Crizotinib for the Treatment of *ALK*-Rearranged Non-Small Cell Lung Cancer: A Success Story to Usher in the Second Decade of Molecular Targeted Therapy in Oncology

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ABSTRACT

Crizotinib, an *ALK*/*MET*/*ROS1* inhibitor, was approved by the U.S. Food and Drug Administration for the treatment of anaplastic lymphoma kinase (*ALK*)-rearranged non-small cell lung cancer (NSCLC) in August 2011, merely 4 years after the first publication of *ALK*-rearranged NSCLC. The crizotinib approval was accompanied by the simultaneous approval of an *ALK* companion diagnostic fluorescent in situ hybridization assay for the detection of *ALK*-rearranged NSCLC. Crizotinib continued to be developed as an *ALK* and *MET* inhibitor in other tumor types driven by alteration in *ALK* and *MET*. Crizotinib has recently been shown to be an effective *ROS1* inhibitor in *ROS1*-rearranged NSCLC, with potential future clinical

applications in *ROS1*-rearranged tumors. Here we summarize the heterogeneity within the *ALK*- and *ROS1*-rearranged molecular subtypes of NSCLC. We review the past and future clinical development of crizotinib for *ALK*-rearranged NSCLC and the diagnostic assays to detect *ALK*-rearranged NSCLC. We highlight how the success of crizotinib has changed the paradigm of future drug development for targeted therapies by targeting a molecular-defined subtype of NSCLC despite its rarity and affected the practice of personalized medicine in oncology, emphasizing close collaboration between clinical oncologists, pathologists, and translational scientists. *The Oncologist* 2012; 17:1351–1375

INTRODUCTION

The era of molecularly targeted therapy in lung cancer began in 2004 with the discovery that activating epidermal growth factor receptor (*EGFR*) mutations in non-small cell lung cancer (NSCLC) correlated with clinical response to *EGFR* tyrosine kinase inhibitors (TKIs) [1–3], although *EGFR* TKIs were developed and approved for clinical use prior to the knowledge of these activating *EGFR* mutations [4, 5]. Subsequently, five

randomized trials in patients with NSCLC with activating *EGFR* mutations have demonstrated statistically significant superior response rates and progression-free survival for *EGFR* TKIs compared with standard doublet chemotherapy [6–10]. Activating *EGFR* mutations provide a partial molecular explanation for the observation that NSCLC in never-smokers is considered a distinct disease with a high proportion of Asian female adenocarcinoma patients who have a better

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survival outcome than that of smokers [11, 12]. This observation also provided the impetus for the identification of other driver mutations in NSCLC. In the Iressa Pan-Asia Study (IPASS) [13, 14] and First-SIGNAL [15] trials, despite the enrichment of Asian female never-smokers with adenocarcinoma and the employment of sophisticated sequencing techniques, only approximately 60% and 44% of patients in each study, respectively, were found to carry activating *EGFR* mutations, indicating that other potential driver mutations remain to be discovered [16].

DISCOVERY OF ANAPLASTIC LYMPHOMA KINASE REARRANGEMENT IN THE PATHOGENESIS OF NSCLC

ALK rearrangements were identified in NSCLC in 2007 by two independent groups. Soda et al. developed retroviral-based cDNA expression libraries to screen for novel oncogenes [17, 18]. They transfected a cDNA library derived from a lung adenocarcinoma from a 62-year-old Japanese male smoker who was prescreened to be negative for *KRAS* and *EGFR* mutations [17]. They identified an echinoderm microtubule associated protein-like 4 (*EML4*)-anaplastic lymphoma kinase (*EML4-ALK*) fusion transcript that possessed transforming activity in 3T3 cells [17]. 3T3 cells transfected with *EML4-ALK* and implanted in nude mice resulted in rapid tumor growth [17]; an *ALK* inhibitor inhibited the growth of BA/F3 cells transfected with *EML4-ALK*. Finally, a preliminary survey of a panel of 33 NSCLC tumors revealed that *EML4-ALK* rearrangement occurs independently of *EGFR* and *KRAS* mutations [17]. To further demonstrate the role of *EML4-ALK* in the pathogenesis of NSCLC, Soda et al. generated transgenic mice engineered to specifically express *EML4-ALK* in lung alveolar cells, which resulted in hundreds of lung adenocarcinoma nodules. Treatment of these transgenic mice with an *ALK* inhibitor resulted in reduced tumor burden compared with untreated mice. Intravenous injection of *EML4-ALK*/3T3 cells resulted in massive infiltration of *EML4-ALK*/3T3 cells in the lungs of nude mice and rapid death of the majority of the mice within 1 month [18]. Treatment with the same *ALK* inhibitor resulted in the absence of *EML4-ALK*/3T3 cells in the lung and prolonged survival [18]. In summary, Soda et al. convincingly demonstrated that *EML4-ALK* is a unique driver mutation in NSCLC and that inhibition of *EML4-ALK* activity in vivo led to the reduction of lung cancer burden.

Contemporaneously, Rikova et al. characterized the phosphotyrosine profile in 191 NSCLC cell lines and tumor samples using a phosphoproteomic approach to identify “driver kinases” in NSCLC [19]. They identified a high level of *ALK* phosphorylation in several NSCLC tumor samples and H2228 NSCLC cells. Rapid amplification of the 5′ complementary DNA ends (5′ RACE) of the RNA transcripts isolated from these samples with highly phosphorylated *ALK* revealed the *EML4-ALK* fusion transcript [19]. No mutations were found in the *ALK* kinase domain [19]. Thus, two groups using two different approaches independently identified *ALK* translocation, the first of its kind in a common solid malignancy.

THE NORMAL PHYSIOLOGICAL ROLE OF *ALK* AND SIGNAL TRANSDUCTION PATHWAYS ACTIVATED BY *EML4-ALK*

The full-length *ALK* cDNA contains 29 exons. The full-length *ALK* protein contains 1,620 amino acids, with a predicted molecular weight of 177 kilodaltons (kDa) [20]. The 254-amino acid kinase domain comprises amino acid residues 1123–1376 preceded by a short transmembrane region of amino acids 1031–1058 [20]. *ALK* is temporally and spatially expressed during development of the murine neonatal central nervous system with its expression highest in the neonatal brain, and is not expressed in any non-neural tissues during any stage of development of the mouse [20, 21]. *ALK* was so named when it was discovered to be translocated in anaplastic large cell lymphoma (ALCL) [22]. Subsequently, *ALK* rearrangement with various fusion partners has been discovered in diffuse large B-cell lymphoma and inflammatory myofibroblastic tumor (IMT) prior to the discovery of *ALK* rearrangement in NSCLC. All three of the major signaling pathways (PI3K-AKT, RAS-ERK, JAK-STAT3) have been identified as being engaged by the various *ALK* fusion proteins [23, 24].

Much less is known about the normal function of the native *ALK* protein. Based on its expression pattern [21, 22], *ALK* is believed to be involved in early neurogenesis. In *Drosophila*, the ligand for *ALK* is jelly belly, but no human homolog of jelly belly has been identified [25]. Instead, there are two known *ALK* ligands: pleiotrophin [26] and midkine [27]. Both are polypeptide nerve growth factors that can bind to other receptors besides *ALK*. Binding of pleiotrophin to *ALK* led to both MAPK- [28] and phosphatidylinositol 3-kinase (PI3K) mediated cell growth and MAPK-mediated antiapoptotic signals [28, 29]. The MAPK signaling pathway, especially MEK, is important for the promotion of cell growth and neurite outgrowth of the SK-N-SH neuroblastoma cell line [30].

ALK is also negatively regulated by receptor protein tyrosine phosphatase (RPTP) β/ζ , which dephosphorylates *ALK*. RPTP β/ζ is also a receptor for pleiotrophin and is inactivated by pleiotrophin upon binding to RPTP β/ζ , allowing the continual autophosphorylation of *ALK* [30]. Thus, pleiotrophin can activate *ALK* both directly and indirectly. Expression of midkine during central nervous development correlates with the expression of *ALK* [31]. Midkine activates *ALK*, resulting in increased N-myc and trkB expression and the proliferation of immature sympathetic neurons. Midkine also (through a different pathway) increases the expression of a transcription factor, Hand2, that results in the upregulation of *ALK* expression, resulting in a positive autoregulatory pathway [31].

ALK has recently been recognized as a “dependence receptor,” where in its inactive form (without ligand binding) it is proapoptotic, whereas *ALK* is antiapoptotic in its active form (with ligand binding or aberrantly translocated in malignancies) [32, 33]. *ALK* can enhance apoptosis following caspase-mediated cleavage at position D1160 in the *ALK* juxtamembrane region, disrupting the *ALK* kinase domain and exposing a proapoptotic region encompassing amino acids 1090–1125 in the intracellular juxtamembrane domain [33].

Figure 1. Signaling pathways transduced by *EML4-ALK*.

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

Activation/phosphorylation prevents caspase-mediated cleavage, resulting in an autoregulatory cycle. Overexpression of ALK led to apoptosis of primary cortical neural cultures and neuroblasts [33]. *ALK* knockout mice are viable and exhibited no phenotypic abnormalities. They do exhibit an age-dependent increase in basal hippocampal neurogenesis consistent with its normal role in proapoptotic enhancement and the restriction of ALK expression largely to the central nervous system [34]. Consistent with the role of ALK in neurogenesis, activating mutations in *ALK* lead to both familial [35] and sporadic neuroblastoma [36–38], and provide the rationale for an ALK inhibitor in the treatment of neuroblastoma [37].

One of the major differences between the signaling pathways engaged by native ALK and *EML4-ALK* is the difference in the subcellular location. Native ALK is transmembrane and generally not activated, whereas *EML4-ALK* is cytoplasmic in location and in a constitutively activated form. *EML4-ALK* had been shown to engage all three major signaling pathways involved in receptor tyrosine kinases (RTKs): MAPK/MEK/ERK, PI3K/AKT, and RAS/STAT3 [39–41], but it remains relatively unclear which pathway(s) are critical to pathogenesis of NSCLC by *EML4-ALK*. Takezawa et al. recently demonstrated that both the MAP kinase (MEK/ERK) and JAK/STAT3 pathways but not the PI3K/AKT pathway are engaged by *EML4-ALK* to induce apoptosis. Cell lines with stably transfected *EML4-ALK* variants 1 and 3 had markedly increased phosphorylation of MEK, ERK, and STAT3 but not AKT [42].

