The treatment of advanced non-small-cell lung cancer with anaplastic lymphoma kinase genetic alterations: reality and hopes

Targeted therapies based on molecular diagnostics have opened a new era of personalized medicine in lung cancer treatment. Recently, anaplastic lymphoma kinase (ALK) fusion gene emerged as an important biomarker for identifying a small proportion of non-small-cell lung cancer (NSCLC) patients that will benefit from ALK kinase inhibitor crizotinib, like EGFR activating mutations and EGFR tyrosine kinase inhibitors. Despite the remarkable initial responses, acquired resistance to crizotinib develops within a year and promising second generation of ALK inhibitors are in current development to overcome it. This review will focus on the basic molecular pathology of ALK gene rearrangement in NSCLC, current testing methods, treatment strategies against ALK-positive and crizotinib-resistant NSCLCs.

Keywords: alectinib • anaplastic lymphoma kinase • ceritinib • crizotinib • Hsp90 inhibitors • mechanisms of resistance • non-small cell lung cancer • ROS1 • targeted therapy

Despite promising new agents and therapeutic approaches, 5-year lung cancer survival rates have lagged far behind those of most other malignancies (6.1% for small cell lung cancer [SCLC] and 17.1% for non-small-cell lung cancer [NSCLC]) [1]. In the past decade, the advent of targeted therapy led to a silent revolution in the war against lung cancer, leading to a survival improvement in selected population screened for specific biomarkers. For example, the identification of somatic mutations (short in-frame deletions of exon 19 and point mutation in exon 21, L858R) in the EGFR gene in a subset of patients with NSCLC led to the treatment of these patients with EGFR tyrosine kinase inhibitors (TKIs), such as gefitinib, erlotinib or afatinib. These mutations occur more frequently in East Asians, in women, never or light smokers, and in adenocarcinoma histology. Several randomized Phase III clinical trials, addressed to patients selected by clinical or molecular features, showed a higher progression-free survival (PFS) and overall response rate (ORR) and better quality of life of EGFR-TKIs compared with platinum-based chemotherapy [2]. Recently, a new molecular target was discovered in NSCLC cells, comprising a fusion gene between echinoderm microtubule-associated protein like 4 (EML4) and anaplastic lymphoma kinase (ALK). ALK fusion gene emerged as an important biomarker for identifying a small proportion of NSCLC patients that will benefit from ALK inhibitors: the accurate and timely identification of these patients will have important therapeutic implications. In this review, we will examine the basic molecular pathology of ALK gene rearrangement in NSCLC, current testing methods and treatment strategies directed at ALK-rearranged and crizotinib-resistant NSCLCs.

ALK rearrangements in NSCLC: from molecular pathology to diagnostic tools

ALK gene is found at chromosome 2 and encodes a classical insulin superfamily tyrosine kinase. The mature ALK-protein undergoes post-translational N-linked glycosylation and comprises an extracellular ligand-binding domain, a transmembrane
domain and a single intracellular tyrosine kinase domain. Normally, ALK protein is expressed only in the CNS, small intestine and testis and is activated by dimerization with subsequent trans-autophosphorylation of three tyrosine moieties [3]. Transforming rearrangements of the ALK gene have been reported in human hematologic and solid tumors including anaplastic large-cell lymphoma, myofibroblastic tumors as well as NSCLC, suggesting that ALK-mediated signaling might play a role in the development or progression of these tumors [4,5]. In 2007, Soda and colleagues identified the EML4-ALK fusion gene in a Japanese NSCLC patient, resulting from an inversion in the short arm of chromosome 2, fusing the N-terminal domain of EML4 to the intracellular kinase domain of ALK (3′ gene region) [6]. This translocation causes aberrant activation of downstream oncogenic signaling pathways (such as the RAS/RAF/MEK, PI3K/Akt/mTOR and the Janus kinase/signal transducer) and transcription signaling pathway, leading to cell proliferation, invasion and inhibition of apoptosis [7]. Multiple variants of EML4-ALK have since been reported, encoding the same intracellular tyrosine kinase domain of ALK, but different truncations of EML4.

