sex, stage, and treatment preprogression.
[b] Adjusted for stage.
FISH, fluorescence in-situ hybridization; IHC, immunohistochemistry; PFS/RFS, progression- or recurrence-free survival; HR, hazard ratio; CI, confidence interval.

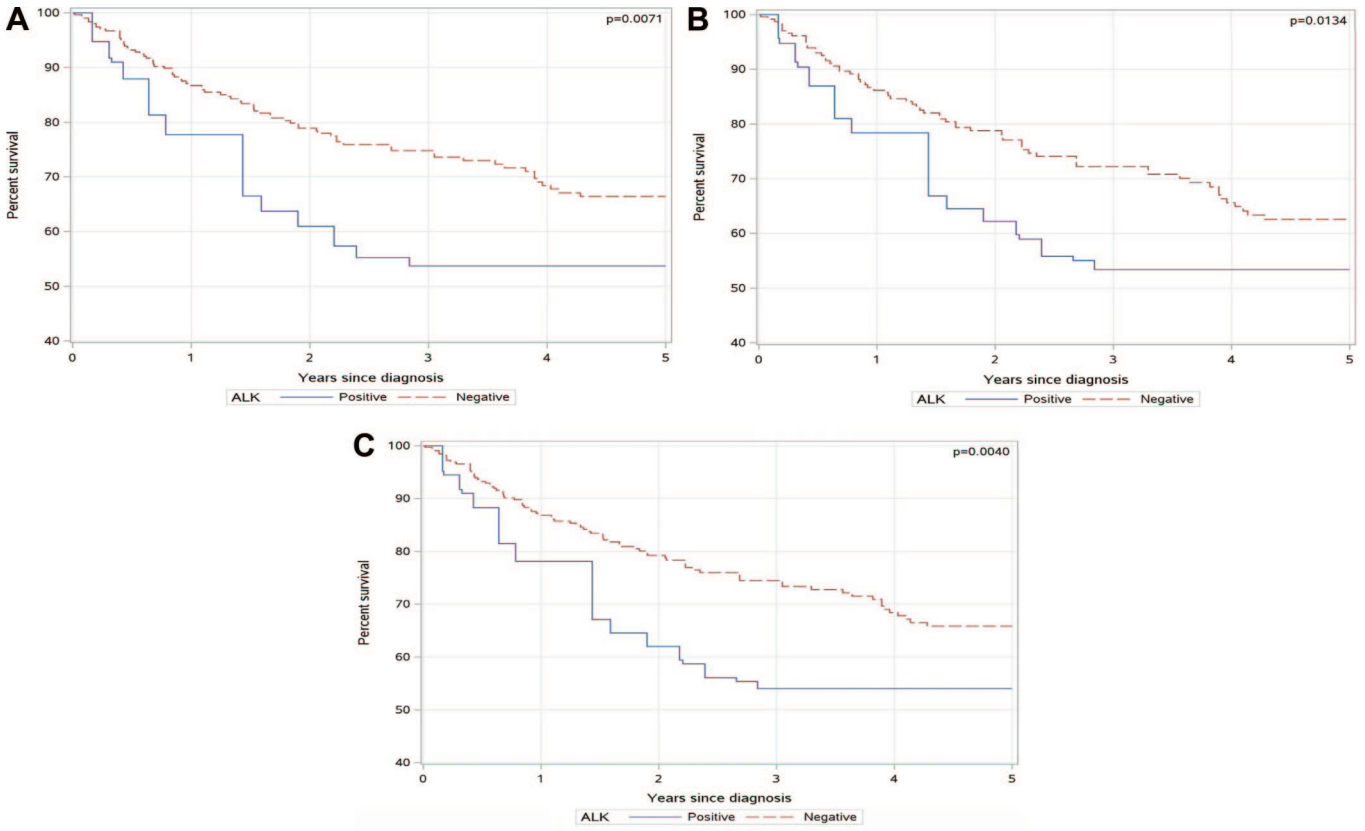

FIGURE 1. *A,* Stage-adjusted progression- or recurrence-free survival (PFS/RFS) curves for immunohistochemistry (IHC) 3+ (positive) versus IHC0/1+ (negative) cases. *B,* Stage-adjusted PFS/RFS survival curves for fluorescence in situ hybridization (FISH)-positive and FISH-negative cases. *C,* Stage-adjusted PFS/RFS survival curves for FISH+/IHC3+ (positive) and FISH-negative/IHC0/1+ (negative) cases (see Select model data in Table 2).

