

Review Article

“ALKoma” in NSCLC- Optimizing Diagnosis and Treatment Strategies

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Abstract

Lung cancer is the main cause of cancer-related death worldwide. In the last decade, certain molecular subgroups were identified, followed by development of different molecular-targeted treatments, which significantly improved the prognosis of these patients. Anaplastic lymphoma kinase echinoderm microtubule-associated protein-like 4 (ALK-EML4) rearrangements occur in 4-7% of patients with lung adenocarcinoma. ALK-EML4 tyrosine kinase inhibitors (TKIs) demonstrated significant clinical efficacy with higher overall response rate (ORR) and longer progression free survival (PFS) as compared with the traditional chemotherapy in the 1st and the 2nd line setting, although resistance development is inevitable and central nervous system (CNS) is a common site of failure. Numerous new agents are currently examined in clinical trials to overcome resistance to ALK inhibitors and improve the CNS penetrance and activity, and second line ALK-EML4 inhibitors are currently in clinical use. ALK-EML4 rearrangements are typically diagnosed by the FDA-approved Fluorescent In-Situ Hybridization (FISH) Break-Apart probe, however can also be detected by immunohistochemistry and next generation sequencing. This review will describe the current and future methods of ALK-EML4-rearrangement detection and treatment options for ALK-EML4-rearranged NSCLC.

Keywords: Lung cancer; Personalized therapy; ALK rearrangement; Crizotinib; Ceritinib

Introduction

Molecular targeted therapy in NSCLC

Lung cancer is the main cause of cancer related death worldwide [1]. Over 70% of lung cancer patients are diagnosed with advanced disease, and receive palliative treatment with main purpose being life prolongation and symptoms management [2]. In the past, all Non-Small Cell Lung Cancer (NSCLC) patients were treated with chemotherapy regimens containing a platinum compound [3]. In the last decade, driver mutations in some of the lung carcinomas have been discovered; the most common drivers are mutations in the Kirsten rat sarcoma (K-RAS) gene, Epidermal Growth Factor Receptor (EGFR) gene, and Anaplastic Lymphoma Kinase-rearrangements. These can be diagnosed in about 60% of lung adenocarcinoma patients, which allows for about 30% of patients to be treated with targeted therapy significantly prolonging their survival (median survival of 3.5 years) [4]. Additionally, these agents typically produce rapid and durable responses and have a favorable toxicity profile. However, acquired resistance inevitably develops. This review focuses on the main pitfalls in the diagnosis of ALK-EML4 rearrangements, clinical characteristics and available treatment options for ALK-EML4-rearranged NSCLC, as well as the acquired resistance mechanisms and overcoming strategies.

The anaplastic lymphoma kinase gene rearrangement

The ALK is a 200 kDa receptor tyrosine kinase, a member of the insulin receptor superfamily. The protein is encoded by the ALK gene located on chromosome 2p239, and has a large extracellular domain, a lipophilic transmembrane segment, and a cytoplasmic tyrosine

kinase domain. Murine RNA blot hybridization analysis revealed expression of ALK in specific regions of the developing brain [5]. In humans, ALK protein is normally expressed in the small intestine, testis, and brain, but not in other tissues [6]. ALK is a dependent receptor, which has a pro-apoptotic activity in the absence of a ligand, and an anti-apoptotic activity in the presence of its ligand and when the kinase is intrinsically activated [7]. The translocation involving the ALK gene was first described in 1994 in anaplastic large-cell non-Hodgkin's lymphoma cells arising from activated T lymphocytes. This rearrangement fused the nucleophosmin (NPM) nucleolar phosphoprotein gene on chromosome 5q35 to ALK. About 60% of the lymphoma cells were associated with the translocation [6]. Mutations involving ALK as a suspected driver mutation was also found in familial and sporadic neuroblastoma, mainly by point mutations and gene amplification [8,9], and in inflammatory myofibroblastic tumors, mainly by gene rearrangement with Tropomyosin (TMP3 and TMP4) fusion oncoproteins [10].

In 2007 [11], Soda, et al. demonstrated that approximately 6% of NSCLC tumors carry a novel translocation in which the echinoderm microtubule-associated protein-like 4 (EML4) gene is fused to ALK, chromosomal translocation inv(2) (p21; p23). This translocation was proved to play a key role in the development of NSCLC, by the rapid growth of NSCLC lesions in transgenic mice that express EML4-ALK specifically in lung alveolar epithelial cells, and rapid response of these lesions to oral administration of small-molecule inhibitor of the kinase activity of ALK [11]. Another evidence to the driver role of ALK-EML4 rearrangement was found in a large-scale survey of tyrosine kinase activity in lung cancer [12]. Using a phosphoproteomic

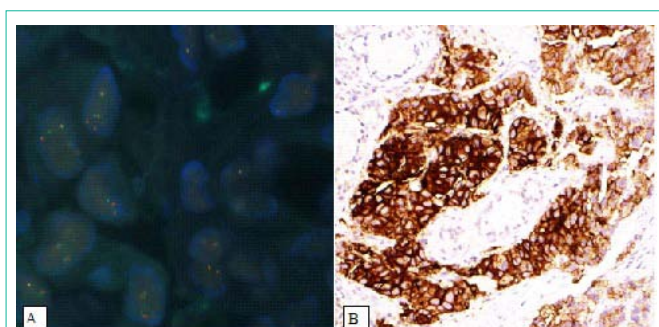


Figure 1: Panel A, shows ALK-EML4 rearrangement in a paraffin section from a patient with lung adenocarcinoma. Hybridization was performed using the VysisALK-EML4 Break Apart FISH probe (Vysis, Downers Grove, IL). The specimen displayed a typical pattern of 3'ALK signals (red) not fused with the 5'ALK signal (green), which was classified as positive for ALK-EML4 gene rearrangement. Panel B, ALK immunohistochemistry was performed using the rabbit monoclonal anti-ALK antibody clone D5F3 (Ventana Medical Systems, Tucson, Arizona). The tumor cells show strong membranous staining (3+) in the vast majority of tumor cells, and were classified positive for ALK-EML4 rearrangement (Courtesy of Dov Hershkovitz, Department of pathology, Rambam Health Care Campus).

approach, Rikova, et al. [13] characterized phosphotyrosine signaling of 41 NSCLC cell lines and 150 NSCLC tumors, and identified a high level of ALK phosphorylation in several NSCLC tumor samples and in one cell line [13]. The ALK-EML-4 translocation caused ligand-independent dimerization of the receptor kinase domain, leading to uncontrolled proliferation and inhibition of apoptosis, although the exact pathways involved in the pathogenesis are yet to be determined [12]. Several other ALK fusion partners, including CLIP4-ALK and SOCS5-ALK have been identified later [14], while EML4 still represents the most common one.

Methods of detection of ALK-EML4 rearrangement

The guidelines for molecular testing recommend ALK-EML4 screening for every patient with adenocarcinoma of lung origin following a negative EGFR testing result [15]. Patients should not be excluded from testing on the basis of clinical characteristics. EGFR and ALK-EML4 testing is not recommended for lung cancer patients that lack any adenocarcinoma component, such as pure squamous cell carcinomas, pure small cell carcinomas, or large cell carcinomas lacking any immunohistochemistry (IHC) staining suggestive for adenocarcinoma differentiation.

Aiming to increase the pre-test probability, and based on the Israeli experience of ALK-EML4 testing in NSCLC patients, our group developed a predictive model. This model demonstrated that the ALK-EML4 fusion was significantly more prevalent in younger male patients (52.1 vs. 61.3 years, $p=0.049$), in whom every additional year reduced the chance to find the fusion by 7% [CI=0.93 (0.88 - 0.99), $p=0.03$] [16].

The ALK-EML4 rearrangement can be detected using several methods. The only FDA-approved method for patient selection for an ALK-EML4 inhibitory therapy is Fluorescent In-situ Hybridization (FISH) Break-Apart probe. Other methodologies also include immunohistochemistry (IHC), RT-PCR and next generation sequencing (NGS) [17].

The FISH method allows direct visualization of the ALK-EML4

gene break apart. The dual color probes stain the DNA segments around the break apart site; therefore, a split signal indicates the presence of an ALK-EML4 rearrangement (Figure 1). This method is FDA-approved; although it is time consuming, expensive and not routinely available in all laboratories. For the more, Rodig, et al. revealed [18], that the probe does not identify all the positive cases, and the interpretation of the results can be especially difficult when the translocation is intra-chromosomal and the break apart points are close together [17,18]. In addition, it is not sensitive for intron abnormalities, which can only be detected by IHC and NGS [19]. Still, Crizotinib has been registered in the US, with the FISH test as an approved companion diagnostic test.

