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Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

ALK Rearrangements Are Mutually Exclusive with Mutations in *EGFR* or *KRAS*: An Analysis of 1,683 Patients with Non–Small Cell Lung Cancer

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Abstract

Purpose: Anaplastic lymphoma kinase (*ALK*) gene rearrangements define a distinct molecular subset of non–small cell lung cancer (NSCLC). Recently, several case reports and small series have reported that *ALK* rearrangements can overlap with other oncogenic drivers in NSCLC in crizotinib-naïve and crizotinib-resistant cancers.

Experimental Design: We reviewed clinical genotyping data from 1,683 patients with NSCLC and investigated the prevalence of concomitant *EGFR* or *KRAS* mutations among patients with ALK-positive NSCLC. We also examined biopsy specimens from 34 patients with ALK-positive NSCLC after the development of resistance to crizotinib.

Results: Screening identified 301 (17.8%) *EGFR* mutations, 465 (27.6%) *KRAS* mutations, and 75 (4.4%) *ALK* rearrangements. *EGFR* mutations and *ALK* rearrangements were mutually exclusive. Four patients with *KRAS* mutations were found to have abnormal ALK FISH patterns, most commonly involving isolated 5′ green probes. Sufficient tissue was available for confirmatory ALK immunohistochemistry in 3 cases, all of which were negative for ALK expression. Among patients with ALK-positive NSCLC who acquired resistance to crizotinib, repeat biopsy specimens were ALK FISH positive in 29 of 29 (100%) cases. Secondary mutations in the *ALK* kinase domain and *ALK* gene amplification were observed in 7 of 34 (20.6%) and 3 of 29 (10.3%) cases, respectively. No *EGFR* or *KRAS* mutations were identified among any of the 25 crizotinib-resistant, ALK-positive patients with sufficient tissue for testing.

Conclusions: Functional *ALK* rearrangements were mutually exclusive with *EGFR* and *KRAS* mutations in a large Western patient population. This lack of overlap was also observed in ALK-positive cancers with acquired resistance to crizotinib. *Clin Cancer Res; 19(15); 4273–81. ©2013 AACR.*

Introduction

Treatment paradigms for non–small cell lung cancer (NSCLC) have recently shifted away from stratification of patients based upon histology alone and toward molecular classifications based on genetic alterations within "driver" oncogenes. NSCLCs harboring such alterations are often dependent on a single oncogenic pathway for cell survival, a concept known as oncogene addiction (1). In recent genotyping efforts, approximately 50% of pulmonary adenocarcinomas were identified as having at least one genetic alteration in an oncogenic driver, with higher rates observed among never-smokers (2–4). Notably, mutations within driver oncogenes are largely mutually exclusive with one another, overlapping in only 3% to 5% of cases (2, 3).

Identified in NSCLC in 2007, chromosomal rearrangements involving the anaplastic lymphoma kinase (*ALK*) gene define a new molecular subset of lung cancer (5). With an estimated frequency of 3% to 5% of NSCLC, *ALK* rearrangements are associated with unique clinical and pathologic features, including younger age, never or light smoking history, and adenocarcinoma histology (6–9). *ALK* rearrangements are also regarded as essentially mutually exclusive with mutations in other driver oncogenes (7, 10). However, several recent reports suggest an unexpectedly high degree of overlap between *ALK* rearrangements and mutations in *KRAS* or *EGFR* (3, 11–19). For example, in a recent analysis of 95 EGFR-mutant patients who participated in the phase III EURTAC trial, 15.8% were

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Translational Relevance

Lung cancers harboring chromosomal rearrangements involving the anaplastic lymphoma kinase (*ALK*) gene are associated with unique clinicopathologic features, including sensitivity to the tyrosine kinase inhibitor (TKI) crizotinib. Recently, several studies have suggested that *ALK* rearrangements co-occur with mutations in *EGFR* or *KRAS* at clinically relevant frequencies. If confirmed, this would have important consequences for therapeutic decision-making and may alter clinical laboratory workflow, as many centers reserve ALK testing for tumor specimens that are negative for mutations in *EGFR* and *KRAS*. Here, we present clinical genotyping data from 1,683 patients with NSCLC, focusing on the prevalence of *EGFR* and *KRAS* mutations in ALK-positive patients. We also report molecular studies on a cohort of 34 ALK-positive patients who underwent biopsies after the development of resistance to crizotinib to determine the prevalence of acquired *EGFR* and *KRAS* mutations in this population.