Inhibition of *EML4-ALK* with TAE684, a small-molecule ALK inhibitor, led to decreased phosphorylation of ERK and increased levels of BIM, a pro-apoptotic protein. BIM is a member of the BCL-2 family of proteins and is degraded via phosphorylation by ERK. It has been recently shown that the level of BIM corresponds to the ability of tyrosine kinase inhibitors to induce apoptosis in various cell lines harboring driver mutations with the higher levels BIM associated with higher levels of apoptosis. Simultaneously and by an independent mechanism, TAE684 led to decreased phosphorylation of STAT3 and reduced levels of survivin [42]. Survivin directly and indirectly inhibits caspases, leading to apoptosis. Thus, *EML4-ALK* in NSCLC results in activation of ERK and STAT3, which leads to decreased BIM and increased survivin, allowing for the synergistic effects of antiapoptosis and promotion of tumor growth (Fig. 1).

CLINICOPATHOLOGIC CHARACTERISTICS OF IN PATIENTS WITH ALK-REARRANGED NSCLC

Many retrospective tissue bank studies have demonstrated the incidence of *ALK*-rearranged NSCLC to be approximately 3%–5% in NSCLC, with no apparent difference in incidence by ethnicity. Patients with *ALK*-rearranged NSCLC tend to be young (approximately 50 years of age at diagnosis) and never-smokers (~70%–75%) or light smokers. The vast majority present with adenocarcinoma and there is no sex preference for males or females. Compared to patients with *EGFR* mutations, *ALK*-positive patients had a younger median age of diagnosis

Table 1. Patients with simultaneous *ALK* and *EGFR* rearrangements reported in the literature

Reference	n of patients	Type of <i>EGFR</i> mutation	Frequency of <i>EGFR</i> mutations in <i>ALK</i> -positive NSCLC	Patient characteristics	Method of <i>ALK</i> detection	Response to <i>EGFR</i> TKI	Response to crizotinib
Rimkunas et al. [56]	2	L858R (detected by specific <i>EGFR</i> L858R antibody)	2/22	NR	Biopsy, FISH, and IHC	NR	NA
Lee et al. [55]	3	Exon 19 del/A750P	NA	Man, 73 yrs, former smoker, adenocarcinoma	Biopsy, FISH	PD	PR
		Exon 19 del		Man, 43 yrs, never-smoker	Biopsy, FISH	NA	NA
		L718P		Woman, 46 yrs	Biopsy, FISH	NA	NA
Yang et al. [53]	2		4/42	Never-smokers, adenocarcinoma	RT-PCR variant 1 (3 pts) and variant 6 (1 pt)	2 pts had PR (L858R, exon 19)	NA
Doebele et al. [46]	1	S768I	1/42	Woman, 53 yrs, never-smoker, adenocarcinoma with focal spindle differentiation	Biopsy, FISH	NR	NA
Sasaki et al. [51]	3	L868R	3/50	Adenocarcinoma	Biopsy, FISH	Erlotinib, PR	NA
		Exon 19 del		Adenocarcinoma	Biopsy, FISH	9 months: erlotinib, PR	NA
		A767_V769dup ASV		Adenocarcinoma	Biopsy, FISH	5 months: NA	NA
Kris et al. [47]	2	NR	2/43	Adenocarcinoma	Biopsy, FISH	NR	NA
Popat et al. [50]	1	Exon 19 del	NA	Woman, 65 yrs, white, never-smoker, adenocarcinoma, TTF-1 positive	Biopsy, FISH	Erlotinib, >25 months	NA
Kuo et al. [48]	1	Exon 19 del	NA	Woman, 72 yrs, Asian, never-smoker, adenocarcinoma, TTF-1 positive	RT-PCR (variant 1)	Gefitinib, clinical response, 232 days	NA
Tiseo et al. [52]	1	Exon 19 del	NA	Man, 48 yrs, white, never-smoker, poorly differentiated adenocarcinoma	Biopsy, FISH	Erlotinib, no response	NA
Zhang et al. [54]	1	Exon 19 del	1/12	Woman, Chinese, never-smoker, adenocarcinoma	RT-PCR (variant 3b)	NR	NA

Abbreviations: FISH, fluorescent in situ hybridization; IHC, immunohistochemistry; NA, not applicable; NR, not reported; NSCLC, non-small cell lung cancer; PD, progressive disease; PR, partial response; pt, patient; RT-PCR, reverse-transcriptase polymerase chain reaction; TKI, tyrosine kinase inhibitor.

(61 vs. 57 years old, respectively) and a higher proportion of never-smokers or light smokers (<100 cigarettes per lifetime; 48% vs. 67%) [43]. The vast majority of *ALK*-rearranged NSCLCs possess wild-type *EGFR* and *KRAS* genes [44, 45], although coexisting *ALK* rearrangements and other driver mutations have been described (Table 1) [46–56].

Several large prospective molecular profiling epidemiology studies (e.g., the Lung Cancer Mutation Consortium in the USA [47], the Institut National Du Cancer in France, and LUNGSCAPE in Europe [57]) will provide data on the incidence and clinical characteristics of *ALK* rearrangement as groups begin to report their findings. Most recently, the Lung Cancer Mutation Consortium reported that, among 901 patients using *ALK* breakapart assay, *ALK* positivity was 8.3% in adenocarcinoma in a clinically enriched population (younger, with more women and never smokers) [58]. In addition, Li et al. reported a rate of 3.2% of *ALK*-positivity after large-scale screening 4,500 patients with NSCLC formalin-fixed paraffin-embedded (FFPE) tissue using multiplex reverse transcriptase-

polymerase chain reaction (RT-PCR) assays that included nine known *EML4-ALK* fusion transcripts and *ALK* RNA levels [59].

Many histologic features have been ascribed to *ALK*-rearranged NSCLC, including acinar [60, 61], papillary [60, 62–63], micropapillary [63], bronchioalveolar [60, 62–63], and the presence of signet ring cells [64–66]. Yoshida et al. performed an extensive histology review of 54 *ALK*-rearranged NSCLC resections and identified two major and different histologic patterns: a solid growth pattern with focal signet ring cell component and a mucinous cribriform pattern associated with significant extracellular mucus materials [67]. Several studies have shown that signet ring cell differentiation is a common specific feature of *ALK*-positive NSCLC within up to two-thirds of tumors exhibiting this morphology [64–67]. Interestingly, “mucinous cribriform” component has recently been shown to be much more common in receptor tyrosine kinase fusion-positive NSCLC and may be used as another feature to draw attention to the potential presence of *ALK*

Figure 2. List of *ALK* fusion variants in NSCLC and proportion of various of *EML4-ALK* fusion variants. (A): Schematic of various forms of *ALK* fusions in *ALK*-rearranged NSCLC [17, 19, 39, 63, 66, 68, 70–78, 75a]. Adapted from Sasaki T, Rodig SJ, Chirieac LR et al. The biology and treatment of *EML4-ALK* non-small cell lung cancer. *Eur J Cancer* 2010;46:1773–1780, with permission. (B): Pie chart of distribution of *EML4-ALK* various fusions [17, 39, 41, 49, 61, 63, 66, 71–75, 75a, 79, 80, 90–94].

rearrangement [68]. However, none of the documented histologic features were sufficiently sensitive or specific to predict for *ALK* rearrangement [69]. Likewise, although lymphovascular invasion and necrosis, TTF-1 expression, and p63 expression (generally considered a “squamous cell” marker but found to be expressed in *ALK*-positive adenocarcinoma) were all found significantly more often in *ALK*-positive than in *ALK*-negative samples, such features should not be used to replace current diagnostic tests for *ALK* rearrangement [67].

There are now at least 27 different *ALK* fusion variants in NSCLC reported in the literature: 21 *EML4-ALK* isoforms (in-

cluding one with fusion to exon 19 of *ALK*) [17, 39, 63, 66, 70–75, 75a, 75b], 3 *KIF5B-ALK* isoforms [68, 75, 75a, 76], 1 *KLC1-ALK* isoform [77], 1 *TFG-ALK* isoform [19] and 1 *ALK-PTPN3* isoform [17, 19, 39, 63, 66, 68, 70–75, 75a, 76–78] (Fig. 2A, Table 2). The distribution of *EML4-ALK* variants has been previously reported [89] and we have performed an updated analysis based on a total of 371 published *EML4-ALK* variants. Variant 1 (49.6%) made up the most common type of the *EML4-ALK* fusion transcripts, followed by variant 3a/b (25.6%) and variant 2 (10%) [17, 39, 41, 49, 61, 63, 66, 71–75, 75a, 79, 80, 90–94] (Fig. 2B).

Table 2. List of <i>ALK</i> fusion variants in NSCLC reported in the literature		
Variant	Symbol	References^c
<i>EML4-ALK</i>		
1	E13; A20	Soda et al. [17]
^a	E10del54 E13;A20	Wang et al. [75a]
2	E20; A20	Soda et al. [17]
^a	E20ins18; A20	Takahashi et al. [73]
3a	E6; A20	Choi et al. [70]
3b	E6ins33; A20	Choi et al. [70]
^a	E6ins18; A20	Wang et al. [75a]
4	E14; ins11del49A20	Takeuchi et al. [74]
5a	E2; A20	Takeuchi et al. [74]
5b	E2; ins117A20	Takeuchi et al. [74]
6	E3; ins69A20	Takeuchi et al. [75]
7	E14; del12A20	Takeuchi et al. [75]
^a	E14; del36A20	Yoshida et al. [66]
8a ^b	E17; ins30A20	Sanders et al. [72]
8b	E17ins61; ins34A20	Sanders et al. [72]
^a	E17del58ins39; A20	Wang et al. [75a]
^a	E17ins65; A20	Wang et al. [75a]
^a	E17ins68; A20	Takahashi et al. [73]
“V4”	E15del19; del20A20	Koivunen et al. [39]
“V5”	E18; A20	Wong et al. [63]
^a	E6; A19	Doebele et al. [71]
<i>KIF5B-ALK</i>		
^a	KI24; A20	Takeuchi et al. [75]
^a	KI15; A20	Wong et al. [76]
^a	KI17; A20	Takeuchi et al. [68]
<i>KLC1-ALK</i>		
^a	KL9; A20	Togashi et al. [77]
<i>TFG-ALK</i>		
^a	T3; A20	Rikova et al. [19]
<i>ALK-PTPN3</i>		
^a	Exons 10 and 11 of <i>ALK</i> inserted between exons 2 and 3 of <i>PTPN3</i>	Jung et al. [78]
Only the reference that first reported the variant is cited here.		
^a These variants have not been officially named.		
^b Variant 8a contains an internal stop codon within the 30 bp insertion. Variant 8a was discovered in the same sample as variant 8b.		
^c Only reference that first reported the variant is cited here.		