Recently, several IHC assays for the detection of ALK over expression were developed, including the rabbit monoclonal antibody D5F3 (Cell Signaling, Danvers, MA, USA) and the mouse monoclonal antibody 5A4 (Novocastra, Newcastle upon Tyne, UK). Both are novel and highly sensitive antibodies, which were shown to detect ALK-EML4 rearrangements with sensitivity and specificity of 100% and 96-99%, respectively, for D5F3, and sensitivity and specificity of 87.5-100% and 96-98.6%, respectively, for 5A4 [20,21]. The antibodies bind the portion downstream the kinase domain, which was found to be preserved in all ALK fusions, and allows for the detection of all the variants [20]. The advantages of the IHC method are the low cost, shorter turnaround time, the ability to imply the antibodies on small biopsies, and availability. However, there is no clear cut off for positive reading of the results.

Several studies compared IHC to FISH [17,18,22]. In some studies FISH was considered the gold standard method, while in others reverse transcription polymerase chain reaction (RT-PCR) was considered the most reliable test (Table 1); some discrepancies were noted. Yatabe reviewed the studies comparing FISH with IHC, and found that in large scale studies, about 0.2-21% of NSCLC cases screened for ALK-EML4 show discordance between IHC and FISH results [22]. Ali, et al. [23] has published the results of NGS from 1,070 lung carcinomas. Forty seven patients were diagnosed with ALK-EML4 rearrangements (4.4%). Interestingly, 28 patients were also tested by FISH Break-Apart probe, and 9 out of 28(32%) appeared to be negative by FISH. Pekar-Zlotin, et al. [24] compared FISH and IHC (D5F3 antibody) in 51 lung adenocarcinoma patients, followed by NGS in cases of discordance. Using the NGS as a standard, the sensitivity of FISH and IHC was 42.9% and 100%, respectively, and the specificity of both was 97.7%. IHC showed a higher positive and negative predictive value than FISH.

Those discrepancies in the different assays results have significant clinical implications- Ali, et al. [23] reported that five of the FISH (-)/NGS(+) tumors responded to Crizotinib. In light of the

Table 1: Comparison between FISH and immunohistochemistry assays (using gene sequencing as a standard method).

		[25]	[26]	[27]	[28]	[30]	[24]
Patients		1070	312	200	46	87	51
ALK positive cases ¹		47	13	25	11	5	8
Sensitivity,%	FISH	68	58	36	23	100	42.9
	IHC		91.6	52	23	100	100

ALK positive- tumors detected either by IHC or FISH

Table 2: ALK inhibitors in 1st and 2nd line of treatment.

	Agent	Study Abbreviation	Trial phase	Number of Patients	Response Rate (Drug vs. other arm)	Medial Progression Free Survival (months)	
1 st line ALK inhibitors	Crizotinib	PROFILE 1001	I	82	57%		
		PROFILE 1005[39]	II	439	53%	8.5	
		PROFILE 1014 ⁴²	III	343	74% vs. 45% with chemotherapy	10.9 vs. 7 with chemotherapy	
	Ceritinib[55]	ASCEND-1[55]	I	34	62%	10.4	
	Alectinib	ALEX NCT02075840	III		ongoing	ongoing	
2 nd line ALK inhibitors		Ceritinib	ASCEND-1[55]	I	83	69.5%	6.9
		Alectinib	[63]	II	47	55%	
		Crizotinib	PROFILE 1007 ⁴¹	III	347	65% vs. 20% with chemotherapy	7.7 vs. 3 with chemotherapy

responsiveness of ALK-EML4 NGS+/FISH- tumors to Crizotinib, this study concluded that the use of FISH as the gold standard for ALK-EML4 detection in NSCLC warrants re-investigation [23]. A case published by our group reported on a patient with an IHC(+) FISH(-) tumor harboring an intron 19 mutation in the ALK gene which demonstrated a complete response to ALK TKI-therapy [19].

Another method for detection of ALK-rearrangement is Reverse transcriptase polymerase chain reaction (RT-PCR) assay, which was recently validated in a cohort of 1100 patients. Among 255 tumors simultaneously analyzed by FISH and RT-PCR, the latter successfully detected all the 25 tumors with arrangements, including two cases that were missed by FISH [25].

The 2013 IASLC guidelines [15] indicated, that ALK-EML4 FISH assay should be used for patient selection for ALK-EML4 TKIs therapy. The guidelines also mentioned that ALK IHC, if carefully validated, may be considered as a screening methodology to select specimens for ALK-EML4 FISH testing [15]. In 2015 it may sound reasonable to use validated IHC testing for ALK detection either as a complement to FISH, or as a single test.

In the real life routine, ALK-EML4 rearrangement assay is been tested in adenocarcinomas after getting a negative EGFR testing. However, the practice in several large institutions today is to test both, regardless of the results of the EGFR. As ALK IHC became more frequent, it seems that ALK IHC will be the first test to be carried out (along with other IHC staining, such as TTF1 etc.), and the EGFR molecular testing will follow. When considering additional methods for molecular profiling, such as NGS, or for a sufficient amount of sample, it is feasible to detect both EGFR and ALK at the same time.

Treatment for ALK-rearranged NSCLC

Crizotinib: Crizotinib (PF-2341066) is an oral, ATP-competitive small-molecule inhibitor of NPM-ALK, ROS-1 and MET, which potently inhibits ALK phosphorylation and signal transduction, resulting in G₁-S phase cell cycle arrest and induction of apoptosis [30]. The drug was developed and evaluated for the treatment of anaplastic large-cell lymphoma, and demonstrated dose-dependent anti-tumor activity with IC₅₀ of 4 and 24nmol/L for ALK and MET receptors TK, respectively.

The first evidence to the activity of Crizotinib in NSCLC was demonstrated by Li [31] and McDermott [32] in H2228 and H3122 NSCLC cell lines and xenografts harboring ALK-EML4 re

arrangement, although it was found less potent than NPV-TAE684, another ALK-EML4 small molecule inhibitor.

Phase I clinical studies: The first in-human study was an open label, dose-escalation study which included patients with advanced cancer, third of them harboring MET amplification/gene mutation or ALK-EML4 fusion genes (PROFILE 1001;NCT00585195) (Table 2) [33]. The starting dose was 50 mg once daily, and it was escalated up to 300 mg bi daily. The maximum tolerated dose (MTD) was 250 mg twice a day (BID), the pharmacokinetic of the drug was found to be linear, and the median terminal half-life was 46 hours. Clinical response to Crizotinib was observed in 70% of the 10 patients with NSCLC harboring ALK rearrangement [34]. Later, the cohort was expanded to include 82 patients with ALK-EML4-rearranged NSCLC, 94% of patients enrolled have received previous anti-neoplastic treatment [35]. In this cohort, the response rate was 57%, and the disease control rate at 8 weeks was 87%. The median time to response was 8 weeks, and at a median follow-up of 6.4 months, the estimated 6-month PFS was 72%. Grade 1 nausea and diarrhea were the most commonly reported side effects; grade 3-4 toxicities included elevation of liver enzymes (6%), lymphopenia, pneumonitis, hypophosphatemia and pulmonary embolism (1% each). Additional side effects were mild visual disturbances and elevated liver enzymes (including 5% grade 3) based on these results, Crizotinib has been approved in an accelerated program by the FDA on August 2011, and received regular approval on November 2013, 6 years from the identification of ALK-EML4-rearrangement [36]. Crizotinib was also examined in phase 1 study among patients harboring ROS-1 rearrangement [37]. Among 50 patients with advanced NSCLC harboring ROS-1 rearrangement, the ORR was 72% and the PFS was 19.2 months.

Phase II clinical studies: PROFILE 1005 (NCT00932451) was a global, multicenter, open-label, single-arm phase II study evaluating safety and efficacy of Crizotinib (250 mg BID) in both Caucasian and Asian patients with advanced ALK-EML4-positive NSCLC who progressed after ≥1 chemotherapy [38]. 85% of patients had ≥2 previous lines of chemotherapy. The overall response rate was 53%, disease control rate was 85%, and median PFS was 8.5 months. The toxicity profile was as previously reported; 6% of patients had severe adverse events, mainly dyspnea and pneumonitis.