reported to have concomitant *ALK* rearrangements (20). Additional studies have identified the coexistence of *EGFR* mutations in up to 6% of ALK-positive patients within Western populations and in up to 11.8% among ALK-positive Asian populations (16, 18).

Recognition of ALK-positive patients is clinically important as *ALK* rearrangements are associated with marked sensitivity to the tyrosine kinase inhibitor (TKI) crizotinib. In early clinical studies of crizotinib in ALK-positive NSCLC, the objective response rate was 60% with median progression-free survival of 8 to 10 months (21–23). Despite the initial sensitivity of ALK-positive lung cancer to crizotinib, patients eventually develop resistance to therapy. Mechanisms of acquired crizotinib resistance have been identified *in vitro* and through molecular analysis of repeat biopsy specimens taken at the time of disease progression (16, 24–28). These include secondary mutations in the *ALK* tyrosine kinase (TK) domain, ALK fusion gene amplification, and upregulation of bypass signaling tracts, such as c-KIT and EGFR (16, 24, 26–28). In addition, one study reported loss of the ALK fusion gene and emergence of *EGFR* and *KRAS* mutations in crizotinib-resistant cancers (27).

The possibility of coexistence of *ALK* fusions and either *EGFR* or *KRAS* mutations would have profound effects on therapeutic choices and would also impact clinical laboratory workflow and resource allocation as many centers now reserve ALK FISH testing, a relatively labor-intensive assay, for cases that test negative for *EGFR* and *KRAS* mutations. In this multi-institutional study, we identified patients who underwent clinical genotyping for alterations in *EGFR*, *KRAS*, and *ALK*, examining the prevalence of coexisting *EGFR* or *KRAS* mutations among ALK-positive patients. We also present an updated analysis of a series of repeat biopsies from crizotinib-resistant, ALK-positive patients (26) to determine the frequency of *EGFR* and *KRAS* mutations in TKI-resistant disease.

Materials and Methods

Study populations

Patients ($n = 1{,}683$) with NSCLC who underwent non-sequential testing for mutations in *EGFR*, *KRAS*, and *ALK* between March 2009 and June 2012 were identified. Patients were seen at Massachusetts General Hospital (MGH; $n = 1{,}619$), Memorial Sloan-Kettering Cancer Center ($n = 33$), and the University of California at Irvine ($n = 31$). All patients had biopsy-proven NSCLC. Medical records were reviewed to extract data on clinical and pathologic features. This study was approved by the Institutional Review Board at each of the participating institutions.

Patients with ALK-positive NSCLC ($n = 34$) with acquired resistance to crizotinib underwent biopsies of their resistant tumors at one institution (MGH) between January, 2009 and October, 2012. Specimens were reviewed for histologic confirmation of malignancy. Total nucleic acid was extracted in all specimens for *ALK* TK domain sequencing as outlined below. Repeat ALK FISH and the multiplexed SNaPshot assay were conducted in all specimens with sufficient tissue for analysis.

Tumor pathology and mutation analysis

Tumor histology was classified according to World Health Organization criteria. EGFR and KRAS testing were conducted using SNaPshot (29). In cases where SNaPshot was unavailable, mutation analysis was conducted by a combination of a multiplexed PCR-based sizing assay, allele-specific PCR, direct sequencing and mass spectrometry genotyping.

ALK molecular analysis

ALK FISH was conducted on formalin-fixed and paraffin-embedded (FFPE) tissue using a dual-color break-apart probe specific to the ALK locus (Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe; Abbott Molecular). Samples were considered positive if more than 15% of cells showed split ALK 5′ and 3′ probe signals or isolated 3′ signals (7).