Despite these various *EML4-ALK* fusion transcripts in NSCLC, Li et al. did not find any significant difference in the median age of diagnosis and gender composition among variants V1–5a in 152 patients with *ALK*-rearranged NSCLC [59]. However, there is in vitro evidence that various *ALK* fusions have different sensitivities to *ALK* inhibitors and may have different intrinsic protein stability within the cell [78a], although *EML4-ALK* variants 1 and 3a had similar clinical outcome treated with first-line platinum-based chemotherapy

[78b]. *EML4-ALK* fusion transcripts have been found in solid tumors other than NSCLC [79, 80]. Using a tumor repository, Lin et al. reported *EML4-ALK* in 2.4% of breast cancer (5/209) and colorectal cancer (2/83) [80]. Recently, *EML4-ALK* has also been detected in non-clear cell renal cell carcinoma using an immunohistochemical screening method [81]. The frequency of *KIF5B-ALK* in adenocarcinoma of the lung estimated from the three reports was 4 of 1,331 patients or approximately 0.3% [68, 75, 76]. It is important to note that the

Table 3. Comparison of overall survival of patients with *ALK*-rearranged NSCLC treated with crizotinib as second-line or third-line treatment versus crizotinib-naïve patients with *ALK*-rearranged NSCLC [95]

	Crizotinib-treated patients	Crizotinib-naïve patients	<i>p</i> value
<i>n</i> of patients	30	23	
Median overall survival, mos (95% CI)	NR (14–NR)	6 (4–17)	
1-year survival (95% CI)	70% (50–83)	44% (23–64)	
2-year survival (95% CI)	55% (33–72)	12% (2–30)	.004

Abbreviations: CI, confidence interval; NR, not reached.

Table 4. Comparison of overall survival of crizotinib-treated patients with *ALK*-rearranged NSCLC and tyrosine kinase inhibitor-treated patients with *EGFR*-mutated NSCLC [95]

	Crizotinib-treated patients	TKI-treated patients	<i>p</i> value
Overall			
<i>n</i> of patients	56	63	
Median overall survival, mos (95% CI)	NR (17–NR)	24 (15–34)	
1-year survival (95% CI)	71% (58–81)	74% (61–83)	
2-year survival (95% CI)	57% (40–71)	52% (38–65)	.786
As a second-line or third-line treatment			
<i>n</i> of patients	30	19	
Median overall survival, mos (95% CI)	NR (14–NR)	15 (12–32)	
1-year survival (95% CI)	70% (50–83)	72% (46–87)	
2-year survival (95% CI)	55% (33–72)	43% (20–64)	.578

Abbreviations: CI, confidence interval; NR, not reached; TKI, tyrosine kinase inhibitor.

three fusion partners to *ALK*—*EML4*, *KIF5B*, and *KLC1*—all interact with the microtubule apparatus [82, 86]. Therefore, agents that interrupt the assembly of microtubules such as taxanes or vinca alkaloids may be useful in the treatment of *ALK*-rearranged NSCLC alone and/or in combination with an *ALK* inhibitor.

Finally, an *ALK-PTPN3* variant has been observed from the insertion of genomic DNA encompassing exons 10 and 11 of *ALK* into the intronic region between exons 2 and 3 of protein tyrosine phosphatase, nonreceptor type 3 gene (*PTPN3*) [78]. Of note, *ALK-PTPN3* can theoretically generate a split-apart fluorescent in situ hybridization (FISH) signal but will not respond to crizotinib as the *ALK* kinase domain is absent.

The clinical presentation and course of patients with *ALK*-rearranged NSCLC have not been extensively described. One study reported that patients with *ALK*-rearranged NSCLC tended to present with a higher frequency of pericardial and pleural-based disease, an increased propensity for liver but not brain metastases, and an increased number of sites of metastasis compared with *EGFR*+ and *ALK*–/*EGFR*– patients [46]. Similarly of the 901 patients with stage IIIB/IV adenocarcinoma profiled to date for *ALK* rearrangement by the Lung Cancer Mutation Consortium, *ALK*-positive disease was significantly associated with liver metastasis compared with *ALK*-negative disease (23% vs. 10%; *p* = .004), but no differ-

ence was detected in bone, brain, or adrenal gland metastases [58].

Patients with *ALK*-rearranged NSCLC may also present with uncommon sites of metastasis such as retinal metastasis [46]. In a retrospective study, Shaw et al. have demonstrated among contemporaneously diagnosed patients with *ALK*-rearranged NSCLC that those treated with crizotinib had a significant survival advantage over those who did not receive crizotinib (Table 3) [95]. In fact, crizotinib-treated patients with *ALK*-rearranged NSCLC achieved excellent survival outcomes comparable with *EGFR* mutation-positive patients who received *EGFR* TKIs (Table 4) [95]. Taken together, these studies indicate that it is important to diagnose patients with *ALK*-rearranged NSCLC early in the treatment course to initiate effective antitumor treatment.

Recently, several retrospective studies have shown that pemetrexed-based chemotherapy may confer high tumor response either as first-line combination with platinum [96], as single agent in second-line or beyond treatment [97, 98] or longer progression-free survival when first-line platinum/pemetrexed combination is used in patients with *ALK*-rearranged NSCLC [98a]. However, these analyses may have confounded by some of the clinical demographics of the *ALK*-positive population such as younger age and/or never-smoking status [98a]. On the other hand, a larger retrospective analysis of treatment

histories from patients enrolled in the PROFILE 1005 study, which is a global phase II single arm study of crizotinib in patients with *ALK*-rearranged NSCLC, showed that among the 711 patients who had received pemetrexed as combination therapy or as a single agent, overall response rate (ORR) and time-to-progression outcomes were similar to unselected patients with NSCLC who were enrolled in phase III randomized trials comparing the use of first-line platinum/pemetrexed or second-line pemetrexed and treated with pemetrexed [99]. It will be important to complete the enrollment and await the results of the randomized trials comparing crizotinib to platinum pemetrexed-based chemotherapy in the first-line treatment setting (PROFILE 1014) or to standard second-line chemotherapy (pemetrexed or docetaxel; PROFILE 1007), which was recently reported to meet its primary endpoint [100].

The prognostic significance of *ALK* rearrangement in NSCLC has not been settled. In two separate reports [65, 95], Shaw et al. did not demonstrate any significant differences in overall survival (OS) for patients with NSCLC by *EML4-ALK* status in the era before crizotinib. Similarly, Zhang et al. reported no survival difference according to *ALK* status after adjusting for disease stage, histology, and *EGFR/KRAS* mutant status [101]. Lee et al. showed that patients with *ALK*-rearranged NSCLC had the shortest overall survival compared to wild-type patients, but the difference was not significant [102]. On the other hand, Kim et al. were able to demonstrate that patients with *ALK*-rearranged NSCLC had a significant worse OS outcomes after factoring in age, sex, histology, stage, and performance status [103]. Similarly, Yang et al. found that *EML4-ALK* status is a poor prognostic factor for relapse-free survival after factoring in stage, sex, age, and treatment [104].

Conversely, Wu et al. found that patients with *EML4-ALK* NSCLC identified from pleural effusion cytology had a significantly improved survival outcome compared with patients without *EML4-ALK* NSCLC [94]. Furthermore, Takeuchi et al. found that receptor kinase fusion-positive NSCLC is an independent favorable prognostic factor after taking into consideration age, sex, stage, and smoking status [68]. All of these studies are limited by the small number of *ALK*-positive patients, different comparison group of patients, the heterogeneous treatment patients received, and differences in the balance of prognostic factors (e.g., smoking status, surgical treatment, age) compared with *ALK*-rearranged patients.

ORIGINAL DESIGN AND SYNTHESIS OF CRIZOTINIB

Crizotinib Development and Preclinical Activity

Crizotinib is a competitive ATP inhibitor against both wild-type MET and against MET ATP-binding site mutants (V1092I, H1094R), P-loop mutant (M1250T), and juxtamembrane domains mutants (R988C, T1010I), but is not active against activation loop mutants (Y1230C, Y1235D) [105]. The cellular effects from MET inhibition by crizotinib are multi-fold: induction of apoptosis, decrease in proliferation, and decrease in angiogenesis [106]. Crizotinib inhibited the growth of the MET-dependent GTL-16 gastric carcinoma cell line,

and inhibited cell migration and invasion of the HGF-stimulated NCI-H441 lung cancer cell line and the growth of multiple human carcinoma xenograft models including gastric carcinoma (GTL-16), renal cell (Caki-1), glioblastoma (U87MG), prostate (PC-3), and NSCLC (NCI-H441) [106].

After its synthesis as a MET inhibitor [107–109] (Figures 3A and 3B [107–109]), crizotinib was evaluated against a panel of >120 kinases in biochemical assays and 12 cell-based phosphorylation assays revealed that it also inhibited phosphorylation of NPM-ALK in both Karpas 299 and SU-DHL-1 ALCL cells with a mean IC_{50} of 24 nmol/L [110]. Since both Karpas 299 and SU-DHL-1 cell lines express detectable MET levels, the lack of inhibitory activity of two specific MET inhibitors (PHA-665752 and PF-4217903) indicated that the inhibitory effect of crizotinib on these two cell lines was not due to its anti-MET activity. Crizotinib also resulted in dose-dependent growth inhibition of the Karpas299 xenograft in SCID-Beige mice. This inhibition was again accomplished by inhibition of NPM-ALK phosphorylation and the phosphorylation of NPM-ALK's downstream signaling mediators: PLC- γ and STAT3 over a wide range of crizotinib concentrations, and AKT and ERK kinases at a relatively high concentration of crizotinib [110].

CLINICAL DATA FOR CRIZOTINIB

First In-Human Phase I Trial of Crizotinib

The first in-human crizotinib trial (PROFILE 1001, NCT00585195) was written as an open-label, multicenter trial that was activated in 2006 with an initial standard dose-escalation pharmacokinetic (PK) portion followed by a clinical efficacy portion that aimed to enroll a small number of molecularly enriched patients to assess the antitumor activity of crizotinib. The initial PK portion used the standard dose-escalation finding schema to determine the maximal tolerated dose (MTD) and the recommended phase II dose (RP2D). There were several substudies to investigate the effect of food on crizotinib PK, a midazolam substudy to assess the effect of CYP3A isozyme inhibition by crizotinib, and a study to assess the feasibility of using fluoro-L-thymidine positron emission tomography to monitor response to crizotinib as an alternative to fluorodeoxyglucose positron emission tomography [111].