Phase III clinical studies: PROFILE 1007 was a first phase III randomized controlled open-label study comparing Crizotinib with

Table 3: Characteristics of different ALK inhibitors.

Agent	MTD	Additional targets	Mechanism of resistance	IC50 (nM)	Side effects
Crizotinib (PF-02341066, Xalkori, Pfizer) [16-20]	250 mg twice daily	C-MET ROS1	Mutations in L1196M (Gatekeeper), L1152R, C1156Y, 1151Tins, G1202R, S1206Y, and G1269A, F1174L ALK/EGFR/KIT amplification, KRAS mutation	24	Visual disturbance, nausea, diarrhea, elevated liver enzymes, lymphopenia, pneumonitis, hypophosphatemia, pulmonary embolism
Ceritinib (LDK378, Zykadia, Novartis) [26-28,74]	750 mg once daily	IGF-1R STK22D PLT3 Alectinib resistant mutation I1171T/N/S		0.2	diarrhea, vomiting, nausea, dehydration, elevated liver enzymes, hypophosphatemia
Alectinib (CH5424802, Roche) [34-37]	600 mg twice daily	RET Crizotinib resistant mutation L1196M	I1171T/N/S mutations	1-3.5	Headache, neutropenia, fatigue, myalgia, peripheral edema, liverenzyme abnormality, hypophosphatemia.
Brigatinib-AP26113	90/180 mg once daily	ROS1 EGFR STK22D PLT3		0.62	Nausea, diarrhea, fatigue, dyspnea, increased lipase, hypoxia, elevated liver enzymes and amylase. pneumonia, pyrexia, pulmonary embolism
X-396 [68]	250 mg once daily			0.15-1	Rash, fatigue, nausea, vomiting, edema
Lorlatinib-PF-06463922	100 mg	ROS1 Crizotinib resistant mutation L1196M LTK (TYK1)		<0.07 nM	Hypercholesterolemia, peripheral edema

either Docetaxel or Pemetrexed in the second-line setting in advanced NSCLC harboring ALK-EML4 rearrangement [39]. Among 347 patients who were randomized, the median PFS was 7.7 months in the Crizotinib arm and 3.0 months in the chemotherapy group (hazard ratio 0.49; 95% confidence interval [CI], 0.37 to 0.64; $P < 0.001$) (Table 3). Treatment with Pemetrexed was superior to treatment with Docetaxel in this group of patients. The rate of crossover to Crizotinib was 64% in the chemotherapy group, and no significant overall survival benefit was observed. The safety profile was similar to earlier studies, and there were 4 treatment-related deaths in the Crizotinib group- 1 due to ventricular arrhythmia, 2 due to pneumonitis and 1 due to hepatic failure.

Crizotinib efficacy and safety was compared to Platinum/Pemetrexed in the PROFILE 1014 trial [40]. This was a phase III, randomized, open-label study conducted in untreated NSCLC patients with advanced disease harboring an ALK-EML4 rearrangement (Figure 2). The primary end point was PFS, and crossover to the other treatment arm was allowed [41]. 343 patients were enrolled. PFS comprised 10.9 months and 7 months for patients treated with Crizotinib and chemotherapy, respectively (HR: 0.454; 95% CI: 0.346–0.596; $P < 0.0001$). The overall response rate was higher with Crizotinib (74% vs. 45%; $P < 0.0001$), and no overall survival advantage was demonstrated.

Following the publication of the above mentioned study results Crizotinib(Xalkori) has received approval in more than 60 countries, including US, EU, Canada, China, Korea, Japan and Australia [36].

Acquired resistance to Crizotinib

Following initial response to Crizotinib disease eventually progresses; it typically occurs within the first 8-10 months of treatment. Data regarding continuing treatment with Crizotinib following progression of disease according to RECIST is scarce, although a retrospective trial found prolonged overall survival among patients who continued Crizotinib beyond documented disease

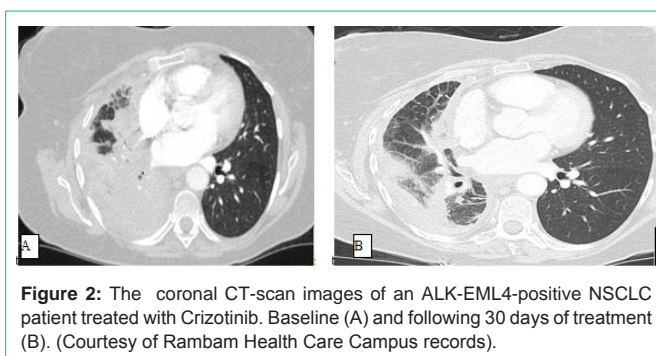


Figure 2: The coronal CT-scan images of an ALK-EML4-positive NSCLC patient treated with Crizotinib. Baseline (A) and following 30 days of treatment (B). (Courtesy of Rambam Health Care Campus records).

progression, while there was continuing clinical benefit [42].

There are two main mechanisms of acquired resistance to be distinguished: molecular and pharmacokinetic. The latter develops due to the pharmacokinetic limitation of the drug to cross the blood-brain barrier (see section Brain metastases in ALK-EML4-positive NSCLC). The molecular resistance is derived by several mechanisms, as summarized in Table 4.

1. Secondary mutations in the inhibited kinase domain. These are detected in up to one third of tumors. Secondary mutations occurring in the ALK gene can be both gatekeeper mutations (e.g. L1196M [43,44], the most common resistance mutation), or non-gate-keeper mutations (e.g. L1152R, C1156Y, 1151Tins, G1202R, S1206Y, F1174L, and G1269A). Secondary mutations can occur in multiple sites [45]. Additionally, ALK amplification may serve one of the acquired resistance mechanisms. However, cells harboring these secondary alterations are still addicted to ALK signaling, suggesting that a more potent ALK-inhibitor would be able to overcome, or even prevent the occurrence of this mechanism of resistance [43,46,47].

2. Activation of alternative signaling pathways [43].

- a) EGFR pathway activation (by amplification of EGFR and

Table 4: Ongoing trials for ALK-rearranged NSCLC.

Agent	Target	Trial phase	NCT identifier	Status	Sponsor	Treatment line
Crizotinib	ALK	III	NCT02201992	Recruiting	Eastern Cooperative Oncology Group	Adjuvant stage Ib-IIIa
Crizotinib+Pembrolizumab	ALK+PD-1	Ib	NCT02511184	Recruiting	Pfizer	1 st
Ceritinib+Evarolimus	ALK+MTOR	Ib	NCT02321501	recruiting	M.D. Anderson Cancer Center	2 nd +
Ceritinib+LEE011	ALK+CDK4/6	Ib/II	NCT02292550	recruiting	Novartis Pharmaceuticals	1 st /2 nd
Ceritinib+Nivolumab	ALK+PD-1	III	NCT02393625	recruiting	Novartis Pharmaceuticals	2 nd +
Alectinib+Bevacizumab	ALK+VEGF	I/II	NCT02521051	recruiting	Massachusetts General Hospital	1+
AUY922	HSP90-I ^a	Ib	NCT01772797	Recruiting	Novartis Pharmaceuticals	
		II	NCT01752400	Recruiting	Massachusetts General Hospital	1 st
		II	NCT01124864	Active, not recruiting	Novartis Pharmaceuticals	3 rd +
TSR-011	ALK/TRK TKI ^b	I/IIa	NCT02048488	Recruiting	Tesaro, Inc.	
Ganetespib	HSP90-I ^a	II	NCT01562015	Active, not recruiting	Synta Pharmaceuticals Corp.	Up to 3 rd
		I/II	NCT01579994	Recruiting	Pfizer/Astex	+/- Crizotinib
PF-6463922	ALK/ROS1 TKI	I/II	NCT01970865	Recruiting	Pfizer	1 st /2 nd
Brigatinib	ALK/EGFR TKI	I/II	NCT01449461	Recruiting	Ariad Pharmaceuticals	1 st /2 nd
		II	NCT02094573	Recruiting	Ariad Pharmaceuticals	Post Crizotinib
		III	NCT02737501	Recruiting	Ariad Pharmaceuticals	Brigatinib vs. Crizotinib 1 st lime
AT13387	HSP90-I ^a	I/II	NCT01712217	Recruiting	Astex Pharmaceuticals	+/- Crizotinib
RXDX-101	ALK/ROS/TRK TKI ^b	I	NCT02097810	Recruiting	Ignyta Operating, Inc.	
ASP3026	ALK/ROS TKI	I	NCT01284192	Active, not recruiting	Astellas Pharma Inc	Post Crizotinib
X-396	ALK TKI	I	NCT01625234	Recruiting	Xcovery Holding Company, LLC	Post Crizotinib/Crizotinib naïve
		III	NCT02767804	Recruiting		X-396 vs. Crizotinib

^aHeat-shock protein inhibitor^bTRK- tropomyosin-related kinases.

its ligands - EGF and amphiregulin) [43,46] is detected in up to 44% of patients. *In vitro* experiments demonstrated that this type of resistance was potentially reversible with EGFR TKIs [46,48].

b) KIT gene amplification is detected in up to 15% of Crizotinib-resistant tumors [48].

c) K-RAS path way activation may also play a role in development of acquired resistance to Crizotinib [43].