In ALK-positive, crizotinib-resistant biopsy specimens, total nucleic acid was extracted and the *ALK* TK domain (exons 20–28) was sequenced as previously described (26).

Immunohistochemistry

Immunohistochemical staining was conducted on representative tissue sections from FFPE tissue blocks. Immunohistochemistry (IHC) was conducted with an anti-ALK monoclonal antibody (clone 5A4, Novacastra) at 1:50 dilution (30).

Statistical analysis

Fisher exact test and Wilcoxon rank-sum test were used to assess the association of genotype status with clinicopathologic features. An exact calculation of the binomial distribution is used to obtain the upper bound of a one-sided

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Table 1. Clinicopathologic features of EGFR, KRAS, and ALK-positive patients

Clinical characteristic	EGFR ($n = 301$)	KRAS ($n = 465$)	ALK ($n = 75$)	*P*-value for ALK vs. EGFR	*P*-value for ALK vs. KRAS
Age at diagnosis				<0.001	<0.001
Median	64	66	56		
Range	26–92	26–92	29–87		
Sex				<0.001	0.022
Male	86 (29%)	170 (37%)	38 (51%)		
Female	215 (71%)	295 (63%)	37 (49%)		
Ethnicity[a]				0.192	<0.001
Caucasian	227 (76%)	436 (96%)	64 (85%)		
Asian	47 (16%)	8 (2%)	9 (12%)		
Other	23 (8%)	11 (2%)	2 (3%)		
Smoking History[b]				0.239	<0.001
Never	173 (57%)	18 (4%)	49 (65%)		
Smoker	128 (43%)	445 (96%)	26 (35%)		
Pathology				0.104	0.072
Adenocarcinoma	287 (95%)	432 (93%)	71 (95%)		
Adenosquamous	6 (2%)	9 (2%)	0 (0%)		
Squamous	2 (1%)	4 (1%)	3 (4%)		
Other NSCLC	6 (2%)	20 (4%)	1 (1%)		
Stage[c]				<0.001	<0.001
Stage I	78 (26%)	175 (38%)	4 (5%)		
Stage II	16 (5%)	55 (12%)	8 (11%)		
Stage III	43 (14%)	69 (15%)	18 (24%)		
Stage IV	163 (55%)	165 (35%)	45 (60%)		
Stage at Testing[d]				0.002	<0.001
Stage I	68 (23%)	158 (34%)	4 (5%)		
Stage II	14 (5%)	47 (10%)	4 (5%)		
Stage III	33 (11%)	56 (12%)	12 (16%)		
Stage IV	185 (62%)	204 (44%)	55 (74%)		

[a]Other includes "Hispanic," "African-American," and "Native American" ethnicities. Ethnicity was unavailable in 14 patients.
[b]Never smokers have smoked less than100 cigarettes per lifetime. Smokers have smoked more than100 cigarettes (current or former). Smoking status was unavailable in 2 patients.
[c]Clinical stage represents stage at initial diagnosis. Stage was determined according to current American Joint Commission on Cancer Guidelines. Stage was not available for 2 patients.
[d]Represents stage at time of molecular testing. Stage was not available for 1 patient.

95% CI for the frequency of ALK-positive cases with a second driver mutation. Data analysis was computed by SAS 9.2 (SAS Institute), and all *P* values were two-sided.

Results

Screening patient characteristics

We identified 1,687 NSCLC specimens that had undergone clinical genotyping for abnormalities in *EGFR*, *KRAS*, and *ALK*. Testing for each genetic alteration was conducted in 100% of specimens. A total of 1,683 patients were included, with 4 patients having 2 separate primary NSCLCs analyzed. Genotyping for *EGFR* and *KRAS* mutations was conducted using the SNaPshot assay in 1,603 (95.1%) cases. Mutations in *EGFR* and *KRAS* were identified in 301 (17.8%) and 465 (27.6%) specimens, respectively. ALK FISH was conducted on all samples. *ALK* rearrangements were found in 75 (4.4%) cases. The clinical and pathologic features of these patients are summarized in Table 1.