Pharmacokinetics of Crizotinib

Crizotinib was first dosed at 50 mg orally once daily and eventually escalated to 300 mg orally b.i.d., at which dose two patients experienced grade 3 fatigue [112]. The dose of crizotinib was reduced to 250 mg orally b.i.d. and was found to be tolerable; this dose was determined to be the MTD and RP2D dose [112].

Eleven patients were enrolled onto the food effect substudy. The absorption of a single dose of 250 mg of crizotinib after a high-fat, high-calorie meal was 14% lower than the concentration of a single dose of 250 mg of crizotinib taken on an empty stomach [112, 113]. Thus crizotinib could be taken with or without food. The peak plasma concentration was reached

Figure 3. Synthesis of crizotinib. **(A):** Design and synthesis of crizotinib. Figure 3A provided by Jean Cui. Please refer to reference 107 for detailed synthesis of crizotinib. **(B):** Crystallography of crizotinib in unphosphorylated *MET* and *ALK* [108, 109]. PDB ID 2wgj for PF-02341066/c-MET complex; PDB ID 2xp2 for PF-02341066/ALK complex. Figure 3B provided Jean Cui.

Abbreviation: MW, molecular weight.

4–6 hours after a single dose of crizotinib [113]. There was linear PK from 100 mg of crizotinib once daily to 300 mg of crizotinib b.i.d. [114]. Steady-state concentration of crizotinib was reached within 15 days of repeated administration of crizotinib at 250 mg orally b.i.d., with a half-life of approximately 43–51 hours [114]. The mean steady-state trough plasma level for 250 mg crizotinib b.i.d. (the recommended phase II dose) is 274 ng/mL or 57 nM of free drug, which exceeded the target efficacy levels predicted for the inhibition of

MET (~13 nM) and ALK (~26 nM) based on preclinical mouse models [114].

Metabolism and Drug Interactions

Thirteen patients were enrolled in the midazolam substudy. The PK of a single 2 mg midazolam dose was evaluated before and 28 days after 250 mg of crizotinib orally b.i.d.. Midazolam is metabolized by CYP3A isozymes and there was a 3.6-fold increase in the midazolam level (90% confidence interval [CI]: 2.7–

Figure 4. Steady-state crizotinib levels in Asians and non-Asians receiving repeated doses of crizotinib 250 mg twice daily [115].

4.9) after 28 days of crizotinib administration, indicating that crizotinib is a moderate CYP3A inhibitor [114]. Coadministration of strong CYP3A inhibitors or inducers should be avoided.

Ethnicity

The phase I study revealed higher exposure to crizotinib in Asian patients at steady state after crizotinib 250 mg b.i.d. than in non-Asian patients (Fig. 4) [115]. Exposure to crizotinib remained higher in Asian patients even after correction for ideal body weight, body surface area, or body mass index [115]. Recently, exposure-response analyses from both PROFILE 1001 and 1005 showed that Asian patients had higher ORR and longer progression-free survival (PFS) times, associated with higher crizotinib exposure than non-Asian patients [116].

CLINICAL EFFICACY OF CRIZOTINIB IN PATIENTS WITH ALK-REARRANGED NSCLC

Following the discovery of the *ML4-ALK* translocation in NSCLC in 2007 [17, 19] and in the context of crizotinib's ALK inhibitor activity [110], a commercially available break-apart FISH assay for detecting *ALK* rearrangement in ALCL was modified to detect *ALK* rearrangement in NSCLC. The first patient with *ML4-ALK* NSCLC was treated with crizotinib on the phase I dose-escalating cohort (300 mg b.i.d.) at Massachusetts General Hospital on December 26, 2007 with rapid clinical benefit, but was taken off trial due to liver enzyme elevations despite dose reduction. Six months later, the second patient with *ML4-ALK* NSCLC was treated with crizotinib, with clinical benefit and stable disease. Based on the clinical benefit from crizotinib for these two patients, there was a concerted shift among the phase I clinical sites to screen for NSCLC patients with *ALK*-rearrangement.

The clinical efficacy data for crizotinib in *ALK*-rearranged NSCLC patients have been remarkably consistent at multiple data cutoff dates, with an ORR of approximately 60% over the 4 years since the first report from 19 patients in 2009 (Table 5) [90, 112, 117–118]. The updated estimated PFS is stable at approximately 9.7 months [118]. The clinicopathologic characteristics of patients with *ALK*-rearranged NSCLC enrolled onto the study remained remarkably constant with a median

age of 51, although patients as young as 21 and as old as 86 were enrolled again underlying the heterogeneous population of patients with NSCLC harboring *ALK* rearrangement. Only ~70% of these patients were never- or light-smokers. The vast majority of patients with *ALK*-rearranged NSCLC presented with adenocarcinoma (Table 5).

An ongoing single-arm, global phase II study of crizotinib was launched in 2009 (PROFILE 1005, NCT00932451) based on the initial data from the phase I study, with central diagnosis of *ALK* rearrangement using the Vysis FISH test (Abbott Molecular, Abbott Park, IL; at the time also under evaluation by the U.S. Food and Drug Administration [FDA]) for the first 250 patients enrolled. (The protocol was subsequently amended to allow NSCLC patients with any *ALK*-positive test performed outside the central laboratory to be enrolled on a case-by-case basis.) This trial also provides an avenue for patients with *ALK*-rearranged NSCLC randomized to the control chemotherapy arm of the second-line phase III PROFILE 1007 (NCT00932893) trial to access crizotinib if they experience progression.

Data from PROFILE 1005 showed that patients had very similar clinicopathologic characteristics (Table 6) to those enrolled in PROFILE 1001, and the first 261 patients enrolled (whose *ALK* positivity was centrally confirmed by FISH) achieved a response rate of 60% (95% CI: 54%–66%) with a median PFS of 8.1 months (95% CI: 6.8–9.7) and median duration of response of 46 weeks [119]. In addition, best overall response appeared to be independent of the percentage of cells positive for *ALK* rearrangement by FISH in the diagnosis sample [120]. ORR was independent of age, sex, and number of prior metastatic treatment regimens. Responses were achieved rapidly, with approximately half of the observed responses occurring at the time of first follow-up scan: within 8 weeks (PROFILE 1001) and 6 weeks (PROFILE 1005) [118, 119].

Adverse Events with Crizotinib

As of January 2012, more than 1,000 patients had been treated in PROFILE 1001 and PROFILE 1005, and the safety profile for crizotinib has been consistent between these studies [118, 119]. Crizotinib was generally well tolerated with the majority of adverse events being grade 1 or 2, and low rates of with-

Table 5. Summary of clinicopathologic characteristics of patients with *ALK*-rearranged NSCLC and clinical activity of crizotinib in patients enrolled in the first in-human trial (PROFILE 1001) over a period of 4 years

Calendar year reported	2009 [112]	2010 [90]	2011 [117]	2012 [118]
Data cutoff date	March 9, 2009	April 7, 2010	October 10, 2010	June 1, 2011
<i>n</i> of patients (total/evaluable)	19/19	82/82	119/116	149/143
Median age, yrs (range)	50 (28–73)	51 ^a (25–78)	51 (21–79)	52 (21–86)
Never-smokers (%)	74	76	72	71
Adenocarcinoma (%)	90	96	97	97
Men (%)	47	52	50	49
Ethnicity (%)				
White	NR	56	62	64
Asian		35	29	27
Other		9	9	9
No prior treatment regimen (%)	0	6	13	16
ECOG PS ≥ 2 (%)	16	17	13	12
ORR (95% CI)	53	57	61 (51.7–70.1)	60.8 (52.3–68.9)
SD (%)	26	33	15	21
DCR at 8 weeks (95% CI)	79	87	79.3 (70.8–86.3)	82.5 (75.3–88.4)
Median time on treatment (95% CI)	NR	6.4 mos ^a	31.9 wks (0.7–101.7)	43.1 wks (0.1–138.6)
Median follow-up time (mos)	NR	NR	9.9 mos	16.3 mos
Estimated PFS, mos (range)	NR	NR	10.0 (8.2–14.7)	9.7 (7.7–12.8)
Median duration of response (wks)	NR	NR	48	49.1

^aMean.
Abbreviations: CI, confidence interval; DCR, disease control rate; ECOG PS, Eastern Cooperative Oncology Group performance status; NR, not reported; PFS, progression-free survival; ORR, overall response rate; SD, stable disease.

drawal due to adverse events. The most commonly reported treatment-emergent, all-causality adverse events were nausea, diarrhea, vomiting, constipation, and visual effects [113]. Other events reported at frequencies $\geq 20\%$ in one or both studies included peripheral edema, dizziness, fatigue, and decreased appetite [113]. Visual effects with crizotinib are particularly noteworthy as such events are not commonly associated with cancer therapies; however, no patient required dosing interruption, dose reduction, or permanent discontinuation of crizotinib treatment because of visual effects. Patient-reported symptom data reported from patients enrolled on PROFILE 1005 showed that visual events were short-lived (the majority of events lasted <1 minute), occurred less frequently as treatment continues, and were not bothersome, with no effect on patient's activities of daily living [119]. Further detail will be elucidated as these data mature and more patients are included. Based on data from ophthalmologic examinations performed at baseline and post-treatment in clinical trials, no clinically meaningful changes in visual acuity, biomicroscopy, or ophthalmoscopy in patients experiencing visual disturbances occurred [121].

Hepatic and Pulmonary Adverse Events with Crizotinib

Transaminase elevations have been noted with crizotinib, generally occurring within the first 2 months of treatment, with a

median onset of 40 days for any grade ALT elevation. Among 1,054 patients treated with crizotinib in both PROFILE 1001 and PROFILE 1005, ALT elevations of greater than 3, 5, and 10 times the upper limit of normal occurred in 15%, 7.4%, and 3% of patients, respectively. The incidences of alanine aminotransferase (ALT), aspartate aminotransferase, and alkaline phosphatase (AP) elevation were comparable for grade 1 and grade 2 events, but grade 3 and grade 4 events were more frequent for ALT elevations. Total bilirubin elevation was less common, suggesting that hepatic function is less affected in most cases of liver injury.

Temporary discontinuation or dose reduction occurred in 5.3% of all patients, and permanent discontinuation rate due to hepatic adverse events was 1.3%. Transaminase elevations were usually reversible and patients were able to resume the treatment at the same or at a lower dose. Fatal drug-induced hepatotoxicity occurred in $<1\%$ of patients in these studies [122]. Concurrent elevations in ALT greater than 3 times the upper limit of normal and total bilirubin greater than 2 times the upper limit of normal, with normal AP, occurred in less than 1% of patients in clinical trials. Liver function test monitoring is recommended, including ALT and total bilirubin once a month and as clinically indicated, with more frequent repeat testing for increased liver transaminases, AP, or total bilirubin in patients who develop transaminase elevations [113].