Repeated biopsy following progression on Crizotinib has no clinical implication yet, but hopefully will aid in choosing the treatment strategy in the near future.

1. Discovery of the mechanisms responsible for the development of acquired resistance to Crizotinib prompted adoption of several approaches in order to overcome it. These are as follows: Alternative dosing/schedule (see section on brain metastases).

2. Use of next-generation agents: several agents or combination therapies have been proposed to overcome Crizotinib resistance, mainly second-generation ALK-EML4 TKIs and Heat Shock Protein-90 (HSP90) inhibitors (Table 2,3 and 4).

Continuing treatment with Crizotinib beyond disease progression is controversial. Discontinuation of Crizotinib upon roentgenological progression can induce a “flare” of Crizotinib-sensitive clones [49]. One retrospective trial found a prolonged overall survival among

patients who continued Crizotinib beyond documented disease progression [43], but the data is scarce and do not allow to draw firm conclusions.

Second-generation of ALK inhibitors

Ceritinib (LDK378): Ceritinib (LDK378, Novartis Pharmaceuticals) is an oral, ATP-competitive, tyrosine kinase inhibitor of ALK-EML4 [50], which was found to be 20 times more potent than Crizotinib in enzymatic assays. Ceritinib does not inhibit MET, but inhibits insulin-like growth factor-1 (IGF-1) [51], and ROS-1 [52]. An ongoing phase II trial is currently studying Ceritinib in patients with NSCLC harboring ROS-1 rearrangement (NCT01964157) (Table 2). In xenograft models of ALK-EML4-rearranged NSCLC, Ceritinib showed antitumor activity against both Crizotinib-sensitive and Crizotinib-resistant tumors [50,51]. Ceritinib might also be active in tumors harboring aL117IT mutation which is known to be Alectinib resistant [53] (Table 4).

ASCEND-11 [54] is a phase I trial which tested Ceritinib in patients with advanced cancers harboring genetic alterations in ALK. The entire cohort included 130 patients, 68% were previously treated by Crizotinib. The MTD was determined to be 750 mg daily. Dose-limiting side effects observed with the compound were diarrhea, vomiting, nausea, dehydration, elevated alanine aminotransferase level, and hypophosphatemia. There were 4 cases of pneumonitis, and 62% required at least one dose reduction. The pharmacokinetic of

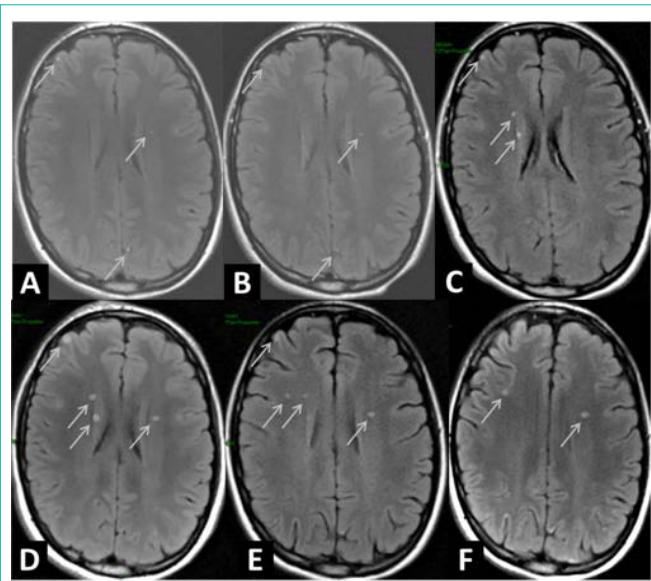


Figure 3: Dynamics of brain metastases on Crizotinib and Ceritinib therapy. Axial T₂-FLAIR magnetic resonance imaging sequences in a patient with ALK-rearranged NSCLC and brain metastases (arrows): baseline (A); stabilization of lesions 3 months after initiation of standard-dose Crizotinib (B); minimal enlargement of lesions 6 months after initiation of standard-dose Crizotinib (C); enlargement of the known right-hemispheric lesions and appearance of a new left-hemispheric lesion 4 months after initiation of pulse-dose Crizotinib - (D) following a 2 month-period of disease stabilization (not shown); size reduction of all the brain lesions 1.5 months after initiation of Ceritinib (E); complete resolution of two of the four brain lesions 3.5 months after initiation of Ceritinib (F).

the drug was linear, with mean terminal half-life of 40 hours. Among 114 patients with NSCLC treated with at least 400 mg daily, the overall response rate was 58% and 56% among Crizotinib-naïve and Crizotinib pre-treated patients, respectively. The median duration of response was 8.2 months. In the subgroup of patients who previously received ALK-inhibitor therapy, median PFS was 6.9 months [54]. An updated analysis of the trial was recently reported; among 246 patients treated with Ceritinib, duration of response was 9.7 months. Based on this data, on April 2014, the FDA granted accelerated approval to Ceritinib for the treatment of ALK-positive, metastatic NSCLC with disease progression on Crizotinib (Table 4). The ASCEND-2 is a phase-2 clinical trial evaluating the safety and efficacy of Ceritinib among ALK-rearranged NSCLC patients, pre-treated with Crizotinib [55]. All 140 patients had at least two lines of systemic treatment, including Crizotinib. The response rate was 38.6%, and the disease control rate was 77.1%, with PFS of 5.7 months. The main side effects included gastrointestinal toxicity, with 81% nausea, 80% diarrhea and 62.9% vomiting, mostly grade 1-2. Ongoing clinical trials are exploring the role of Ceritinib in treatment of CNS metastases (NCT02336451), the food effect on the dosage (NCT02299505) and the combination of Ceritinib with other targeted agents (Table 2).

Alectinib: Alectinib (CH5424802) is a novel potent selective oral ALK inhibitor. It is active against ALK-EML4- rearrangement, ALK L1196M [56], and also shows activity against mutation in RET gene [57]. Resistance mechanisms to Alectinib were recently discovered, these include secondary ALK mutations V1180L and I1171T [58] and *in vitro* bypass signaling (such as EGFR and cMET [59]). A multi-

center, single-arm, open-label, phase I and II studies evaluated Alectinib in Crizotinib-naïve ALK-rearranged NSCLC. In the dose escalation phase of the trial, doses of 20–300 mg twice daily were administered, and MTD has not been reached [60]. In the phase II portion of the study, the response rate was 93.5%. The interim analysis of this study demonstrated 73% response rate for the 15 evaluable patients [61]. Adverse events observed were liver function test abnormalities, neutropenia, rash, nausea and myalgia; none of those caused dose reduction. A second phase I trial evaluated Alectinib in Crizotinib-resistant ALK-rearranged NSCLC. Forty-seven patients were treated with doses up to 900 mg twice daily. DLTs were observed in 2 patients (headache and neutropenia), while common adverse events were fatigue, myalgia and peripheral edema, and t common grade 3/4 toxicities were GGT increase, neutropenia and hypophosphatemia. ORR was 55%; complete response (CR), partial response (PR), and stable disease (SD) were achieved in 2%, 52%, and 36% of patients, respectively [62]. In this trial, 21 patients had CNS metastases at baseline; 52% of patients had an objective response with Alectinib, and 29% of patients achieved a complete response in the brain. Based on this data, the drug received Breakthrough Therapy Designation by the FDA and has been recently approved in Japan for the treatment of ALK-positive, advanced NSCLC patients. The global phase II of Alectinib in Crizotinib-refractory patients included 138 patients, and demonstrated overall response rate of 50%, and median duration of response of 11.2 months with Alectinib [63]. The median PFS was 8.9 months, and the safety profile was favorable, with side effects of constipation (33%), fatigue (26%), peripheral edema (25%) and myalgia (23%), mostly grade 1-2. The first phase III trial in first line evaluated Alectinib versus Crizotinib in Japan, and primary results demonstrated superiority of Alectinib in PFS with HR=0.34 (99.6826% CI: 0.17-0.70, stratified log-rank p<0.0001), and better tolerability profile, with grade 3-4 adverse events 27% with Alectinib versus 51% in the Crizotinib arm [64]. Another ongoing phase II study is evaluating the addition of Erlotinib to Alectinib in patients with disease progression following treatment with Crizotinib (NCT01801111). Phase III study comparing Alectinib vs. Crizotinib in the 1st-line setting is currently recruiting patients (ALEX trial-NCT02075840) (Tables 2 and 3).