Table 3 provides descriptions of the location and number of events in the survival cohort ($n = 299$) by four *ALK* status subgroups. With the exception of adrenal glands, the *ALK*-positive group reported higher percentages of events for all individual locations than the combined *ALK*-negative groups, whereas the 12 IHC2+ cases (considered as *ALK* negative unless also FISH-positive) had an event occurrence pattern which fell between patterns seen for the *ALK*-positive and the two other *ALK*-negative subgroups (IHC0, IHC1+). More detailed event data by specific *ALK* test results are provided in Supplemental Tables 5 and 6. Specified adjusted Cox model results indicated that a significantly higher risk of extrathoracic events (brain and liver) was observed in patients harboring *ALK*-positive tumors versus *ALK*-negative tumors (Table 4).

DISCUSSION

This study confirms earlier results by our group that IHC scores 3 and 0 were 100% concordant with FISH-positive and FISH-negative status, respectively, whereas IHC scores 1 and 2 may require further confirmatory testing.[12] In addition, we report the first *ALK*-positive prevalence estimate in an enriched sample of never-smokers with adenocarcinoma. Prevalence rates ranged from 6.0% (by IHC3+) to 12.2% (FISH-positive/IHC3+/2+). This study also suggests, through controlled and matched analyses, a less favorable clinical outcome for *ALK*-positive compared with *ALK*-negative cases. Of note, we found that the risk of lung cancer progression or recurrence in the 5 years postdiagnosis doubled in *ALK*-positive cases compared with *ALK*-negative cases (as defined by IHC, FISH, or combined IHC and FISH). Furthermore, despite the small sample size of *ALK*-positive patients and the small number of events, patients with *ALK*-positive tumors appeared to have a higher risk of developing tumor progression and/or recurrence in the brain and liver than patients with *ALK*-negative tumors.

We initially hypothesized that a high proportion of IHC2+ patients would also be FISH-positive. However, IHC–FISH concordance in this group was low, with only 14.3% of the 14 IHC2+ cases being FISH-positive. Therefore, we recommend confirmatory testing with FISH for any case showing IHC2+. As the patient and clinical characteristics of the IHC2+ group appeared intermediate between those for the IHC3+ and IHC1+ groups (see Tables 1 and 3 and Supplemental Table 5), we grouped IHC3+ and IHC2+ patients together as an exploratory scenario for IHC–*ALK* positivity. Patients with IHC1+ had a very low rate of FISH positivity, with only 3.1% of IHC1+ cases showing *ALK* rearrangement. For practical purposes, IHC1+ cases can be considered *ALK* negative. However, in an attempt to capture

TABLE 3. Number of Lung Cancer Recurrences and/or Progressions by Location and ALK Status[a]

		ALK− (n = 277)		
	ALK+ (n = 22)[b]	IHC2+ (n = 12)	IHC1+ (n = 63)	IHC0 (n = 202)[b]
No. recurrence or progression, n (%)	6 (30.0)	7 (58.3)	46 (73.0)	153 (76.1)
No. in intrathoracic region[c], n (%)				
1	4 (20·0)	1 (8.3)	8 (12.7)	17 (8.5)
2+	4 (20.0)	3 (25.0)	6 (9.5)	21 (10.4)
No. in same lung, n (%)				
1	4 (20.0)	3 (25.0)	9 (14.3)	26 (12·9)
2+	2 (10.0)	0 (0.0)	4 (6.4)	8 (4.0)
No. in other lung, n (%)				
1	1 (5.0)	2 (16.7)	3 (4.8)	14 (7·0)
2+	2 (10.0)	1 (8.3)	3 (4.8)	7 (3.5)
No. in chest cavity[d], n (%)				
1	3 (15.0)	2 (16.7)	2 (3.2)	6 (3.0)
2+	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.5)
No. in extrathoracic region[e], n (%)				
1	6 (30.0)	2 (16.7)	6 (9.5)	16 (8.0)
2+	3 (15.0)	1 (8.3)	3 (4.8)	9 (4.5)
No. in brain, n (%)				
1	4 (20.0)	3 (25.0)	3 (4.7)	9 (4.5)
2+	1 (5.0)	0 (0.0)	0 (0.0)	1 (0.5)
No. in bones[f], n (%)				
1	2 (10.0)	1 (8.3)	3 (4.8)	8 (4.0)
2+	0 (0.0)	0 (0.0)	1 (1.6)	4 (2.0)
No. in liver, n (%)				
1	2 (10.0)	0 (0.0)	0 (0.0)	3 (1.5)
2+	0 (0.0)	0 (0.0)	1 (1.6)	0 (0.0)
No. in kidneys, n (%)				
1	1 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)
No. in adrenal glands, n (%)				
1	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)
No. in other sites[g], n (%)				
1	3 (15.0)	0 (0.0)	1 (1.6)	6 (3.0)
2+	0 (0.0)	0 (0.0)	1 (1.6)	2 (1.0)

n = 299 cases in the survival cohort.