Lack of overlapping mutations in driver oncogenes

The overall frequency of coalterations among any of the 3 tested oncogenes was 0.06% (1/1,687). Only 1 case of overlap, involving concomitant mutations in *EGFR* and *KRAS*, was observed by allele-specific PCR. Insufficient tumor tissue was available for confirmatory SNaPshot testing of this specimen. Among 376 patients found to have *EGFR* mutations ($n = 301$) or *ALK* rearrangements ($n = 75$), there were no cases of coexisting *EGFR* and *ALK* alterations identified.

Four patients with *KRAS* mutations were noted to have abnormal ALK FISH patterns that did not meet criteria for the presence of an *ALK* rearrangement (Table 2). Such

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Table 2. Molecular analysis of patients with *KRAS* mutations and abnormal ALK FISH

Patient no.	*EGFR* mutation	*KRAS* mutation	ALK FISH pattern	ALK IHC
1	WT	Gly12Cys	Isolated green 5′ probe	Negative
2	WT	Gly12Ser	Isolated green 5′ probe	Negative
3	WT	Gly13Cys	Isolated green 5′ probe	N/A
4	WT	Gly12Val	Isolated red 3′ probe[a]	Negative

[a]ALK FISH—isolated red 3′ probe considered "unusually small."

abnormal ALK FISH patterns involved single isolated green 5′ probes in 3 (75%) cases. The last case involved an ALK FISH pattern with a single isolated red 3′ probe that was considered unusually small. To determine whether these cases harbored functional *ALK* rearrangements leading to expression of an ALK fusion protein, ALK immunohistochemistry (clone 5A4) was conducted. Previous immunohistochemical studies using the ALK 5A4 antibody have shown a sensitivity and specificity of 100% among 29 ALK FISH-positive adenocarcinoma specimens and 110 ALK FISH-negative samples (30). Sufficient tissue was available for ALK immunohistochemical testing in 3 of the KRAS-positive, abnormal ALK FISH cases. Immunohistochemistry was negative for ALK expression in 3/3 (100%) specimens, including the case with an isolated, unusually small red 3′ probe on ALK FISH (Fig. 1). Thus, these abnormal ALK FISH patterns were not consistent with functional *ALK* gene rearrangements. Therefore, altogether 0 of 75 (0%; upper 95% CI: 4%) patients with *ALK* rearrangements were found to have concomitant mutations in *EGFR* or *KRAS* (Table 3).

Repeat biopsies in ALK-positive, crizotinib-refractory NSCLC

We previously reported an analysis of crizotinib resistance in 18 ALK-positive patients who underwent repeat biopsies at the time of progression (26). Here, we present an updated and expanded analysis of 34 repeat biopsy specimens from ALK-positive, crizotinib-refractory patients. Specimens were examined for mechanisms of acquired resistance. In all cases, patients had initially responded to crizotinib but subsequently developed progressive disease after a median of 9 months (range 3–34 months). A majority of repeat biopsies were obtained before or within 1 month of crizotinib discontinuation (Table 4). If sufficient tissue was available, specimens underwent repeat ALK FISH, direct sequencing of the *ALK* TK domain, and SNaPshot testing.

Persistence of the ALK fusion gene at the time of acquired crizotinib resistance

Our earlier report of acquired resistance to crizotinib in patients with *ALK* rearrangements showed the continued presence of the ALK fusion transcript upon rebiopsy (26). However, it has recently been reported that patients may lose the *ALK* gene rearrangement upon treatment with crizotinib (27). We therefore conducted repeat ALK FISH testing on the additional crizotinib-resistant specimens. Among 34 specimens, a total of 29 were evaluable. Tumor material in the remaining 5 samples was exhausted due to sequencing efforts. Notably, in 29 of 29 (100%) cases, repeat ALK FISH confirmed the continued presence of an ALK fusion gene despite acquired resistance to crizotinib (Tables 4 and 5).