AP26113 (Brigatinib): AP26113 (Brigatinib, ARIAD Pharmaceuticals) is an oral reversible TKI, active against ALK-rearrangement and ALK mutations L1196M. It is also active against mutant EGFR, including T790M-resistance mutation and ROS-1 [65]. In pre-clinical trials using ALK-rearranged cell line Ba/F3, AP26113 potently inhibited both Crizotinib-native and -Crizotinib-resistant ALK mutants, and inhibited ROS-1 fusions [65]. In phase I trial with AP26113, the maximal tolerated dose was 300 mg, and the dose for phase II was set at 90 and 180 mg daily. The common side effects were nausea, fatigue and diarrhea. Two dose limiting toxicities were observed—elevation in transaminases and pneumonitis [66]. In the single arm phase I/II trial testing AP26113, 137 patients were enrolled. Among 72 patients previously treated with an ALK inhibitor, ORR was 72% and median duration of response was 49 weeks [67]. The median PFS was 56 weeks. Among 12 patients with measurable brain metastases, 6 responded, and 8/26 patients had CNS complete responses. Pulmonary side effects were noted in 10% of patients, starting as early as 7 days from the treatment onset. These

were dose-dependent, and therefore, enrollment continued at the lower dose level of 90 mg daily [68]. Based on the results listed above, AP26113 has received Breakthrough Therapy designation by the FDA for the treatment of patients with ALK-positive metastatic NSCLC who are resistant to Crizotinib.

X-396: X-396 (Xcovery) is aminopyridine-based kinase inhibitor. *In vitro* dose-response profiling suggested that X-396 is approximately 10-fold more potent against ALK rearrangement, but less specific for MET as compared to Crizotinib. In cell-based assays, X-396 was 3-10 times more potent than Crizotinib, and demonstrated the ability to overcome the ALK acquired resistance mutations- L1196M and C1156Y [68]. In an ongoing phase I trial, X-396 demonstrated activity in both treatment-naïve and pre-treated ALK-rearranged NSCLC patients. Among 6 ALK-rearranged NSCLC patients enrolled, PR was observed in 83%, and SD was seen in another 17% of patients; median duration of treatment was 20 weeks [69]. Doses of up to 250 mg once daily were well tolerated, frequent adverse events were rash, fatigue, nausea, vomiting, and edema, the majority of adverse events were grade 1-2 [69] (Tables 2 and 4).

TSR-011: TSR-011 (Tesaro) is an orally available inhibitor of both ALK and the tropomyosin-related kinases (TRK) TRKA, TRKB, and TRKC. In a phase I/II dose escalation clinical trial testing the compound, 3/5 Crizotinib-resistant ALK-rearranged NSCLC patients achieved PR. The dose was set at 60 mg daily, and adverse events included dysesthesia and QTc interval prolongation [70] (Tables 2 and 4).

Lorlatinib (PF-06463922): Lorlatinib (PF-06463922Pfizer), is a novel small-molecule ROS-1/ALK inhibitor that was optimized for robust brain penetration [71]. PF-06463922 demonstrated significant cell activity against native and mutant ALK with IC₅₀ ranging from 0.2 -77 nM. Additionally, PF-06463922 is a highly selective ROS-1 inhibitor with exquisite potency against ROS-1 kinase, but no substantial activity against MET [72]. Clinical phase I/II trial testing PF-06463922 in ALK- and ROS1-mutated NSCLC is currently ongoing (NCT01970865) (Table 2), and the phase I part of this trial reported ORR of 50%, and intra-cranial ORR of 60%, with 25% of CR among 54 pre-treated ALK-rearrange patients [73]. The most common adverse events noted were hypercholesterolemia (54%) and peripheral edema (37%).

Entrectinib (RXDX-101): Entrectinib (RXDX-101, Ignyta), formerly known as NMS-E628, is an orally available small-molecule inhibitor of TRKA, TRKB, TRKC, ROS-1 and ALK. It is currently being evaluated in phase I/II clinical studies (Table 2). In pre-clinical models, Entrectinib potently inhibits the growth of ALK-driven tumors, and demonstrates an ability to effectively penetrate the blood-brain-barrier [74]. In a phase I dose escalation trial, among 19 patients with various tumors, 2 patients with NSCLC (1 ALK-rearranged, 1 ROS-1 mutated) achieved PR, no dose limiting toxicity was observed [75]. On February 2015, the FDA has granted Orphan Drug designation to Entrectinib for the treatment of TRKA-positive, TRKB-positive, TRKC-positive, ROS-1-positive and ALK-positive NSCLC (Table 2).

Brain metastases in ALK-positive NSCLC

Approximately 25% of ALK-rearranged patients with newly diagnosed advanced NSCLC have CNS metastases [41]. Additionally,

CNS is the most frequent site of treatment failure in Crizotinib-treated ALK-rearranged NSCLC patients. In fact, the pooled analysis of PROFILE 1005 and PROFILE 1007 data demonstrated that further CNS progression during Crizotinib occurs in more than 70% of patients diagnosed with brain metastasis before enrollment, whereas another 20% of patients developed brain metastasis while on treatment [76]. Frequently, CNS also comprises the only site of disease progression [77]. Being a P-glycoprotein-substrate, Crizotinib is a subject for the active drug-efflux in the endothelial cells forming the BBB. As a result, the drug has an extremely low cerebro-spinal fluid (CSF)-to-plasma ratio of only 0.0026 [78]. This pharmacokinetic phenomenon is thought to represent the main mechanism responsible for the impaired control of the disease in the CNS. However, Crizotinib activity in the brain is not negligible. In fact, durable (more than 6 months) intracranial response and disease stabilization was reported with Crizotinib in 18% and 56% of radiation-naïve ALK-rearranged NSCLC patients, respectively [76]. The intra-cranial disease control rate in the brain was found to be significantly higher with Crizotinib compare with chemotherapy (85% v 45%, at 12 weeks; $P < .001$, and 56% v 25% at 24 weeks; $P = .006$) at the phase III PROFILE 1014 [79], and the time to intra-cranial tumor progression was higher in the Crizotinib group for both patient with and without brain metastases, although not statistically significant. High inter-patient variability in terms of drug metabolism and CNS drug penetration may exist, but has never been assessed appropriately.

Brain irradiation is administered to the vast majority of patients whose disease progresses in the brain which frequently results in long-term cognitive decline [80]. Systemic strategies lacking long-term morbidity might be an effective alternative, and these are rapidly evolving [62,69, 81-86].

The second generation ALK inhibitors, Ceritinib and Alectinib, demonstrated CNS activity. The ASCEND-1 trial included 124 patients with ALK-rearranged NSCLC and brain metastasis treated with Ceritinib. Ten out of 29 patients with measurable disease in the CNS demonstrated a partial response, and additional 5/45 patients with non-measurable brain metastases demonstrated a CR to Ceritinib, with median intracranial PFS of 8.3 months [84]. The ASCEND-2 trial reported results of 20 patients with active target brain lesion, with intra-cranial response rate of 45%.

Alectinib demonstrated activity in CNS on earlier trials, and on phase II trial on Crizotinib-refractory patients, Alectinib demonstrated overall CNS response rate of 57%, with CNS disease control rate of 83% [63]. Among 23 patients with untreated CNS metastases, 10 patients (43%) achieved CNS complete response.