Crizotinib has been associated with severe, life-threaten-

Table 6. Clinicopathologic characteristics of patients with *ALK*-rearranged NSCLC enrolled in the PROFILE 1005 trial [119]

Patient characteristics	First patients enrolled, with all confirmed to be <i>ALK</i> -positive via centralized FISH (mature cohort)	Enrolment to date, with <i>ALK</i> -positivity confirmed centrally or locally
<i>n</i> of patients	261	901
Age, median yrs (range)	52 (24.0–82.0)	53 (>18–83.0)
Women (%)	142 (54.4)	514 (57)
Ethnicity (%)		
White	154 (59)	485 (53.8)
Black	8 (3.1)	18 (2.0)
Asian	94 (36)	379 (42.1)
Other	5 (1.9)	19 (2.1)
Baseline ECOG PS (%)		
0	68 (26.1)	225 (25.0)
1	148 (56.7)	511 (56.7)
2	42 (16.1)	134 (14.9)
3	3 (1.1)	31 (3.4)
Adenocarcinoma (%)	245 (93.9)	826 (91.7)
Smoking classification (%)		
Never smoker	176 (67.4)	592 (65.7)
Former smoker	73 (28.0)	271 (30.1)
Smoker	12 (4.6)	38 (4.2)
Prior therapies for locally advanced/metastatic disease (%)		
0	0 (0)	3 (<1.0) ^a
1	32 (12.3)	248 (27.5)
2	91 (34.9)	299 (33.2)
≥3	138 (52.8)	351 (39.0)

Data are *n* (%) unless noted.
^aThree patients were not eligible due to prior adjuvant treatment only.
Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; FISH, fluorescence in situ hybridization.

ing, or fatal treatment-related pneumonitis/interstitial lung disease (ILD). Four cases among 255 patients (1.6%) in PROFILE 1005 and PROFILE 1001 have been reported [113]. Patients should therefore be monitored for pulmonary symptoms indicative of pneumonitis/ILD in addition to NSCLC progression. Other causes of pulmonary symptoms, such as other pulmonary disease, infection, or radiation effects, should be excluded [113].

Endocrine Adverse Events

Recently, the University of Colorado reported that 13 men with NSCLC experienced a rapid decrease in total testosterone levels after initiating crizotinib potentially via a hypothalamic or pituitary effect, which may account for some of the fatigue experience by some patients [123]. Testosterone promptly returned to the normal level after crizotinib discontinuation. However, hypogonadism is multifactorial in its etiology in ad-

vanced lung cancer. Further investigation in an appropriate control setting will provide a more comprehensive description and understanding of this phenomenon.

Specific Clinical Situations (Treatment Beyond Progression/Brain Metastasis)

Both the PROFILE 1001 and 1005 protocols allow crizotinib to be continued if the investigators deem the patient will continue to derive “continual clinical benefit.” Of 138 patients who continued on crizotinib for >2 weeks postprogression on PROFILE 1001 and PROFILE 1005, a total of 53 patients (46%) had progression in the brain as a new lesion or nontarget lesion [125]. Among the 42 patients who received more than 6 months treatment postprogression, half had brain metastases and some patients had received 40 weeks of postprogression treatment, with treatment still ongoing [125]. The level of crizotinib achieved in cerebrospinal fluid (CSF) has been re-

ported in one patient who received crizotinib 250 mg orally b.i.d. [124]. The CSF level was only 0.26% of the plasma level (0.616 ng/mL; 0.0014 μ mol/L), a level expected to be insufficient to inhibit ALK. Thus, progression in the brain alone likely reflect a “sanctuary site” for crizotinib rather than true resistance to crizotinib given many patients continued to be on crizotinib after progression in the brain.

Additionally, PROFILE 1001 and PROFILE 1005 protocols empirically specify that crizotinib be withheld the day before, during, and the day after any radiation treatment, allowing patients who received definitive radiation to the brain to continue crizotinib without undue interruption. This strategy has been successfully employed by the University of Colorado to provide additional 6 months of PFS for selected patients with *ALK*-rearranged NSCLC who presented with oligoprogressive disease [126].

FDA APPROVAL OF CRIZOTINIB AND THE BREAK-APART FISH ASSAY

The FDA agreed to accept clinical efficacy and safety data of crizotinib from the PROFILE 1001 and PROFILE 1005 trials as the basis for the new drug application (NDA) filing in April 2010. Crizotinib was granted orphan drug designation for *ALK*-positive NSCLC in September 2010. Fast-track designation for crizotinib was granted in December 2010 and review of the NDA on a rolling basis began in January 2011.

The break-apart FISH assay kit manufactured by Abbott Molecular was required to satisfy two criteria: an analytical component and clinical utility. The exclusive use of the Abbott kit in PROFILE 1005 satisfied the clinical utility requirement based on response rates in that study [115]. For the analytical component, Abbott Molecular and the Department of Pathology at Massachusetts General Hospital (which served as the central laboratory for PROFILE 1001) simultaneously and independently re-evaluated the patients enrolled in PROFILE 1001 using the Abbott Vysis probes with excellent concordance (>90%). The Abbott Molecular break-apart FISH testing regulatory package was submitted in May 2011, completing the regulatory submission package to the FDA.

Crizotinib was approved in the USA on August 26, 2011, primarily based on response rates of 50% from the first 136 patients with *ALK*-rearranged NSCLC enrolled on PROFILE 1005 [127] and secondarily on a response rate of 61% from the first 119 patients with *ALK*-rearranged NSCLC enrolled on PROFILE 1001 [117]. The approval language states: “Xalkori is a kinase inhibitor indicated for the treatment of patients with locally advanced or metastatic non-small cell lung cancer (NSCLC) that is anaplastic lymphoma kinase (*ALK*)-positive as detected by an FDA-approved test. This indication is based on response rate. There are no data available demonstrating improvement in patient reported outcomes or survival with Xalkori” [113], with no restriction as to which line of treatment crizotinib could be used to treat patients with *ALK*-positive NSCLC. Crizotinib is now also approved in many countries, including Argentina, Canada, Israel, Japan, Korea, Macau, Mexico, and Switzerland and imminently by the European Union.

WHAT IS THE BEST COMPANION DIAGNOSTIC TEST FOR DETECTING *ALK* REARRANGEMENTS?

Break-apart FISH Assay as the “Gold Standard”

Molecularly targeted therapy in oncology is critically dependent on a validated test to detect the molecular alteration in question, especially when molecular alterations constitute a small subgroup of patients. Ideally, the test should be both highly sensitive and highly specific, relatively inexpensive, and feasible in most diagnostic laboratories to facilitate worldwide adoption. FISH is routinely used in oncology to detect chromosomal translocations which play an important role in soft tissue and hematologic malignancies, including the diagnosis of *ALK*-positive ALCL. Until recently, chromosomal translocations were considered uncommon in epithelial tumors [128]. The same *ALK* break-apart FISH assay used for ALCL was the basis for the *ALK* diagnostic test eventually approved by the FDA to detect *ALK*-rearranged NSCLC in conjunction with the approval of crizotinib in the USA [113].

The performance of the FISH assay has been rigorously evaluated and shown to be effective in analyzing FFPE NSCLC specimens. Technically, *ALK* FISH can be more challenging than most break-apart assays, because *ALK* and its most common fusion partner *EML4* are situated very near to each other on chromosome 2 (approximately 12 megabases apart). *EML4-ALK* rearrangement is generated most commonly from an intrachromosomal inversion, which results in “split” signals that may not be very far apart in positive cases and thus occasionally difficult to interpret. FISH positivity is defined by the separation between the 5' and 3' signals of greater than two signal diameters (Fig. 5A, 5B) [129] and at least 50 cells to be counted to ensure 100% sensitivity and specificity [130].

A second common pattern in positive cases is the presence of isolated *ALK* 3' probes (red signals only; Fig. 5C), likely reflecting the presence of an *ALK* rearrangement with loss of the nonfunctional derivative chromosome. A careful analysis of known *ALK*-negative cases revealed a cutoff of 15% of tumors cells to have split signals, above which a case will be called positive (15% is 2 standard deviations above the mean cell count in negative cases). Some cells may be falsely positive because the tumor cells counted may have artificial split signals due to sectioning artifact or poor nucleus morphology. The mean proportion of cells with split signals in *ALK*-positive NSCLC ($n = 13$) was 53.8% (range: 22.25%–86.62%) and 5.98% (range: 3.51–9.45) in *ALK*-negative NSCLC ($n = 56$; $p < .0001$; Table 7, Fig. 5D) [130–132]. With the inclusion of more *ALK*-positive NSCLC samples ($n = 90$), the mean percentage positive cells remained similar at 56% (range: 18%–100%) [133].

The reason the percentage of positive cells in *ALK*-positive samples is not always 100% is due to the inherent technical limitations of the assay to detect a rearrangement on a cell-by-cell basis and not because of tumor heterogeneity. Furthermore, 5' and 3' split signals are more common than isolated 3' signals in FISH-positive samples (Table 8) [131, 133–134]. Interestingly, NSCLC with isolated 3' signals has on average more percentage cells positive than NSCLC with split signals,

Figure 5. *ALK* breakapart FISH assay. **(A):** Greater than 2 signal diameter separation as a criterion for anaplastic lymphoma kinase (ALK) break-apart fluorescence in situ hybridization (FISH) positivity. **(B):** 5'3' break-apart ALK FISH. Figure provided by John Iafrate. **(C):** Isolated 3' break-apart ALK FISH. **(D):** Using the 15% criterion for ALK break-apart, FISH resulted in a clear separation of two groups of NSCLC patients. Adapted and modified from figure 2 of reference [130], with permission.

likely due to the difficulty in calling a split signal with the inversion pattern (Table 9) [133]. Importantly, there was no correlation between the percentage of cells positive by FISH and response to crizotinib from analyses of both PROFILE 1001 [133] and PROFILE 1005 [120].

There are advantages and disadvantages to the break-apart FISH assay. As mentioned, the break-apart FISH assay is currently the only ALK assay that has been clinically validated. This assay can be performed on FFPE tissue, which is how the vast majority of lung cancer tissue is processed. Furthermore, it is not necessary to know the specific fusion partner to perform break-apart FISH, an important factor considering that more fusion partners may be discovered in NSCLC and other solid tumors, such as *VCL-ALK* in sickle cell trait-associated renal medullary carcinoma [135, 136] and *C2orf44-ALK* in

colorectal cancer [84]. A standardized break-apart FISH protocol will therefore allow detection of *ALK* rearrangements beyond NSCLC.