The most common variants were variant 1 (detected in 33% of cases) which leads to the juxtaposition of exon 13 of EML4 to exon 20 of ALK (E13;A20) and variant 3a/b (29% of cases) in which exon 6 of EML4 was joined to exon 20 of ALK (E6a/b;A20) [8]. In addition to EML4-ALK, other ALK fusions have also been reported in lung cancer, including TFG-ALK, KIF5B-ALK and KLC1-ALK [9–11]. EML4-ALK translocation is found approximately in 2–5% of all cases of NSCLC. The ALK fusion genes appear to be more common in younger patients, never or light smokers and in adenocarcinoma with solid pattern and signet-ring cells [12–14].

ALK translocations typically occur independently of EGFR or KRAS mutations, although they are not mutually exclusive, as confirmed by two recent biomolecular studies, the French and the Lung Cancer Mutation Consortium [15,16].

On the other hand, ALK rearrangements were identified as a poor predictive marker for the EGFR TKI response [14,17–19]. About the frequency and impact of the concomitant presence of EML4-ALK rearrangement and EGFR mutation, disappointing data emerged from the Phase III EURTAC, that randomized 173 EGFR-mutant NSCLC patients to receive erlotinib or standard chemotherapy (cisplatin or carboplatin plus docetaxel or gemcitabine). Notably, the EML4-ALK rearrangement was concomitant with EGFR mutations in a considerable number of NSCLC patients (15.8%), with any negative impact on PFS (primary endpoint), with erlotinib scoring statistically better than chemotherapy [20,21]. This study highlights that EGFR-mutated patients and previously responders to EGFR-TKIs should not be excluded from ALK screening and further research on other genetic factors are required.

According to evidence-based guidelines developed by the College of American Pathologists, International Association for the Study of Lung Cancer and Association for Molecular Pathology, the test for ALK rearrangements should be performed in all patients with advanced-stage adenocarcinoma, regardless of sex, race, smoking history or other clinical risk factors, in order to guide patient selection for therapy with an ALK inhibitor [22]. ALK gene rearrangements or the resulting fusion proteins may be detected in tumor specimens using three different testing methods: fluorescence in situ hybridization (FISH), immunohistochemistry (IHC) and reverse transcription polymerase chain reaction of cDNA (RT-PCR).

FISH analysis is considered the gold standard for diagnosing ALK-positive NSCLC. The commercial break-apart probes (approved by Federal US FDA in 2011 for molecular diagnostic testing) include two differently colored (red and green) probes that flank the highly conserved translocation breakpoint within ALK. In nonrearranged cells, the overlying red and green probes result in a yellow (fused) signal; in the setting of an ALK rearrangement, these probes are separated, and splitting of the red and green signals is observed [22]. By definition of this test, observation of more than 15% split nuclei is the indicative cut-off of an ALK rearrangement [23]. This testing method for EML4-ALK translocations allows to detect all ALK rearrangements regardless of the fusion partner and is accurate and reliable. On the other hand, FISH has a high cost, its accurate interpretation requires expertise and experience, it does not identify specific translocation types and often has a lengthy turn-around.

Multiple monoclonal antibodies have been developed to use for the IHC detection of the ALK fusion oncogene. Several studies have shown that the Ventana IHC has a high sensitivity and specificity (> 98%), as well as good concordance with FISH. In addition to its high coherence with FISH, Ventana IHC is quicker, less expensive, easier to implement and interpretation by general pathologies and has a good repeatability [24–27]. Thus, IHC can potentially be used to screen and identify the presence of ALK positivity. In the United States, FISH is the only approved test to diagnose ALK-positive NSCLC, and thus FISH should be used to confirm the IHC results. In contrast, IHC has been approved by the European Medical Agency as an aid in identifying patients who are eligible for treatment with crizotinib. Future challenges in developing...
ALK-inhibitor-specific therapies for NSCLC

**Crizotinib: from early development to approval in clinical practice**

After identification of the EML4-ALK fusion in NSCLC, a search for effective inhibitors with clinical applications began. The first clinically useful inhibitor PF-2341066 (crizotinib), is now in widespread use for ALK-positive or MET-positive NSCLC.