[a] Anaplastic lymphoma kinase (ALK)+ is fluorescence in-situ hybridization (FISH)+ and/or immunohistochemistry (IHC)3+, and ALK− is FISH− and/or IHC0/1+ (included in ALK+ if FISH+ and IHC1+).

[b] Two ALK+ cases and one IHC0 case have unknown locations for their recurrences and/or progressions; reported percentages are out of n = 20 ALK+ cases and n = 201 IHC0 cases.

[c] Intrathoracic—includes same lung, other lung, and chest cavity.

[d] Chest cavity—includes mediastinum, pleural effusion, pleural nodes, and pericardium.

[e] Extrathoracic—includes brain, bones (including ribs), liver, kidneys, adrenal glands, and other sites.

[f] Bones—includes all bones of the body, including ribs.

[g] Other sites—includes all areas of the body not otherwise specified.

TABLE 4. Five-Year Time-to-Recurrence and/or Progression Cox Proportional Hazards Models for Location of Events

Location of Recurrence/Progression	HR (95% CI) for ALK+	p
Intrathoracic		
Unadjusted	1.98 (0.90–4.35)	0.09
Full model	0.71 (0.30–1.69)	0.44
Select model[a]	0.76 (0.33–1.76)	0.53
Same lung		
Unadjusted	1.57 (0.62–3.96)	0.34
Full model	0.74 (0.24–2.32)	0.61
Select model[b]	1.16 (0.45–2.99)	0.76
Other lung		
Unadjusted	1.57 (0.48–5.17)	0.46
Full model	1.20 (0.20–7.16)	0.85
Select model[c]	2.46 (0.72–8.32)	0.15
Chest cavity		
Unadjusted	3.56 (1.00–12.65)	0.05
Full model	9.56 (1.54–59.40)	0.02
Select model[d]	4.06 (0.98–16.90)	0.05
Extrathoracic		
Unadjusted	3.80 (1.82–7.92)	<0.001
Full model	2.53 (1.11–5.76)	0.03
Select model[e]	2.44 (1.12–5.36)	0.03
Brain		
Unadjusted	4.75 (1.74–12.98)	0.002
Full model	4.46 (1.37–14.51)	0.01
Select model[f]	4.55 (1.50–13.78)	0.007
Liver		
Unadjusted	6.90 (1.26–37.69)	0.03
Full model	Cannot be calculated	—
Select model[g]	8.22 (1.06–63.51)	0.04
Bones, kidneys, or adrenal glands	Samples too small	—
Other sites		
Unadjusted	4.92 (1.33–18.24)	0.02
Full model	3.25 (0.30–35.11)	0.33
Select model[h]	1.14 (0.16–8.12)	0.89

Intrathoracic—includes same lung, other lung, and chest cavity.

Chest cavity—includes mediastinum, pleural effusion, pleural nodes, and pericardium.

Extrathoracic—includes brain, bones, liver, kidneys, adrenal glands, and other sites.

Other sites—includes all areas of the body not otherwise specified.

Treatment includes all treatments before the first event of second primary, recurrence, or progression.

Full model = anaplastic lymphoma kinase (ALK) status, age at diagnosis, sex, stage, treatment, and remaining regions (e.g., same lung event is adjusted for other lung, chest cavity, brain, bones, liver, kidneys, adrenal glands, other sites, etc.).

Select model = ALK status and a stepwise model selection for the above covariates.

Event with unknown location—all location variables were set to "no" to preserve sample size.

Model information: Time = time to first recurrence or progression; Event = event location (e.g., intrathoracic, same lung, brain, etc.) at any time. For example, the first recurrence could be intrathoracic, but if they had an extrathoracic recurrence 2 months later, extrathoracic would be an event and the time for the extrathoracic event would be the same as the intrathoracic event. Extrathoracic event would be a covariate in the intrathoracic model and vice versa.

[a] Adjusted for stage and extrathoracic.

[b] Adjusted for treatment, other lung, chest cavity, and other sites.

[c] Adjusted for same lung.

[d] Adjusted for same lung and liver.

[e] Adjusted for sex, stage, treatment, and intrathoracic.

[f] Adjusted for stage, same lung, and bones.

[g] Adjusted for treatment, other lung, and bones.

[h] Adjusted for sex, stage, treatment, same lung, and kidneys.

all patients with *ALK* rearrangement, targeted FISH testing could be considered for some adenocarcinoma patients with IHC1+, especially if they have other characteristics associated with *ALK* positivity (never-smoker status, young age,

signet ring morphology, negativity for *EGFR* and *KRAS* mutations). The biological basis for the observed discordance in cases showing IHC2+ and FISH negativity is under investigation by our group and may be due to nonspecific IHC staining, a unique *ALK* fusion variant or mutation not identified by one or both of the FISH probes used or could be the result of normal *ALK* protein aberrantly expressed by some other mechanisms. These cases could represent a "transitional" phase of an oncogenic process, and it may be important to determine whether patients with an IHC score of 2+ may also benefit from *ALK*-targeted therapy.