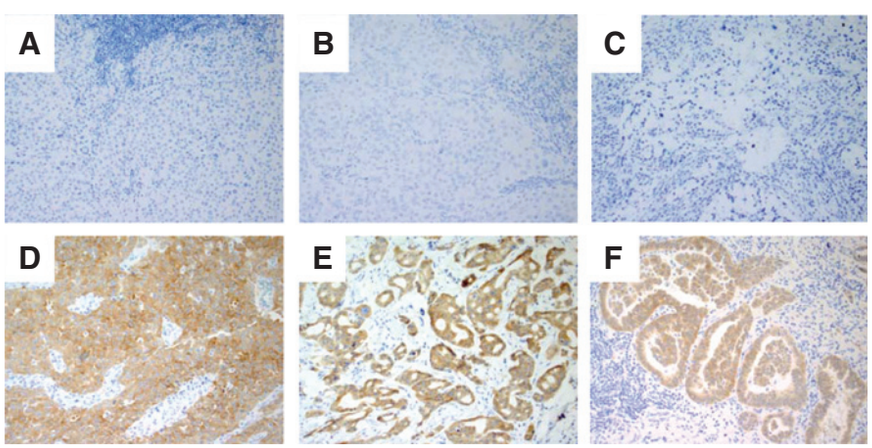

Figure 1. Immunohistochemical staining for ALK. Specimens (A–C) show negative ALK immunohistochemical staining for 3 patients with *KRAS* mutations and abnormal ALK FISH. Specimens (D–F) depict positive ALK immunohistochemical staining from 3 representative patients with positive ALK FISH.

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Table 3. Summary of overlapping mutations in EGFR, KRAS, and ALK

# Single mutations	EGFR	KRAS	ALK
EGFR ($n = 301$)	–	1	0
KRAS ($n = 465$)	1	–	0
ALK ($n = 75$)	0	0	–

Gene amplification has been reported to mediate resistance in a number of oncogene-driven malignancies (31, 32), including ALK-rearranged NSCLC (26–28). In our crizotinib-resistant cohort, repeat ALK FISH also permitted detection of gene amplification. In 2 patients (MGH029 and MGH034), ALK FISH was notable for high-level amplification, and a third specimen (MGH044) showed low-level gene amplification. In total, 3 of 29 (10.3%) patients exhibited amplification of the *ALK* gene at the time of crizotinib resistance.

Secondary mutations in *ALK* TK domain as mediators of crizotinib resistance

We next evaluated crizotinib-resistant specimens for secondary mutations in the *ALK* TK domain. Total nucleic acid was extracted and direct sequencing of the *ALK* TK domain was carried out as described previously (26). Among 34 tested specimens, a total of 8 secondary mutations were identified among 7 (20.6%) patients (Tables 4 and 5). The resistant specimen from MGH021 contained 2 separate secondary mutations: an amino acid substitution (G1269A) and an insertion mutation (1151Tins). A majority of secondary mutations involved missense mutations (S1206Y, G1202R, L1196M, and G1269A), all of which have been previously reported as mediators of crizotinib resistance (24, 26, 27). The most frequently identified secondary mutation was the gatekeeper L1196M mutation. However, this substitution was present in only 3 (9.3%) specimens, underscoring that the *ALK* gatekeeper mutation is not the dominant mechanism of crizotinib resistance.

Absence of *EGFR* and *KRAS* mutations in crizotinib-resistant cancers

Aberrant coactivation of EGFR signaling, in the absence of *EGFR* mutations, has been observed as a mechanism of crizotinib resistance *in vitro* and within patient-derived specimens (16, 26). One recent report also identified the emergence of *EGFR* and *KRAS* mutations among 11 ALK-positive patients following development of resistance to crizotinib (27). In a separate study of 7 ALK-positive patients with acquired crizotinib resistance, 1 patient was found to have *ALK* gene amplification as well as *EGFR* high polysomy and an acquired *EGFR* L858R mutation (28). We therefore tested repeat biopsy specimens from crizotinib-resistant, ALK-positive patients for mutations in *EGFR* and *KRAS* using the SNaPshot assay (Table 4). In 25 patients with available tissue, *EGFR* mutations were identified in 0 of 25 (0%; upper 95% CI: 11%) cases. Similarly, *KRAS* mutations were found in 0 of 25 (0%; upper 95% CI: 11%) specimens.