Crizotinib is a first-in-class, oral, potent and selective small-molecule competitive inhibitor of ALK with additional MET, ROS1 and RON kinase inhibitory activity. This compound induces a G1-S phase cell cycle checkpoint and apoptosis in ALK-positive anaplastic large-cell lymphoma cells, but not ALK-negative lymphoma cells; in addition, it inhibited MET phosphorylation and MET-dependent proliferation, migration or invasion of human tumor cells *in vitro* [29,30].

In the first-in-man dose-escalation Phase I study, begun in May, 2006, 37 patients with advanced stage tumors including colorectal, pancreatic, sarcoma, anaplastic large-cell lymphoma and NSCLCs, were enrolled: crizotinib was administered on a continuous schedule to patients in successive dose-escalating cohorts, at doses ranging from 50 mg once daily (q.d.) to 300 mg twice daily (b.i.d.). Dose-limiting toxicities included grade 3 increased alanine aminotransferase and grade 3 fatigue. The most common mild (grade 1 or 2) side effects were nausea, emesis, fatigue and diarrhea, reversed with drug cessation [31]. Based on these results, 250 mg b.i.d. in 28-day cycle was established as the recommended Phase II study dose.

This part of the study was followed by protocol-defined patient prescreening for evidence of ALK or MET activation in specific tumor types. Patients with ALK-positive or MET-positive tumors were enrolled into a series of molecularly defined expansion cohorts at the proposed recommended Phase II dose.

After the discovery of ALK gene rearrangements in NSCLC and promising results in two patients with ALK-positive NSCLC enrolled during the dose-escalation phase of the study [31,32], the protocol was amended and an additional ALK-positive NSCLC expanded cohort was instigated in 2008. Data from the first 19 evaluable patients with ALK-positive NSCLC within the cohort revealed a high proportion of objective responses (53%) [31]. Subsequent data from the first 82 patients confirmed these findings, with an ORR of 57% (with 46 confirmed partial responses [PR], and one confirmed complete response [CR]) and a 33% of stable disease (SD) [32]. In 2012, an updated analysis of 149 ALK-positive patients, prevalently never smokers, with adenocarcinoma histology and a median age of 52 years, confirmed the efficacy of crizotinib, with a tumor shrinkage in over 90% of patients and with an ORR of 60.8%, including three CR and 84 PR. Time to response was rapid (median time to first documented OR was 7.9 weeks), durable (median duration of response was 49.1 weeks), independent of age, sex, performance status (PS) or line of treatment. Disease control (i.e., CR, PR and SD) was achieved by 118 patients (82.5%) at week 8 and 101 patients (70.6%) at week 16. Although no differences were reported regarding ORR according to the line of treatment, better median PFS was documented in the treatment-naïve subgroup (16% of patients) than in the subgroup with crizotinib as second-line or later treatment (18.3 vs 9.2 months, respectively). Median PFS of the entire group was 10 months, with an estimated 1-year OS of 75% [33]. Also in this larger group of patients, adverse events (AEs) were mainly grade 1 and 2 and reversible with drug interruption: visual effects (visual impairment, photopsia, blurred vision, vitreous floaters, photophobia and diplopia; median time to onset 14.5 days), gastrointestinal events (nausea, diarrhea, vomiting and constipation; generally early, 2–5 days), and peripheral edema (late-onset cumulative AE, with a median time to onset of 85 days).
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most common treatment-related grade 3 or 4 AEs were neutropenia, elevated alanine aminotransferase, hypophosphatemia and lymphopenia [33]. A rapid-onset hypogonadism and lower total serum testosterone levels have been recently reported in male patients treated with crizotinib [34]. Mean total testosterone levels were found lower in crizotinib-treated than crizotinib-naïve patients. The symptoms due to androgen deficiency (fatigue, depression, sexual dysfunction) could be improved with testosterone supplementation. The mechanism is unknown, but it is interesting to note that MET and ALK are both expressed in testes [35].