Although several studies have shown that *ALK*-positive cases were more likely to have never or light/former smoking status and adenocarcinoma histology,[6,8,10] *ALK*-positive cases have been reported in current smokers[1,2,17,18] and in nonadenocarcinoma histology.[9,17,19] Furthermore, *ALK* positivity has been reported along with the *EGFR* exon 19[10] and exon 20 mutations,[20] despite *ALK* positivity and *EGFR* mutations being mutually exclusive in all other studies to date. Therefore, other evaluation procedures should be explored to maximize the opportunity for these "exceptional" cases to also benefit from *ALK*-targeted therapy.

Recent studies, attempting to elucidate the natural history of *ALK*-positive NSCLC in terms of response to standard therapies, have demonstrated no statistically significant difference in platinum-based chemotherapy response rates in *ALK*-positive compared with *ALK*-negative patients.[2,8] Although not statistically significantly different, response rates were numerically smaller in *ALK*-positive patients. Shaw et al.[8] reported a chemotherapy response rate for *ALK*-positive patients ($n = 12$) of 25% versus 50% for *EGFR*-mutated ($n = 8$) and 35% for *ALK*/*EGFR* wild-type patients ($n = 34$), suggestive of a trend toward a poorer response to chemotherapy in the *ALK*-positive patients; however, this analysis did not adjust for between-group differences in age and smoking history. Likewise, Koh et al.[2] reported a first-line response rate to platinum-based chemotherapy of 18.8% in *ALK*-positive versus 34.4% in *ALK*-negative patients ($p = 0.088$). Importantly, these two studies found that patients with *ALK*-positive NSCLC did not respond well to EGFR tyrosine kinase inhibitors, an association that was statistically significant.[2,8] More recently, Camidge et al.[21] and Lee et al.[22] have presented data suggesting that *ALK* is predictive for favorable outcome with pemetrexed-based therapy. Shaw et al.[23] demonstrated significantly prolonged overall survival in *ALK*-positive NSCLC patients treated within a clinical trial of the experimental *ALK* inhibitor, crizotinib, when indirectly compared with nontrial patients with *ALK*-positive NSCLC who were treated with standard therapy. Taken together, these studies show that patients with *ALK*-positive tumors do not have a more favorable clinical outcome with existing standard therapies, perhaps with the exception of pemetrexed-based therapy. *ALK*-specific inhibitors may prove to prolong overall survival, and *ALK* positivity is a negative predictive marker for EGFR tyrosine kinase inhibitor treatment outcomes.

Our current study adds further evidence that *ALK*-positive NSCLC patients do not have a more favorable prognostic course of disease, as is the case in *ALK*-rearranged anaplastic large cell lymphoma.[24,25] Rather, our data clearly demonstrate a less favorable prognosis, as measured by 5-year PFS/RFS. These findings may be due to complete clinical data ascertainment and the thorough consideration of potential confounding variables in all analyses. On the other hand, as with other retrospective studies of this rare gene rearrangement, the small sample size, the observational assessment of progression or recurrence, unknown EGFR status in some cases, and the use of a broad category of treatment modality may have affected our estimates. Our ongoing analyses will assess treatment and stage-specific outcomes with respect to known EGFR status as well as the correlation of signet ring cell morphology, *ALK* status, and clinical outcomes.

Because we have included an all-inclusive patient cohort of never-smokers with testable tissue sample, proportionally we have had a cohort with more early-stage or surgically treated patients, as opposed to most other *ALK*-related studies focusing on late-stage patients. As a consequence, much longer follow-up time is required to obtain mature data for overall survival analysis. In the near future, we will achieve a complete view of treatment modalities and their effects on treatment outcomes including overall survival stratified by *ALK* status.

In conclusion, our results suggest that *ALK*-specific therapies are needed for patients with *ALK*-positive tumors due to significant worse 5-year PFS/RFS survival. In addition, if our data regarding a greater risk of brain or liver metastases are corroborated with more robust data, it will be even clearer that there is, indeed, an unmet and urgent medical need in the *ALK*-positive NSCLC patient population.

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medical record data abstraction. F.N. was responsible for recruitment of cases and controls and participated in manuscript preparation. J.M. was responsible for recruitment of cases and controls and participated in manuscript preparation. M.C.A. participated in study design and pathological review. H.T. provided advice in statistical analysis and participated in results discussion and interpretation. E.S.Y. coled the whole project from study design, pathological review to manuscript preparation.

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