Discussion

In NSCLC, genetic alterations in *EGFR*, *KRAS*, and *ALK* are considered to be largely mutually exclusive (7, 10, 33). However, recent series have suggested that *EGFR* mutations and *ALK* rearrangements coexist within patients at clinically meaningful frequencies (3, 15–19). The question of whether overlap exists between these driver oncogenes is clinically relevant and would impact therapeutic choices. *In vitro* studies suggest that concomitant alterations in *EGFR* and *ALK* lead to mutual resistance to EGFR or ALK TKI monotherapy (16). In limited clinical series of patients reported to have both alterations, isolated responses to erlotinib, gefitinib, and crizotinib have been described (11, 12, 16, 18, 19). However, to the best of our knowledge, there have been no reports in which individual patients with co-existing *EGFR* mutations and *ALK* rearrangements responded to both EGFR and ALK TKIs.

In this study, we examined a large Western population of patients with NSCLC who underwent clinical genotyping. In contrast to the reports mentioned above, we found no cases of coexisting *EGFR* mutations and *ALK* rearrangements across 1,687 screened specimens, supporting the mutual exclusivity of these 2 genetic alterations. We did identify a few cases of concomitant *KRAS* mutations and abnormal ALK FISH. While abnormal, however, these FISH patterns did not meet criteria for an *ALK* rearrangement. Specifically, most cases (3/4) involved single isolated green 5′ probes, rather than the typical split probe or isolated 3′ probe pattern. To confirm the absence of a functional *ALK* rearrangement in these specimens, ALK immunohistochemistry was conducted and found to be negative in 3 of 3 cases tested. In a prior series using this antibody, ALK immunohistochemistry had a negative predictive value of 100% (30). Thus, these findings are unlikely to be due to false-negative staining, as the probability of obtaining 3 false-negative immunohistochemical results among these cases would be less than 0.000001. We therefore conclude that these abnormal ALK FISH patterns are unlikely to represent functional *ALK* gene rearrangements. Altogether, these findings support the lack of overlap between *ALK* rearrangements and either *EGFR* or *KRAS* mutations.

We recognize that these findings are in contrast to some recent reports (14–19). One explanation for such discordant findings may involve ethnic differences between screening populations. Specifically, the higher prevalence of *EGFR* mutations within Asian NSCLC populations may increase the chance of detecting dual *EGFR* and *ALK* alterations (34). Indeed, the highest frequencies of concomitant *EGFR* mutations and *ALK* rearrangements have been reported in Asian populations (17–19). For instance, in one study describing concomitant *EGFR* mutations in 11.8% of patients with *ALK* rearrangements, the overall prevalence of *EGFR* mutations in the study population ($n = 444$) was 51.4% (18).

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Table 4. ALK-positive patients with acquired crizotinib resistance