Concerning survival, median OS was not reached [33]; thus a retrospective analysis compared OS in three different groups of patients: ALK-positive patients treated with crizotinib (crizotinib group), ALK-positive crizotinib naïve patients (ALK-positive controls) and EGFR wild-type patients without ALK rearrangement (ALK-negative controls). Among 82 patients of crizotinib group, median OS has not been reached, with 1-year OS of 74%, and 2-year OS of 54%, independent of age, sex, smoking history or ethnic origin. Survival in 30 ALK-positive patients who were given crizotinib in the second-line or third-line setting was significantly longer than in 23 ALK-positive controls given any second-line therapy (median OS not reached vs 6 months; 1-year OS of 70 vs 44%, and 2-year OS of 55 vs 12%; hazard ratio [HR]: 0.36, 95% CI: 0.17–0.75; p = 0.004). Survival in 56 ALK-positive crizotinib-treated patients was similar to that in 63 ALK-negative, EGFR-positive patients given EGFR TKI therapy (median OS not reached vs 24 months, 1-year OS of 71% vs 74% and 2-year OS of 57% vs 52%; p = 0.786). Finally, 36 crizotinib-naïve, ALK-positive controls reported a similar median OS to 253 wild-type controls, lacking EGFR or ALK alterations (median OS of 20 and 15 months, respectively; p = 0.244) [36]. However, this survival analysis has several limitations, including that it was a retrospective, nonrandomized study with unmatched and potentially unbalanced study populations. Survival differences between treated patients and historical or historical-like controls can be difficult to interpret because of confounding by differences in patient selection, in standard and supportive care treatments. These differences would be minimized in a randomized controlled study [36].

Based on promising Phase I data, several Phase II–III trials have been performed. PROFILE 1005, an open-label, single-arm Phase II study, has evaluated the efficacy and safety of crizotinib in pretreated patients (failed more than two lines of chemotherapy) with advanced NSCLC harboring translocation or inversion involving the ALK gene locus detected by FISH.

As of January 2012, 901 patients received crizotinib, but the first 261 patients were considered to be the mature population. Crizotinib demonstrated a high response rate (60%), durable (median 46 weeks), with a disease control rate at 6 weeks of 86% and at 12 weeks of 75%, with a median PFS of 8.1 months. Crizotinib has a favorable tolerability: among all 901 patients, 15% discontinued treatment due to AEs and 10% had a dose reduction due to an AE. Vision disorder (54%), nausea (51%), diabetes (44%), vomiting (44%) and constipation (37%) were the most frequent AEs, generally grade 1–2 [37].

In a later prospective, randomized, Phase III study, crizotinib treatment (250 mg b.i.d.) was compared with standard second-line chemotherapy (docetaxel and pemetrexed, every 3 weeks) in 347 advanced ALK-positive NSCLC, previously treated with platinum-based regimen [38]. The median PFS (primary endpoint) was significantly longer for the crizotinib group than pemetrexed or docetaxel-based treatment group (PFS: 7.7 vs 3 months, HR: 0.49; 95% CI: 0.37–0.64; p < 0.001). Also the response rates were impressively higher in these second-line setting patients: 65% in the crizotinib group versus 19.5% in the chemotherapy group. The patients receiving crizotinib reported a greater quality of life improvement (time to deterioration in lung cancer symptoms significantly longer with crizotinib than chemotherapy: 5.6 vs 1.4 months, respectively; HR: 0.54, 95% CI: 0.40–0.71; p < 0.0001) and reduction in lung cancer symptoms compared with the chemotherapy group [38].