The major challenge with the *ALK* FISH assay is that interpretation of the *ALK* break-apart FISH assay requires experience, patience, and technical expertise. For example, there is potential to misinterpret *ALK* amplification (copy number gain) as positive for *ALK* rearrangement due to the existence of multiple single red signals; however, this is not an indication for crizotinib use based on current evidence. The close involvement or supervision by an anatomic pathologist familiar with NSCLC morphology and FISH techniques will allow optimal FISH assessment.

The College of American Pathologists (CAP) has recommended that break-apart FISH analysis be performed by two independent observers and confirmed by a cytogeneticist or

Table 7. Mean percentage of FISH-positive cells in *ALK*-positive and *ALK*-negative non-small cell lung cancer

	University of Colorado Cancer Center (USA) [130]		National Cancer Center (Tokyo, Japan) [132]		Grenoble University Hospital (France) [131]	
	ALK+	ALK–	ALK+	ALK–	ALK+	ALK–
<i>n</i> of patients	90	56	15 ^a	30	19	58
Mean percentage of positive cells (range)	56 (18–100)	6 (3.5–9.5)	41 (17 ^b)	5 (3 ^b)	74.5	NR

^aPositive FISH signal was defined as >1 signal diameter apart.^bStandard deviation.

Abbreviations: FISH, fluorescence in situ hybridization; NR, not reported.

Table 8. Distribution of patterns of break-apart FISH-positivity in patients with *ALK*-rearranged non-small cell lung cancer

	University of Colorado Cancer Center (USA) [133]	Grenoble University Hospital (France) [131]	Seoul National University Bundang Hospital (South Korea) [134]
<i>n</i> of patients	90	21	19
Split signals pattern (%)	49 (54)	19 (90.5)	14 (73.7)
Single red (3' <i>ALK</i>) pattern (%)	33 (37)	2 (9.5)	5 (26.3)
Both split signal and single red pattern (%)	8 (9)	0	0

Abbreviation: FISH, fluorescence in situ hybridization.

Table 9. Correlation of FISH pattern positivity and percentage of *ALK*-positive cells (*n* = 90) [133]

Pattern of FISH positivity	Mean percentage of ALK+ cells (range)	<i>p</i> value
Overall positivity	56	
Split signals pattern	48 (18–82)	
Single red (3' <i>ALK</i>) pattern	74 (26–100)	<.0001

Abbreviation: FISH, fluorescence in situ hybridization.

pathologist with training in FISH [137]. The Internal System of Human Cytogenetic Nomenclature (ISCN) may also be recommended for use with all *ALK* break-apart reports (examples of ISCN reports are given in Table 10) [137]. The break-apart FISH assay is generally more expensive than some other assays, and so its primary role in the detection of *ALK* rearrangement will need to be carefully evaluated for both performance and cost-effectiveness against other techniques that are currently being introduced.

ALK FISH has been validated in pathologic specimens but not on cytologic specimens as part of the PROFILE 1005 trial, although the package insert of the Abbott *ALK* FISH Vysis test kit does not make this fine distinction. If the FISH test is to be performed on cell blocks from tumor cells spun down and collected from pleural, pericardial, or ascitic fluids and not from tumor cells, it is recommended to use cell blocks made of spun down tumor cells or to spread the specimens isolated from fine needle biopsy on glass slides to prevent cells from overlapping one another.

Dual-Color Break-apart Chromogenic In Situ Hybridization

Chromogenic in-situ hybridization (CISH) has been developed in recent years to simplify FISH as a detection assay for HER2 overexpression in breast cancer; it has shown high concordance with FISH in clinical studies [138]. Instead of using fluorescent probes as in FISH, CISH uses probes that can be detected by routine cytochemical techniques. The advantage of CISH is that it has been automated, can be assessed under light microscope (thus enabling simultaneous cytomorphologic examination), and the signals are stable in room temperature, meaning slides can be stored as permanent records.

Using the adapted standard criteria of FISH positivity, Kim et al. demonstrated 94% sensitivity (17/18) and a 100% specificity (425/425) for dual-color break-apart CISH compared with break-apart FISH [139]. Separately, Yoshida et al. also compared CISH with FISH using RT-PCR as the criterion standard. In 15 *ALK*-positive NSCLC and 30 *ALK*-negative NSCLC samples identified by RT-PCR, CISH and FISH were able to detect the same 14 *ALK*-positive samples and all of the 30 *ALK*-negative samples were FISH- and CISH-negative [132]. However, one of the criteria for FISH/CISH positivity in the study was separation of the probes by greater than only one signal diameter rather than the standard criterion of greater than two signal diameter separations. Based on these two studies, dual-color break-apart CISH has the potential to be an alternative to FISH as the diagnostic choice for *ALK*-rearrangement in NSCLC, but the criteria for positivity will likely have to be different from FISH and will need to be validated to reach a consensus.

Table 10. Examples of Internal System of Human Cytogenetic Nomenclature (ISCN) for <i>ALK</i> FISH results		
ISCN nomenclature	<i>ALK</i> rearrangement	Notes
nuc ish (<i>ALK</i> ×2) [50/50]	Negative	2 copies of normal <i>ALK</i> copies in all 50 cells examined
nuc ish (5' <i>ALK</i> , 3' <i>ALK</i>)×2 (5' <i>ALK</i> con 3' <i>ALK</i> ×2) [50/50]	Negative	2 copies of 5' and 3' <i>ALK</i> probes (fused) in all 50 cells examined
nuc ish (<i>ALK</i> ×2) (5' <i>ALK</i> sep 3' <i>ALK</i> ×1) [30/50]	Positive	2 copies of <i>ALK</i> , but 30 out of 50 cells show separation of the 5' <i>ALK</i> and 3' <i>ALK</i> probes, indicating the presence of <i>ALK</i> translocation
nuc ish (5' <i>ALK</i> ×1, 3' <i>ALK</i> ×2) [30/50]	Positive	30 out of 50 cells show isolated 3' <i>ALK</i> signals, indicating the presence of <i>ALK</i> translocation
nuc ish (5' <i>ALK</i> ×2, 3' <i>ALK</i> ×4)(5' <i>ALK</i> con 3' <i>ALK</i> ×2) [30/50]	Positive	30 out of 50 cells show increased 3' <i>ALK</i> signals, indicating the presence of <i>ALK</i> translocation
nuc ish (<i>ALK</i> ×2) (5' <i>ALK</i> sep 3' <i>ALK</i> ×1) [25/50]/(5' <i>ALK</i> ×1, 3' <i>ALK</i> ×2) [5/50]	Positive	25 out of 50 cells show separation of 5' and 3' <i>ALK</i> signals, with an additional 5 out of 50 cells showing isolated 3' <i>ALK</i> signals, indicating the presence of <i>ALK</i> translocation
nuc ish (5' <i>ALK</i> ×2, 3' <i>ALK</i> ×1) [30/50]	Negative	30 out of 50 cells show isolated 5' <i>ALK</i> signals
nuc ish (5' <i>ALK</i> , 3' <i>ALK</i>)×4~5 [30/50]	Negative	30 out of 50 cells show 4–5 copies of 5' <i>ALK</i> and 3' <i>ALK</i> probes, indicating <i>ALK</i> polysomy
nuc ish (<i>ALK</i> ×3) [30/50]	Negative	30 out of 50 cells show 3 copies of <i>ALK</i> signals, indicating <i>ALK</i> polysomy
nuc ish (<i>ALK</i> ×1) [30/50]	Negative	30 out of 50 cells show one copy of <i>ALK</i> signal, indicating monosomy of <i>ALK</i> signal
Abbreviations: ISCN, International System of Human Cytogenetic Nomenclature; nuc ish, interphase in situ hybridization; 5' <i>ALK</i> con 3' <i>ALK</i> , fusion of <i>ALK</i> probes; 5' <i>ALK</i> sep 3' <i>ALK</i> , separation of <i>ALK</i> probes.		

Reverse-Transcriptase Polymerase Chain Reaction

ALK rearrangement in NSCLC may also be detected by RT-PCR. RT-PCR is easy to perform, and the majority of current *ALK* fusion variants were detected by RT-PCR in fresh frozen tumor tissue [17, 19, 39, 63, 66, 71, 73–75]. However, RT-PCR requires *ALK* fusion variants to be known so that primers to all variants are included in the reaction. With an ever-expanding list of *ALK* fusion variants (Fig. 2A), primers for RT-PCR must be constantly updated to keep pace with published literature. Further, in daily clinical practice, most of the tumor tissue available for molecular profiling is from FFPE tissue, where the integrity of RNAs is likely to be greatly compromised compared with fresh/frozen tissue. Although FISH and immunohistochemistry (IHC) can be performed on a single FFPE slide, RT-PCR requires multiple slides in order to extract sufficient RNA for a successful reaction.

Li et al. demonstrated that RT-PCR can be successfully performed to detect the common *EML4-ALK* fusion variants from 4,750 archived FFPE NSCLC samples at a commercial diagnostic laboratory, but the false-negative rate is unknown [59]. Therefore, head-to-head comparison with FISH and/or IHC is still needed before RT-PCR can become a useful mass screening test. Thus, although RT-PCR remains a critical laboratory technique to investigate the various *ALK* fusion variants, to identify new fusion variants and to ultimately multiplex other markers, it remains to be optimized as a definite method to detect *ALK* rearrangement in NSCLC.

Immunohistochemistry

Although *ALK* FISH is currently considered the criterion standard for diagnosing *ALK*-positive NSCLC, *ALK* IHC holds promise as a rapid and affordable method that could be preferred for routine screening and diagnosis by pathology laboratories worldwide. Similar to *ALK* FISH, IHC requires one unstained slide cut from an FFPE block as long as there are at least a few clusters of viable tumor cells. IHC can be performed successfully on a variety of different tumor specimens, including FNA cell blocks. The major challenge for *ALK* IHC is the low level of *ALK* fusion protein expression in *ALK*-rearranged NSCLC (at least fivefold lower than *ALK* fusion protein expression in ALCL; Fig. 6A–D) [140]. It is most likely due to the weaker transcription activity of the *EML4* promoter compared with the transcriptional activity of the nucleophosmin (*NPM*) promoter in the generation of *NPM-ALK* protein in ALCL. There are three *ALK* antibodies (*ALK1*, *5A4*, and *D5F3*) that have been studied in depth in NSCLC (Table 11) [131, 134].