This compound was also effective in 4 ALK-rearranged NSCLC patients presented with leptomeningeal spread whose disease progressed on Crizotinib and Ceritinib [86]. Alectinib represents the only compound which has the firm pharmaco-kinetic (p/k) data reported. In fact, the extrapolated Alectinib trough concentration (C_{trough}) in the CSF of 2.69 nM, was similar to the unbound systemic C_{trough} of 3.12 nM, and exceeded the *in vitro* IC₅₀ (1.9 nM) for ALK inhibition in cell-free assays, therefore, confirming the good CNS penetration of the compound [62]. CNS responses in different treatment settings were also reported with other novel ALK-inhibitors, but the data is sparse and inconsistent [69,81].

Another evolving treatment approach for treating brain metastasis in ALK-rearranged NSCLC is intermittent high-dose Crizotinib administration which has the potential to overcome pharmacokinetic resistance by achieving higher peak (C_{max}) drug concentrations in the CSF. This strategy was associated with good CNS control in epidermal growth factor receptor (EGFR)-mutated NSCLC patients who failed standard-dose EGFR-TKIs [87] (Figure 3), and might be also beneficial in treating ALK-rearranged NSCLC patients with CNS metastases [82,83]. Moreover, in patients with controlled systemic disease and CNS progression alone, this strategy can defer brain radiotherapy and the delay the use of second-line ALK inhibitors [87].

Overall, the data suggests that although Crizotinib administered at a standard dose has only limited efficacy in treating brain metastasis, the use of novel ALK-inhibitors or Crizotinib p/k manipulation is a promising treatment strategy in ALK-rearranged NSCLC with CNS involvement. It may defer brain radiotherapy administration, therefore, avoiding long-term cognitive decline. The major caveat of the existing data is the absence of a thorough p/k assessment for the majority of the compounds tested. Another major limitation is the fact that the majority of patients in the above reported clinical trials have received brain radiotherapy prior to study enrollment. This, along with the lack of details regarding the nature and timing of brain irradiation, limits the data interpretation, since the effect of brain radiotherapy (either direct, or indirect, through increase of the BBB penetration for the drug) cannot be separated from the effect of the compound tested. Therefore, prospective assessment of these novel agents with thorough p/k assessment while avoiding the contamination of the study population by enrollment of brain-radiotherapy-pretreated patients is highly anticipated.

In summary, ALK-rearranged NSCLC represents a distinct molecularly defined subtype which is characterized by the marked sensitivity to ALK-TKIs. The molecular diagnosis of ALK rearrangement is done by the FDA-approved FISH break-apart assay, although using immunohistochemistry assay for that purpose is becoming widely accepted. Crizotinib, the only ALK-TKI which is approved by the FDA for the 1st-line use in this tumor subtype, achieves excellent systemic controls. Resistance to Crizotinib, an inevitable event, and poor brain penetrance, required the development of second generation ALK inhibitors- Ceritinib and Alectinib, and the third generation ALK inhibitors are currently tested in clinical trials, granting hope for this small subset of NSCLC patients.

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin*. 2015; 65: 87-108.
2. H.C. Schrump DS, Altorki NK, Cancer: Principles and Practices of Oncology. 9th ed. Philadelphia: Lippincott Williams & Wilkins; 2005.
3. Schiller JH, Harrington D, Belani CP, Langer C, Sandler A, Krook J, et al. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med*. 2002; 346: 92-98.
4. Kris MG, Johnson BE, Berry LD, Kwiatkowski DJ, Iafrate J, Wistuba II, et al. Using multiplexed assays of oncogenic drivers in lung cancers to select targeted drugs. *JAMA*. 2014; 311: 1998-2006.
5. Iwahara T, Fujimoto J, Wen D, Cupples R, Bucay N, Arakawa T, et al. Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. *Oncogene*. 1997; 14: 439-449.
6. Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL, et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. *Science*. 1994; 263: 1281-1284.
7. Mourali JA, Bénard FC, Lourenço FC, Monnet C, Greenland C, Moog-Lutz C, et al. Anaplastic lymphoma kinase is a dependence receptor whose proapoptotic functions are activated by caspase cleavage. *Mol Cell Biol*. 2006. 6209-6222.
8. Janoueix-Lerosey I, Lequin D, Brugières L, Ribeiro A, de Pontual L, Combaret V, et al. Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. *Nature*. 2008; 455: 967-970.
9. George RE, Sanda T, Hanna M, Fröhling S, Luther W, Zhang J et al. Activating mutations in ALK provide a therapeutic target in neuroblastoma. *Nature*. 2008; 455: 975-978.
10. Griffin CA, Hawkins AL, Dvorak C, Henkle C, Ellingham T, Perlman EJ. Recurrent involvement of 2p23 in inflammatory myofibroblastic tumors. *Cancer Res*. 1999; 59: 2776-2780.
11. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature*. 2007; 448: 561-566.
12. Soda M, Takada S, Takeuchi K, Choi YL, Enomoto M, Ueno T, Haruta H. A mouse model for EML4-ALK-positive lung cancer. *Proc Natl Acad Sci U S A*. 2008; 105: 19893-19897.
13. Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell*. 2007. 131: 1190-1203.
14. Drlon A, Wang L, Arcila ME, Balasubramanian S, Greenbowe JR, Ross JS, et al. Broad, Hybrid Capture-Based Next-Generation Sequencing Identifies Actionable Genomic Alterations in Lung Adenocarcinomas Otherwise Negative for Such Alterations by Other Genomic Testing Approaches. *Clin Cancer Res*. 2015; 21: 3631-3639.
15. Lindeman NI, Cagle PT, Beasley MB, Chitale DA, Dacic S, Giaccone G, et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer and Association for Molecular Patho, *J Thorac Oncol*. 2013; 8: 823-859.
16. Shlomi D, Onn A, Gottfried M, Bar J, Biran H, Ilouze M, et al. Better Selection Model for EML4-ALK Fusion Gene Test in Patients with Non-Small-Cell Lung Cancer. 2013; 2013: 54-58.
17. Yi ES, Boland JM, Maleszewski JJ, Roden AC, Oliveira AM, Aubry MC, 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.
18. Rodig SJ, Mino-Kenudson M, Dacic S, Yeap BY, Shaw A, Barletta JA. Unique clinicopathologic features characterize ALK-rearranged lung adenocarcinoma in the western population. *Clin Cancer Res*. 2009; 15: 5216-5223.
19. Peled N, Palmer G, Hirsch FR, Wynes MW, Ilouze M, Varella-Garcia M, et al. Next-generation sequencing identifies and immunohistochemistry confirms a novel crizotinib-sensitive ALK rearrangement in a patient with metastatic non-small-cell lung cancer. *J Thorac Oncol*. 2012; 7: e14-e16.
20. Mino-Kenudson M, Chirieac LR, Law K, Hornick JL, Lindeman N, Mark EJ, Cohen DW, 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: 1561-71.
21. Hutarew G, Hauser-Kronberger C, Strasser F, Llenos IC, Dietze O. Immunohistochemistry as a screening tool for ALK rearrangement in NSCLC: evaluation of five different ALK antibody clones and ALK FISH. *Histopathology*. 2014; 65: 398-407.
22. Yatabe Y. ALK FISH and IHC: you cannot have one without the other. *J Thorac Oncol*. 2015; 10: 548-550.
23. Ali SM, Ignatius Ou SH, He J, Peled N, Chmielecki J, Pinder MC, et al. Identifying ALK rearrangements that are not detected by FISH with targeted next-generation sequencing of lung carcinoma. *J Clin Oncol*. 2014; 32: 5s.