Patient	Timing (months)[a]	Duration (months)[b]	ALK fusion	ALK amplification	ALK secondary mutation	EGFR mutation	KRAS mutation
MGH0NZ	0	20	Positive	No	No	WT	WT
MGH001	4.5	4	Positive	No	No	WT	WT
MGH010	0	8	Positive	No	No	WT	WT
MGH011	0	34	Positive	No	S1206Y	WT	WT
MGH013	0	9	Positive	No	No	WT	WT
MGH016	6	6	Positive	No	No	WT	WT
MGH017	0	23+	Positive	No	No	WT	WT
MGH018	0.5	10	Positive	No	G1202R	N/A	N/A
MGH019	<0.5	8	Positive	No	No	WT	WT
MGH020	0	13	N/A	N/A	L1196M	N/A	N/A
MGH021	3	12	Positive	No	1151Tins, G1269A	WT	WT
MGH022	0	6	Positive	No	No	WT	WT
MGH023	0	12	Positive	No	No	WT	WT
MGH024	0	15	Positive	No	No	N/A	N/A
MGH025	0	11	N/A	N/A	No	WT	WT
MGH027	1	4	N/A	N/A	No	N/A	N/A
MGH028	1	14	N/A	N/A	No	N/A	N/A
MGH029	0	8	Positive	Yes	No	N/A	N/A
MGH030	0	9	Positive	No	No	WT	WT
MGH031	N/A	N/A	Positive	No	No	WT	WT
MGH032	21	9	Positive	No	No	N/A	N/A
MGH033	0	3	Positive	No	No	WT	WT
MGH034	0	8	Positive	Yes	No	WT	WT
MGH035	N/A	14	Positive	No	No	WT	WT
MGH036	0.5	13	Positive	No	No	WT	WT
MGH037	0	11	Positive	No	G1269A	WT	WT
MGH038	0	7	Positive	No	L1196M	WT	WT
MGH040	0	9	Positive	No	No	WT	WT
MGH044	0	5	Positive	Yes[c]	No	WT	WT
MGH045	0	13	Positive	No	L1196M	N/A	N/A
MGH051	<0.5	3	Positive	No	No	WT	WT
MGH054	8.5	3	Positive	No	No	WT	WT
MGH055	0	3	N/A	N/A	No	N/A	N/A
MGH058	0	14	Positive	No	No	WT	WT

[a]Indicates the time interval between last crizotinib dose and repeat biopsy. Repeat biopsies conducted while the patient is still receiving crizotinib are depicted with a "0".

[b]Indicates the approximate duration the patient was treated with crizotinib.

[c]Low level ALK amplification.

In addition to ethnic variations, our findings may differ from other reports due to the stage distribution of our patient population. A significant proportion of patients in this study had early-stage disease at the time of mutation testing. It is therefore possible that we did not capture additional genetic alterations that may have accumulated later during the disease course. Another potential explanation for reports of dual genetic alterations may be the presence of multiple separate primary tumors in patients, each harboring different driver mutations. When

Table 5. Summary of ALK-positive, crizotinib-refractory cases

Resistant cases	ALK fusion	ALK amplification	ALK secondary mutation	EGFR mutation	KRAS mutation
34	29/29 (100%)	3/29 (10.3%)	7/34 (20.6%)	0/25 (0%)	0/25 (0%)

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overlapping genetic alterations in *ALK* and *EGFR* are suspected, ALK and EGFR mutant-specific immunohistochemistry may help distinguish whether both abnormalities are colocalized in the same cells. Our findings may also differ from those of prior studies due to possible reporting bias and choice of screening techniques. ALK testing can be conducted using ALK immunohistochemistry, reverse transcription PCR (RT-PCR), and ALK FISH. While ALK FISH is the only clinically validated assay and current gold standard, this technique is technically challenging (35). In prior reports of coexisting *EGFR* mutations and *ALK* rearrangements, various methods of ALK testing were used, including ALK FISH, ALK immunohistochemistry, RT-PCR, and rapid amplification of cDNA ends (RACE)-coupled PCR (11–19). In contrast, all patients in our study were screened using ALK FISH. Of note, ALK FISH positivity in this study was defined as more than 15% of cells showing positive signals. This is consistent with the definition of ALK positivity used in clinical trials of crizotinib, and this threshold is above the technical background noise of the assay (21, 36).

As the molecular profile of malignancies may evolve over the course of treatment, we also examined the molecular characteristics of ALK-positive cancers after the development of resistance to crizotinib. Crizotinib-resistant specimens were examined for *ALK* gene amplification, secondary *ALK* TK domain mutations, and emergence of *EGFR* and *KRAS* mutations. Of particular note, we found that the presence of the ALK fusion gene as assessed by FISH was preserved across all tested specimens despite the presence of acquired crizotinib resistance. This finding is consistent with the oncogene addiction paradigm as well as results from repeat biopsy series in patients with *EGFR* mutations and acquired resistance to EGFR inhibitors (32, 37). Although we and others have previously identified ligand-dependent activation of EGFR as a potential resistance mechanism to crizotinib (16, 26, 38), we found no cases of *EGFR* or *KRAS* mutations among 25 examined specimens, including those cases with upregulation of activated EGFR by immunohistochemistry. Thus, our findings are different from a previous study reporting that ALK-positive patients develop ALK-negative tumors harboring new mutations in *EGFR* following treatment with crizotinib (27). It remains possible that the patients identified in the other study had 2 separate primary cancers, an *EGFR*-mutant lung cancer and an ALK-positive lung cancer, and the *EGFR*-mutant lung cancer emerged upon treatment with crizotinib. In such cases, more comprehensive genetic analyses may inform whether pretreatment and resistant cancers are derived from a common ancestor clone. Nevertheless, our findings on a larger cohort of patients, suggest that such occurrences will be rare in cancers with acquired crizotinib resistance.