D5F3 is a promising rabbit monoclonal *ALK* antibody (developed by Cell Signaling Technology, Danvers, MA) for the detection of *ALK* rearrangement in NSCLC that has shown high sensitivity and specificity in a recent report [140]. However, the current impediments to *ALK* IHC being widely adopted in screening for *ALK* rearrangement include the lack of rigorous large-scale performance comparison with FISH and the lack of validation of clinical responses to crizotinib with *ALK* IHC. IHC is more likely to yield false-negative results, especially with low-*ALK*-expressing variants [59] or when specimens are small and/or poorly preserved. Unsuccessful FISH will be reported as

Figure 6. Comparison of immunohistochemistry (IHC) using anaplastic lymphoma kinase (ALK) 1 and 5A4 in anaplastic large cell lymphoma and *ALK*-rearranged non-small cell lung cancer. Both 5A4 IHCs were detected with Leica automation. Magnification: $\times 200$. Figure provided by Marie Mino-Kenudson.

Abbreviations: ALCL, anaplastic large cell lymphoma; NSCLC, non-small cell lung cancer.

Table 11. Immunohistochemistry versus fluorescent in situ hybridization				
	IHC 3+	IHC 2+	IHC 1+	IHC 0
Seoul National University Bundang Hospital ^a				
Test set				
Total	16	10	14	413
<i>ALK</i> FISH-positive	16	3	0	0
<i>ALK</i> FISH-negative	0	7	14	413
Validation set				
Total	6	6	2	173
<i>ALK</i> FISH-positive	6	3	0	0
<i>ALK</i> FISH-negative	0	3	2	173
Grenoble University Hospital ^b				
Total	20	0	2	59
<i>ALK</i> FISH-positive	19	0	2	0
<i>ALK</i> FISH-negative	1	0	0	59

^aImmunohistochemistry (5A4) versus *ALK* break-apart FISH study ($\kappa = 0.92$) [134].
^bImmunohistochemistry (5A4) versus *ALK* break-apart FISH study [131]. A total of 19 tumors for which FISH was uninterpretable were not included in the table.
Abbreviations: FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

“technical failure,” whereas unsuccessful IHC will be reported as a negative result.

If IHC methods (with or without amplification or scoring systems) with 5A4 and/or D5F3 are eventually proven to be accurate in detecting *ALK* rearrangements in larger-scale studies, IHC could become the primary screening modality for *ALK*-rearranged NSCLC, with only rare IHC-positive cases requiring confirmation with the FDA-approved *ALK* FISH test. In a research project, Sugawara et al. utilized 5A4 with an intercalated antibody-enhanced method and identified two *ALK* IHC-positive cases of non-clear cell renal cell carcinoma that were confirmed to harbor *ALK* rearrangements (*EML4-ALK*, *TPM3-ALK*) [81].

AFTER FDA APPROVAL OF CRIZOTINIB

The frontline PROFILE 1014 that compares crizotinib to platinum/pemetrexed is ongoing (Table 12). Recently, the second-line PROFILE 1007 study was reported as meeting its primary endpoint [100]. Both trials allow patients to receive crizotinib on disease progression if they are randomized to chemotherapy. Therefore, it is unlikely that significant improvement in OS with crizotinib will be shown in the first- or second-line setting based on these studies. Shaw et al. have performed a retrospective study and demonstrated that OS times in contemporaneously diagnosed patients with *ALK*-rearranged NSCLC who enrolled onto PROFILE 1001 were significantly im-

Table 12. List of major ongoing trials with crizotinib

Trial number	NCT number	Brief description	Primary endpoint
Phase I			
PROFILE 1001	00858195	Original crizotinib phase I trial, now accruing <i>ALK</i> -negative NSCLC (cMET amplified) and <i>ROS</i> -rearranged tumors	RR
A8081002	00965731	Combination of crizotinib and erlotinib	MTD
A8081006	01121575	Combination of crizotinib and dacomitinib (PF-299804)	MTD
COG-ADVL0912	00939770	Children's Oncology Group phase I study in pediatric population with relapsed/refractory solid tumors and ALCL	MTD
Phase II			
PROFILE 1005	00932451	Clinical efficacy and safety of crizotinib in patients with <i>ALK</i> -rearranged NSCLC who were previously treated for their advanced NSCLC	RR
PROFILE 1013	01121588	Clinical efficacy and safety of crizotinib in <i>ALK</i> -rearranged tumors except NSCLC	RR
CREATE (EORTC 91010)	01524926	Cross tumoral study exploring crizotinib in patients with advanced tumors induced by causal alterations of <i>ALK</i> and/or cMET	RR
Phase III			
PROFILE 1014	01154140	Randomized trial comparing platinum/pemetrexed versus crizotinib in treatment-naïve advanced/metastatic <i>ALK</i> -rearranged NSCLC	PFS
PROFILE 1007 ^a	00932893	Randomized trial comparing docetaxel or pemetrexed versus crizotinib as second-line treatment of advanced/metastatic <i>ALK</i> -rearranged NSCLC	PFS

^aClosed to accrual.
Abbreviations: ALCL, anaplastic large cell lymphoma; EORTC, European Organization for Research and Treatment of Cancer; MTD, maximum tolerated dose; NCT, National Clinical Trial; NSCLC, non-small cell lung cancer; PFS, progression-free survival; RR, response rate.

proved compared with patients who did not enroll (Tables 3,4) [95]. Although there are certain limitations to this analysis, such retrospective analyses are likely to be the only evidence that crizotinib confers survival advantage in patients with *ALK*-rearranged NSCLC in the near future.

SCREENING FOR *ALK*-REARRANGED NSCLC

The current draft version of the guidelines from CAP, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology recommends *ALK* testing in all patients with NSCLC exhibiting an adenocarcinoma component, regardless of age, ethnicity, sex, and smoking history [137]. The current National Comprehensive Cancer Network guidelines, developed by an expert opinion panel, recommend reflex testing of all adenocarcinoma, large cell, and NSCLC not otherwise specified for both *EGFR* mutations and *ALK* rearrangement and the use of crizotinib as a first-line treatment of *ALK*-rearranged NSCLC [141, 142]. Of the 24 *ALK*-rearranged patients who received crizotinib as a first-line treatment of their metastatic disease in PROFILE1001, 14 of 22 response-evaluable patients achieved an objective response

(ORR, 64% with 95% CI, 40%–83%). The median PFS was 18.3 months (95% CI, 8.3—not reached) [118].

The French National Cancer Institute recommends break-apart *ALK* FISH assay to screen all *EGFR/KRAS*-negative adenocarcinomas of the lung among the 28 French molecular genetics tumor laboratories [131]. Prior to the approval of crizotinib and the *ALK* companion diagnostic test in the USA and prior to the approval of crizotinib in Canada, a Canadian consensus report did not recommend routine screening for *ALK*-rearrangement in NSCLC with the argument that there was no approved treatment for *ALK*-rearranged NSCLC and no proven diagnostic test [143].

It is likely that individual countries will need to set up screening guidelines, especially in locations where crizotinib is approved. In regions (i.e., East Asia) where the incidence of activating *EGFR* mutations is high, sequential testing may be considered providing the overall turnaround time is within 14 days to allow the first-line use of crizotinib. However, double-positive mutant *EGFR/ALK* has been reported with increasing frequency from Asian tumor samples (Table 1), which could complicate formulation of a screening strategy.

RESISTANCE TO CRIZOTINIB

Resistance to RTK inhibitors can be divided into primary (intrinsic) or secondary (acquired). Accelerated in vitro mutagenesis has identified mutations in six amino acid residues in ALK (C1156, I1171, F1174, G1269, L1196, S1206) that confer resistance at the highest level of crizotinib tested [41]. Mutations at three of these amino acid residues (L1196 in the gatekeeper region, S1206 near the ribose binding pocket, and G1269 in the DFG motif) confer the greatest resistance to crizotinib in vitro [41]. To date, multiple secondary point mutations in *ALK* that confer acquired resistance to crizotinib have been identified in patients with crizotinib-treated NSCLC (L1196M, C1156Y, L1152R, G1269A, S1206Y, G1202R, I151Tins) [51, 71, 144–146] and inflammatory fibroblastic tumor (IMT; F1174L) [147] and they occurred in the same amino acid residues identified in the accelerated in vitro mutagenesis screen [41]. These secondary mutations accounted for approximately one-quarter of the resistance mechanisms. Furthermore, different *ALK* mutations can confer differential resistance according to the specific type of ALK inhibitor [145, 148].

Many “second-generation” ALK inhibitors are being developed with stronger ALK inhibitor properties than crizotinib, but also with the capability to inhibit to a varying degree these secondary acquired mutations, including the L1196M gatekeeper mutation [145, 149]. L1196M is considered a gatekeeper mutation similar to T351I, T670I, and T790M that developed in ABL, KIT, and EGFR, respectively [150]. L1196M is also a mutation that can be generated after exposing *ALK*-dependent NSCLC cell lines to increasing concentrations of crizotinib without the concomitant use of a mutagen [151]. Currently, at least four second-generation ALK inhibitors—AP26113 [41, 151], CH5424802 [152], AS3062 [145], and X396 [153]—have been reported to be able to inhibit the gatekeeper L1196M mutation. Other resistance mechanisms such as *ALK* gene copy number gain [71, 140], loss of the *ALK* fusion gene [71], and EGFR pathway activation [51, 140] including EGFR mutation [71], *KRAS* mutation [71], and *KIT* amplification [140] have so far been reported to confer resistance to crizotinib. Finally, in a substantial number of crizotinib resistance cases, the molecular mechanisms that underlie the resistance remain to be determined [71, 140].

Two heat shock protein (HSP) 90 inhibitors, retaspimycin (IPI-504) [154] and ganetespib (STA-9090) [155], have demonstrated clinical activity in patients with *ALK*-rearranged NSCLC. HSPs are a family of chaperone proteins that shepherd the aberrantly expressed EML4-*ALK* proteins to their subcellular location and substrates. In vitro, the addition of IPI-504 to an *EML4-ALK*-dependent cell line led to the rapid degradation of EML4-*ALK* [156], and this inhibition of EML4-*ALK* kinase activity occurs even in the presence of acquired crizotinib resistance [152, 156]. Therefore, HSP90 inhibitors alone or in combination with other therapy (ALK inhibitors, chemotherapy) have the potential to overcome acquired resistance to crizotinib independent of specific secondary *ALK* mutations and/or *ALK* fusion variants [78a]. Clinical trials testing this hypothesis have commenced.