24. Pekar-Zlotin M, Hirsch FR, Soussan-Gutman L, Ilouze M, Dvir A, Boyle T, et al. Fluorescence In Situ Hybridization, Immunohistochemistry, and Next-Generation Sequencing for Detection of EML4-ALK Rearrangement in Lung Cancer. *Oncologist*. 2015; 20: 316-322.
25. Wang Y, Zhang J, Gao G, Li X, Zhao C, He Y, et al. EML4-ALK Fusion Detected by RT-PCR Confers Similar Response to Crizotinib as Detected by FISH in Patients with Advanced Non-Small-Cell Lung Cancer. *J Thorac Oncol*. 2015; 10: 1546-1552.
26. Wu YC, Chang IC, Wang CL, Chen TD, Chen YT, Liu HP, et al. Comparison of IHC, FISH and RT-PCR methods for detection of ALK rearrangements in 312 non-small cell lung cancer patients in Taiwan. *PLoS One*. 2013; 8: e70839.
27. Teixidó C, Karachaliou N, Peg V, Gimenez-Capitan A, Rosell R. Concordance of IHC, FISH and RT-PCR for EML4-ALK rearrangements. *Transl Lung Cancer Res*. 2014; 3: 70-74.
28. 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, *Arch Pathol Lab Med*. 2012; 136: 796-803.
29. Tuononen K, Sarhadi VK, Wirtanen A, Rönty M, Salmenkivi K, Knuutila A, et al. Targeted resequencing reveals ALK fusions in non-small cell lung carcinomas detected by FISH, immunohistochemistry, and real-time RT-PCR: a comparison of four methods. *Biomed Res Int*. 2013; 2013: 757490.
30. Christensen JG, Zou HY, Arango ME, Li Q, Lee JH, McDonnell SR, et al. Cytoreductive antitumor activity of PF-2341066, a novel inhibitor of anaplastic lymphoma kinase and c-Met, in experimental models of anaplastic large-cell lymphoma. *Mol Cancer Ther*. 2007; 6: 3314-3322.
31. Li Y, Ye X, Liu J, Zha J, Pei L. Evaluation of EML4-ALK fusion proteins in non-small cell lung cancer using small molecule inhibitors. *Neoplasia*. 2011; 13: 1-11.
32. Mc Dermott U, Iafrate AJ, Gray NS, Shioda T, Classon M, Maheswaran S, et al. Genomic alterations of anaplastic lymphoma kinase may sensitize tumors to anaplastic lymphoma kinase inhibitors. *Cancer Res*. 2008; 68: 3389-3395.
33. Kwak EL, Camidge DR, Clark J, Shapiro GI, Maki RG, Ratain MJ, et al. G6 Clinical activity observed in a phase I dose escalation trial of an oral c-met and ALK inhibitor, PF-02341066. *Eur J Cancer*. 2009; 7: 8.
34. Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med*. 2010; 363: 1693-1703.
35. Kazandjian D, Blumenthal GM, Chen HY, He K, Patel M, Justice R, et al. FDA approval summary: crizotinib for the treatment of metastatic non-small cell lung cancer with anaplastic lymphoma kinase rearrangements. *Oncologist*. 2014; 19: e5-e11.
36. Shaw AT, Ou SH, Bang YJ, Camidge DR, Solomon BJ, Salgia R, et al. Crizotinib in ROS1 -Rearranged Non-Small-Cell Lung Cancer. *N Engl J Med*. 2014; 371: 1963-1971.
37. Kim D, Ahn MJ, Shi Y. Results of a global phase II study with crizotinib in advanced ALK-positive non-small cell lung cancer (NSCLC). *J Clin Oncol*. 2012.
38. Shaw AT, Kim DW, Nakagawa K, Seto T, Crinó L, Ahn MJ. Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. *N Engl J Med*. 2013; 368: 2385-2394.
39. Mok T, Kim D, Wu Y, Solomon B. First-line crizotinib versus pemetrexed-cisplatin or pemetrexed-carboplatin in patients (pts) with advanced ALK-positive non-squamous non-small cell. *J Clin Oncol*. 2014; 32: 5.
40. Solomon BJ, Mok T, Kim DW, Wu YL, Nakagawa K, Mekhail T, et al. First-line crizotinib versus chemotherapy in ALK-positive lung cancer. *N Engl J Med*. 2014; 371: 2167-2177.
41. Ou SH, Jänne PA, Bartlett CH, Tang Y, Kim DW, Otterson GA, et al. Clinical benefit of continuing ALK inhibition with crizotinib beyond initial disease progression in patients with advanced ALK-positive NSCLC. *Ann Oncol*. 2014; 25: 415-422.
42. Katayama R, Khan TM, Benes C, Lifshits E, Ebi H, Rivera VM, et al. Therapeutic strategies to overcome crizotinib resistance in non-small cell lung cancers harboring the fusion oncogene EML4-ALK. *Proc Natl Acad Sci U S A*. 2011; 108: 7535-7540.
43. Choi YL, Soda M, Yamashita Y, Ueno T, Takashima J, Nakajima T, et al. EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. *N Engl J Med*. 2010; 363: 1734-1739.
44. Gainor JF, Shaw AT. Emerging paradigms in the development of resistance to tyrosine kinase inhibitors in lung cancer. *J Clin Oncol*. 2013; 31: 3987-3996.
45. Sasaki T, Koivunen J, Ogino A, Yanagita M, Nikiforow S, Zheng W, et al. A novel ALK secondary mutation and EGFR signaling cause resistance to ALK kinase inhibitors. *Cancer Res*. 2011; 71: 6051-6060.
46. Katayama R, Shaw AT, Khan TM, Mino-Kenudson M, Solomon BJ, Halmos B, et al. Mechanisms of acquired crizotinib resistance in ALK-rearranged lung Cancers. *Sci Transl Med*. 2012; 4: 120ra17.
47. Pop O, Pirvu A, Toffart AC, Moro-Sibilot D. Disease flare after treatment discontinuation in a patient with EML4-ALK lung cancer and acquired resistance to crizotinib. *J Thorac Oncol*. 2012; 7: e1-e2.
48. Marsilje TH, Pei W, Chen B, Lu W, Uno T, Jin Y, et al. Synthesis, structure-activity relationships, and in vivo efficacy of the novel potent and selective anaplastic lymphoma kinase (ALK) inhibitor 5-chloro-N2-(2-isopropoxy-5-methyl-4-(piperidin-4-yl)phenyl)-N4-(2-(isopropylsulfonyl)phenyl)pyrimidine-2,4-diam, *J Med Chem*. 2013; 56: 5675-5690.
49. Shaw A, Mehra R, Kim DW, Felip E, Chow LQM, Camidge DR, et al. Clinical activity of the ALK inhibitor LDK378 in advanced, ALK-positive NSCLC. *J Clin Oncol*. 2013.
50. Anjum R, Vodala S, Kohlmann A, Rivera VM, Garner AP. Abstract A98: An *in vitro* mutagenesis screen identifies L1951R and G2032R as drug-resistant mutants of CD74-ROS1. *Mol Cancer Ther*. 2014; 12: A98-A98.
51. Ou SH, Greenbowe J, Khan ZU, Azada MC, Ross JS, Stevens PJ, et al. I1171 missense mutation (particularly I1171N) is a common resistance mutation in ALK-positive NSCLC patients who have progressive disease while on alectinib and is sensitive to ceritinib. *Lung Cancer*. 2015; 88: 231-234.
52. Shaw AT, Kim DW, Mehra R, Tan DS, Felip E, Chow LQ, et al. Ceritinib in ALK-rearranged non-small-cell lung cancer. *N Engl J Med*. 2014; 370: 1189-1197.
53. Crinó L, Ahn MJ, De Marinis F, Groen HJ, Wakelee H, Hida T, et al. Multicenter Phase II Study of Whole-Body and Intracranial Activity With Ceritinib in Patients With ALK-Rearranged Non-Small-Cell Lung Cancer Previously Treated With Chemotherapy and Crizotinib: Results From ASCEND-2. *J Clin Oncol*. 2016; 34: 2866-2873.
54. Sakamoto H, Tsukaguchi T, Hiroshima S, Kodama T, Kobayashi T, Fukami TA, et al. CH5424802, a selective ALK inhibitor capable of blocking the resistant gatekeeper mutant. *Cancer Cell*. 2011; 19: 679-690.
55. Kodama T, Tsukaguchi Y, Satoh Y, Yoshida M, Watanabe Y, Kondoh O, et al. Alectinib shows potent antitumor activity against RET-rearranged non-small cell lung cancer. *Mol. Cancer Ther*. 2014; 13: 2910-2918.
56. Katayama R, Friboulet L, Koike S, Lockerman EL, Khan TM, Gainor JF, et al. Two novel ALK mutations mediate acquired resistance to the next-generation ALK inhibitor alectinib. *Clin Cancer Res*. 2014; 20: 5686-5696.
57. Tanimoto A, Yamada T, Nanjo S, Takeuchi S, Ebi H, Kita K, et al. Receptor ligand-triggered resistance to alectinib and its circumvention by Hsp90 inhibition in EML4-ALK lung cancer cells. *Oncotarget*. 2014; 5: 4920-4928.
58. Seto T, Kiura K, Nishio M, Nakagawa K, Maemondo M, Inoue A, et al. CH5424802 (RO5424802) for patients with ALK-rearranged advanced non-small-cell lung cancer (AF-001JP study): a single-arm, open-label, phase 1-2 study. *Lancet Oncol*. 2013; 14: 590-598.
59. Nishio M, Kiura K, Nakagawa T, Seto A, Inoue M, Maemondo T, et al. 4410-phase I/II study of ALK inhibitor CH5424802 in patients with ALK-positive