Our study has several implications. In rare cases of suspected overlapping genetic alterations in driver oncogenes, our findings underscore the importance of using confirmatory molecular testing, preferably using different genotyping techniques. Our findings may also affect clinical laboratory workflow and resource allocation since the lack of concomitant genetic alterations in *EGFR*, *KRAS*, and *ALK* may permit sequential genetic testing in centers where ALK FISH is not readily available. However, this must be balanced against the need for timely acquisition of genotyping data to guide rapid initiation of therapy, particularly at the time of diagnosis. For this reason, a simultaneous molecular testing strategy also remains a viable approach. Finally, this study has implications for our understanding of crizotinib resistance. Consistent with prior reports, we observed ALK fusion gene amplification and secondary mutations in the *ALK* TK domain in a subset of ALK-positive patients with acquired crizotinib resistance. Nonetheless, the number of crizotinib-resistant patients examined in this and other studies have been relatively small, and a significant proportion of these patients still have unknown mechanisms of resistance to date. Resistance is likely to be a molecularly complex process, and studies of repeat biopsies in larger cohorts of crizotinib-resistant patients are needed.

There are several potential limitations to our study. It is possible that we did not detect genetic alterations present at very low allelic frequencies within our study population. To minimize this likelihood, all ALK testing was conducted using the current gold-standard assay, ALK FISH. Moreover, *EGFR* and *KRAS* mutation testing was conducted using the SNaPshot assay, which has analytic sensitivity to detect mutations present at a frequency of approximately 5% (29). Another consideration is that mutations in *EGFR* or *KRAS* may have been present, but they were outside of the mutation hotspot regions designated in our multiplexed assays. This is less likely as more than 95% of mutations in both *EGFR* and *KRAS* are captured by these assays. One last consideration is the presence of intratumoral heterogeneity. In this study, molecular testing was conducted on a single biopsy or resection specimen and thus, may not be reflective of all sites of disease. This is mainly relevant to treatment resistant specimens, as studies have shown clonality of driver gene alterations in pretreatment samples (39, 40). However, if this were a common occurrence in resistance, one would have expected to observe an *EGFR* or *KRAS* mutation in at least one of the resistant biopsies examined.

Finally, while this manuscript was under review, 2 smaller screening studies were published, confirming similar findings in 208 and 99 patients, respectively (41, 42). Together, our analyses provide support for the mutual exclusivity of alterations in *EGFR*, *KRAS*, and *ALK* within the Western population. By examining a series of biopsy specimens from ALK-positive patients after development of resistance to crizotinib, we also show that this lack of overlap persists in the resistant cancers.

Disclosure of Potential Conflicts of Interest

A.J. Iafrate is employed (other than primary affiliation; e.g., consulting) as a consultant in Pfizer and Bioreference Labs. J.A. Engelman has a commercial research grant from Novartis, Sanofi-Aventis, and AstraZeneca; has

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ownership interest (including patents) in Gatekeeper; and is a consultant/advisory board member of Novartis, Sanofi-Aventis, Chugai, and AstraZeneca. D. Dias-Santagata is a consultant/advisory board member of BioReference Laboratories, Inc. A.T. Shaw is a consultant/advisory board member of Pfizer, Novartis, Ariad, Chugai, and Daiichi sankyo. No potential conflicts of interest were disclosed by the other authors.

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