In the PROFILE 1001 crizotinib study, 8 of 133 evaluable

patients with *ALK*-rearranged NSCLC did not experience tumor shrinkage with crizotinib and 5 patients had disease progression as a best response, indicating the potential existence of primary resistance to crizotinib [118]. EML4-*ALK* is postulated to lead to antiapoptosis by decreasing the level of proapoptotic BIM through activation of ERK and increasing the level of antiapoptotic survivin through the activation of STAT3 [42]. Indeed, the pretreatment BIM level has been shown to predict response to a wide array of RTK inhibitors, with high pretreatment BIM level associated with a better response to RTK inhibitors and improved clinical outcome [157]. Another potential primary mechanism is the existence of bypass pathways. It has been demonstrated in vitro that activation of the EGFR pathway can lead to crizotinib resistance [51, 146]. In the future, it may be important to measure BIM levels and to ascertain the presence of *EGFR* mutations in *ALK*-rearranged NSCLC prior to crizotinib treatment [51, 71].

CRIZOTINIB ACTIVITY IN OTHER *ALK*-DEPENDENT TUMORS AND *MET*-AMPLIFIED TUMORS

Crizotinib was initially developed as a *MET* inhibitor, and the molecular enriched cohort of PROFILE 1001 was initially designed to screen for *MET*-amplified tumors (e.g., gastric carcinoma, non-Barrett's gastroesophageal junction [GEJ] cancer) or *MET*-mutated tumors (e.g., squamous cell carcinoma of the head and neck, sporadic and hereditary papillary renal cell carcinoma). Other *MET*-dependent tumors, such as alveolar soft part sarcoma and alveolar rhabdomyosarcoma, were also eligible upon histologic confirmation.

The criteria for *MET* amplification in PROFILE 1001 followed the American Society of Clinical Oncology/CAP guidelines for definition of gene amplification (*MET/CEP7* copy number ratio >2.2). As it turned out, true *MET* amplification in GEJ is an extremely rare event (2%), with aggressive tumor behavior that severely limited patient enrollment into the crizotinib clinical trial. To date, only two patients with *MET*-amplified GEJ derived clinical benefit on crizotinib, with time to progression being 105 and 112 days, respectively [158]. Similarly, de novo *MET*-amplified NSCLC is rare; no obvious clinicopathologic characteristics for this molecular subset of NSCLC have been identified.

Recently, the U.S. Lung Cancer Mutation Consortium reported a rate of 4.4% with *MET/CEP7* cutoff at 2.2 and association with female sex [58]. To date, there is a report of one patient with a de novo highly *MET*-amplified NSCLC achieving confirmed partial response with crizotinib [159], with ongoing response for >19 months (S.-H.I. Ou, unpublished data). Additionally, one patient with *MET*-amplified glioblastoma achieved rapid radiographic response on crizotinib [160].

Promising antitumor activity has also been observed with crizotinib in patients with treatment-resistant *ALK*-positive ALCL [161]. The PROFILE 1013 study (NCT01121588) is enrolling patients with *ALK*-positive tumors other than NSCLC to better assess the activity of crizotinib in a wide range of malignancies. In addition, the European Organization for Research and Treatment of Cancer are in the process of initiating a study to explore crizotinib in patients with advanced

tumors induced by casual alternations of *ALK* and *MET* (Table 12).

Currently, the U.S. Children's Oncology Group is conducting a phase I study in pediatric patients with refractory solid tumors and ALCL. A recent report showed that seven of eight enrolled patients with ALCL experienced a complete response. Among seven patients with *ALK*-positive IMT, three achieved partial response, one achieved prolonged stable disease (>24 months), and the other three were too early in treatment to be assessed. Two pediatric patients with *ALK*-positive NSCLC were enrolled. One achieved partial response and one achieved stable disease. Among the eight patients with neuroblastoma and a known *ALK* mutation, one experienced a complete response and two achieved stable disease. Of 19 patients with *ALK*-unknown neuroblastoma, one experienced a complete response and six had prolonged stable disease [162].

***ROS1* REARRANGMENT IN NSCLC**

Rearrangements in *ROS1*, another human RTK, in NSCLC were discovered by Rikova et al. in 2007 with the same screening strategy for activated tyrosine kinases that identified *ALK*-rearranged NSCLC [19], with the initial incidence of *ROS*-rearranged NSCLC estimated to be <2% [163]. *ROS1* is one of 58 human receptor tyrosine kinase RTKs [164] and is evolutionarily related to *ALK* [165]; the mouse *ALK* and *ROS1* proteins share 52% amino acid homology within the kinase domain [20]. No biological ligand has been identified for *ROS1* in humans, and its normal physiologic function in human is not well defined. Male *ROS1* knockout mice are infertile due to disruption of the differentiation of the epididymal epithelium [166].

Rikova et al. identified two *ROS1* fusion variants in NSCLC, including *SLC34A2-ROS1* in HCC78 cell line. More recently, five additional fusion partners (*TPM3*, *SDC4*, *EZR*, *LRIG3*, *FIG*) to *ROS1* have been identified in NSCLC [56, 68, 166a], representing 12 *ROS1* fusion variants in NSCLC. More importantly, the identification of *SCL34A2-ROS1* in the HCC78 cell line gave the first indication that an *ALK* inhibitor may also serve as a *ROS1* inhibitor. The potential *ROS1* inhibitory potential by *ALK* inhibitors was discovered by McDermott et al., who attempted to identify potential response mechanisms to *ALK* inhibition by screening the activity of TAE684, an *ALK* inhibitor in 603 different cell lines derived from a variety of human tumors [40]. They identified 10 cell lines derived from NSCLC, ALC, and neuroblastoma that showed maximum inhibition by TAE684, including the HCC78 NSCLC cell line. No *ALK* abnormality or detectable *ALK* protein expression was found in HCC78 [40].

The discovery by Rikova et al. and the amino acid homology in the kinase domain between *ALK* and *ROS1* led to the hypothesis that *ALK* inhibitors could act as *ROS1* inhibitors. Consequently, *ROS1*-rearranged tumors based on a positive break-apart FISH assay were added as an additional eligibility criterion for molecularly enriched cohorts of PROFILE 1001 since May 2010. This modification provided the impetus to identify the clinicopathologic characteristics of patients with

ROS1-rearranged NSCLC in order to implement a screening strategy to identify such patients.

Using a "home-grown" break-apart FISH assay at Massachusetts General Hospital, Bergeth et al. [167] screened 1,073 NSCLC tumor samples and identified 18 (1.7%) with *ROS1*-rearranged NSCLC and 31 (2.9%) with *ALK*-rearranged NSCLC, consistent with the previously reported low incidence of *ROS1*-rearranged NSCLC [163]. Perhaps not surprisingly, the clinicopathologic characteristics of *ROS1*-rearranged NSCLC are very similar to *ALK*-rearranged NSCLC: patients are young (a similar median age of diagnosis of approximately 50 years of age) never-smokers with adenocarcinoma [167]. These findings were independently demonstrated by Takeuchi et al. [68].

It seems that there is no difference in the prevalence of *ROS1*-rearranged NSCLC between Asian and non-Asian patients, with *ROS1*-rearranged NSCLC found in approximately 1% of East Asian patients with adenocarcinoma [56, 68, 168]. Among the first 15 patients (14 responses evaluable) enrolled in the *ROS1* NSCLC cohort of A8081001, an ORR of 57% and disease control rate of 79% at 8 weeks suggest that crizotinib has marked antitumor activities in *ROS1*-rearranged NSCLC [169], similar to the activity of crizotinib initially reported for *ALK*-rearranged NSCLC [112]. Taken together, these data suggest that *ROS1* rearrangement defines another unique molecularly defined subtype of lung cancer with heterogeneity, as well as a validated "driver mutation" and therapeutic target.

Whether the activity of crizotinib will be able to be extended to other tumors with *ROS1* rearrangement remains to be determined [170, 171]. This will serve as a proof of principle demonstration that *ROS1* rearrangement also function as a driver mutation in those tumors. In addition, optimized testing methods such as multiplexing are needed.

SUMMARY

In 2011, we marked the beginning of the second decade of targeted therapy in oncology. The development and approval of crizotinib indicates that breathtaking advances are in store for the next 10 years. Already, novel chromosomal translocations involving receptor tyrosine kinases are now being discovered in other major epithelial malignancies [68, 81, 83–84, 135–136]. Although it took almost 20 years from the identification of the translocated *ABL* kinase in the Philadelphia chromosome in chronic myeloid leukemia [172] to the initial approval of imatinib as an *ABL* inhibitor in 2001 [173], it took only 4 years from the discovery of *ALK* rearrangement in NSCLC to the approval of crizotinib for the treatment of advanced *ALK*-positive NSCLC. The development of crizotinib put into focus several previously rare and relatively unknown molecular subsets of malignancy, such as *ALK*-rearranged NSCLC and IMT, in a similar way to the role of imatinib in defining gastrointestinal stromal tumor as a unique molecular subset of tumor. Similar to imatinib, crizotinib is also a multitargeted kinase inhibitor, and its emerging role as a *MET* and *ROS1* inhibitor will likely spotlight further unique molecular tumor subsets underpinned by these driver mutations. The realization of the

increasing incidence of chromosomal aberrations in solid tumors was actually predicted by Mitelman et al. [174].

Going forward, the emerging complexities of resistance mechanisms found in crizotinib-treated *ALK*-rearranged NSCLC will create a daunting task [71, 145–146]. To complicate the task further, progression on crizotinib occurred in diverse clinical scenarios, such as development of new metastasis, progression of pre-existing lesions, or both. Therefore, the importance of rebiopsying after treatment progression cannot be overemphasized [71, 144, 175]. Rebiopsying on treatment progression will likely be a major paradigm switch in solid tumor oncology as we go forward in the second decade of personalized therapy. Although rebiopsying in solid tumor malignancies will not be easy compared to repeated blood draws in chronic myeloid leukemia, it is only then that rational combination with targeted therapies can be implemented to delay or overcome resistance in order to prolong survival.

The approval of crizotinib was dependent on the simultaneous approval of a validated diagnostic assay. In contrast, when imatinib was conditionally approved for CD117-positive gastrointestinal stromal tumor in 2001, one of the post approval commitments was to develop a CD117 IHC assay in order to identify patients for imatinib treatment [176]. The codevelopment of drug with a companion diagnostic assay has spurred rapid development in the area of diagnostic assays for chromosomal translocation in solid tumors; it engenders an on-

going healthy and vigorous debate as to the most sensitive, specific, and cost-effective assay for the screening of chromosomal aberrations. With the advent of the second decade of molecular targeted therapy, the continual development of crizotinib points to more molecular subsets of solid tumors being discovered and defined, the increasing importance of repeat tissue acquisition and analysis during the clinical management of solid tumors, rapid advances in the diagnostic area of oncology, expanded translational research in oncology in the future, and the need for close collaboration between clinical oncologists, pathologists, and translational scientists.

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