- NSCLC; safety and efficacy interim results of the phase II portion. *Oncology*. 2012.
60. Gadgeel SM, Gandhi L, Riely GJ, Chiappori AA, West HL, Azada MC, et al. Safety and activity of alectinib against systemic disease and brain metastases in patients with crizotinib-resistant ALK-rearranged non-small-cell lung cancer (AF-002JG): results from the dose-finding portion of a phase 1/2 study. *Lancet. Oncol*. 2014; 15: 1119-1128.
 61. Ou SH, Ahn JS, De Petris L, Govindan R, Yang JC, Hughes B, et al. Alectinib in Crizotinib-Refractory ALK-Rearranged Non-Small-Cell Lung Cancer: A Phase II Global Study. *J Clin Oncol*. 2016; 34: 661-668.
 62. Nokihara H, Hida T, Kondo M, Kim Y, Azuma K, Seta T, et al. Alectinib (ALC) versus crizotinib (CRZ) in ALK-inhibitor naive ALK-positive non-small cell lung cancer (ALK+ NSCLC): Primary results from the J-ALEX study. *ASCO Annu Meet*. 2016.
 63. Squillace R, Anjum R, Miller D, Vodala S. AP26113 possesses pan-inhibitory activity versus crizotinib-resistant ALK mutants and oncogenic ROS1 fusions. *Cancer Res*. 2013.
 64. Camidge D, Bazhenova L, Salgia R, Weiss GJ, Langer CJ, Shaw AT, et al. First-in-human dose-finding study of the ALK/EGFR inhibitor AP26113 in patients with advanced malignancies: updated results. *J Clin Oncol*. 2013.
 65. Camidge D, Bazhenova L, Salgia R, Langer CJ, Gold KA, Rosell R, et al. Safety and efficacy of brigatinib (AP26113) in advanced malignancies, including ALK+ non-small cell lung cancer (NSCLC). *ASCO Annu Meet*. 2015.
 66. Lovly CM, Heuckmann JM, de Stanchina E, Chen H, Thomas RK, Liang C, et al. Insights into ALK-driven cancers revealed through development of novel ALK tyrosine kinase inhibitors. *Cancer Res*. 2011; 71: 4920-4931.
 67. Horn L, Jeffrey R, George R, Blumenschein G, Wakelee HA, Arkenau HT, et al. A Phase 1 Trial of X-396, a Novel ALK Inhibitor, in Patients With Advanced Solid Tumors. *J Clin Oncol*. 2014.
 68. Weiss G, Sachdev J, Infante J. TSR-011, a potent ALK inhibitor with clinical activity in phase I/IIa development. *J Thorac Oncol*. 2013.
 69. Johnson TW, Richardson PF, Bailey S, Brooun A, Burke BJ, Collins MR, et al. Discovery of (10R)-7-amino-12-fluoro-2,10,16-trimethyl-15-oxo-10,15,16,17-tetrahydro-2H-8,4-(metheno)pyrazolo[4,3-h][2,5,11]-benzoxadiazacyclotetradecine-3-carbonitrile (PF-06463922), a macrocyclic inhibitor of anaplastic lymphoma kinase (ALK) and c-ros oncogene 1 (ROS1) with preclinical brain exposure and broad-spectrum potency against ALK-resistant mutations. *J Med Chem*. 2014; 57: 4720-4744.
 70. Zou HY, Li Q, Engstrom LD, West M, Appleman V, Wong KA, et al. PF-06463922 is a potent and selective next-generation ROS1/ALK inhibitor capable of blocking crizotinib-resistant ROS1 mutations. *Proc Natl Acad Sci U S A*. 2015; 112: 3493-3498.
 71. Solomon BJ, Bauer TM, Felip E, Besse B, James LP, Clancy JS, et al. Safety and efficacy of lorlatinib (PF-06463922) from the dose-escalation component of a study in patients with advanced ALK+ or ROS1+ non-small cell lung cancer (NSCLC). *ASCO Annu Meet*. 2016.
 72. Ardini E, Menichincheri M, Banfi P, Casero D, Giorgini ML, Saccardo MB, et al. The ALK inhibitor NMS-E628 also potently inhibits ROS1 and induces tumor regression in ROS-driven models. *Cancer Res*. 2013; 73.
 73. De Braud FGM, Pilla L, Niger M, Damian S, Bardazza B, Martinetti A, et al. 448PD * RDX-101, An Oral Pan-Trk, ROS1, And ALK inhibitor, in patients with advanced solid tumors with relevant molecular alterations. *Ann. Onc*. 2014; 25: 148-149.
 74. Costa DB, Shaw AT, Ou SH, Solomon BJ, Riely GJ, Ahn MJ, et al. Clinical Experience With Crizotinib in Patients With Advanced ALK-Rearranged Non-Small-Cell Lung Cancer and Brain Metastases. *J Clin Oncol*. 2015; 33: 1881-1888.
 75. Chun SG, Choe KS, Iyengar P, Yordy JS, Timmerman RD. Isolated central nervous system progression on Crizotinib: an Achilles heel of non-small cell lung cancer with EML4-ALK translocation? *Cancer Biol Ther*. 2012; 13: 1376-1383.
 76. Costa DB, Kobayashi S, Pandya SS, Yeo WL, Shen Z, Tan W, et al. CSF concentration of the anaplastic lymphoma kinase inhibitor crizotinib. *J Clin Oncol*. 2011; 29: e443-445.
 77. Solomon BJ, Cappuzzo F, Felip E, Blackhall FH, Costa DB, Kim DW, et al. Intracranial Efficacy of Crizotinib Versus Chemotherapy in Patients With Advanced ALK-Positive Non-Small-Cell Lung Cancer: Results From PROFILE 1014. *J Clin Oncol*. 2016; 34: 2858-2865.
 78. Attia A, Page BR, Lesser GJ, Chan M. Treatment of radiation-induced cognitive decline. *Curr Treat Options Oncol*. 2014; 15: 539-550.
 79. Gettinger S, Bazhenova L, Salgia R, Langer CJ, Gold KA, Rosell R, et al. Updated efficacy and safety of the ALK inhibitor AP26113 in patients (pts) with advanced malignancies, including ALK+ non-small cell lung cancer (NSCLC). *ASCO Annu Meet*. 2014.
 80. Peled N, Zach L, Liran O, Ilouze M, Bunn PA Jr, Hirsch FR et al. Effective crizotinib schedule for brain metastases in ALK rearrangement metastatic non-small-cell lung cancer. *J Thorac Oncol*. 2013; 8: e112-113.
 81. Kim YH, Ozasa H, Nagai H, Sakamori Y, Yoshida H, Yagi Y, et al. High-dose crizotinib for brain metastases refractory to standard-dose crizotinib. *J Thorac Oncol*. 2013; 8: e85-86.
 82. Shaw A, Mehra R, Tan D. Evaluation of Ceritinib-treated Patients with Anaplastic Lymphoma Kinase Rearranged (ALK+) Non-small Cell Lung Cancer (NSCLC) and Brain Metastases in the ASCEND-1 Study. *Ann Oncol*. 2014; 25.
 83. Seto T, Hida T, Nakagawa K, Satouchi M, Nishio M, Hotta K, et al. 12240 Anti-tumor activity of alectinib in crizotinib pre-treated ALK-rearranged NSCLC in JP28927 study. *Ann Onc*. 2014; 25.
 84. Gainor JF, Sherman CA, Willoughby K, Logan J, Kennedy E, Brastianos PK, et al. Alectinib salvages CNS relapses in ALK-positive lung cancer patients previously treated with crizotinib and ceritinib. *J. Thorac Oncol*. 2015; 10: 232-236.
 85. Grommes C, Oxnard GR, Kris MG, Miller VA, Pao W, Holodny AI, et al. "Pulsatile" high-dose weekly erlotinib for CNS metastases from EGFR mutant non-small cell lung cancer. *Neuro Oncol*. 2011; 13: 1364-1369.
 86. Dudnik E, Siegal T, Zach L, Allen AM, Flex D, Yust-Katz S, et al. Durable brain response with pulse-dose crizotinib and ceritinib in ALK-positive non-small cell lung cancer compared with brain radiotherapy. *J Clin Neurosci*. 2016; 26: 46-49.
 87. Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. 2010; 363: 1693-1703.