The efficacy of second-line docetaxel in patients with ALK-positive NSCLC was modest (ORR: 7%), consistent with previous studies involving the general population of NSCLC [39,40]. In contrast, the response rate to pemetrexed was higher than expected (ORR: 29%), as compared with 12.8% reported in the general population of patients with lung adenocarcinoma pretreated with chemotherapy [39,41], though the median PFS among pemetrexed group was only 4.2 months. Thus, patients with ALK-positive NSCLC may have a higher response rate with pemetrexed than does the general population, although the benefit of pemetrexed was less than that originally suggested in retrospective studies [42,43] and, importantly, less than that of crizotinib, as shown in this randomized trial. In a prespecified interim analysis, as expected, OS was shown to be similar in the crizotinib and chemotherapy groups (median OS: 20.3 vs 22.8 months, respectively; HR: 1.02, 95% CI: 0.68–1.54; p = 0.54). This analysis was immature, and it is likely that it was confounded by the high crossover rate among patients in the chemotherapy group.

Both crizotinib and chemotherapy were associated with toxic effects that were primarily grade 1 or 2: visual
disorder, gastrointestinal side effect, elevated liver aminotransferase levels, edema, upper respiratory infection, dysgeusia and dizziness for crizotinib, fatigue, alopecia, dyspnea and rash for chemotherapy group. Two serious toxic effects crizotinib-related were elevated aminotransferase levels (16%) and interstitial lung disease (2%, two of three cases were fatal) [38].

Other interesting data emerging from PROFILE 1007 were the relationship between ALK positivity and the sensitivity to pemetrexed treatment. Of note, these results should be interpreted with caution because patients were not randomized to a chemotherapy regimen (left to investigator choice), and prior pemetrexed might have confounded the results. This correlation might be explained with the lower concentration of thymidylate synthase (TS), the main target of pemetrexed, in ALK-positive tumors [44,45]. Disappointing results emerged from a larger retrospective analysis, which reported any statistical advantages for ALK-positive patients treated with pemetrexed-based therapy [46].

After a rapid clinical development period, in August, 2011, Crizotinib was approved by FDA as the first NSCLC personalized therapy in which treatment is determined by clinically validated ALK testing [47]. Subsequently, the EMA-approved crizotinib following further analysis of randomized data in July 2012 [48].

Finally, at 2014 ASCO Annual Meeting, important data on efficacy and safety of crizotinib, as first-line treatment, in 343 ALK-positive nonsquamous NSCLC patients have been presented. This multicenter, open-label Phase III study (PROFILE 1014) randomized 1:1 patients to receive crizotinib (250 mg b.i.d.) or pemetrexed–platinum chemotherapy, allowing the continuation of/cross-over to crizotinib after PD [49]. The study met its primary objective and demonstrated the superiority of crizotinib over chemotherapy in prolonging PFS (median 10.9 vs 7.0 months; HR: 0.454; 95% CI: 0.346–0.596; p < 0.0001). The ORR was significantly higher with crizotinib (74 vs 45%; p < 0.0001). With 68% of patients still in follow-up, a statistically significant improvement in OS was not demonstrated (HR: 0.821; 95% CI: 0.536–1.255; p = 0.1804). At the time of data cut-off (July 2013), 109 patients treated with chemotherapy had crossed over to crizotinib. Toxicity profile was consistent with previously reported, confirming vision disorder and gastrointestinal symptoms as the most common all-causality AEs [49].

Beyond crizotinib: from mechanisms of resistance to second generation of ALK inhibitors

Despite the remarkable initial sensitivity, the long-term effectiveness of crizotinib is universally limited by the development of resistance, generally within 1 year. These mechanisms of acquired resistance in oncogene-driven malignancies are broadly divided into two categories. The first involves the development of additional genetic alterations in the primary oncogene, which facilitates continued downstream signaling. This commonly arises through secondary mutations in the kinase target or through gene amplification of the kinase itself (called ALK-dominant group). Alternatively, resistance can develop independent of genetic changes in the target. This occurs through activation of downstream signaling pathways, changes in tumor histology or alterations in drug metabolism (called ALK nondominant) [50–52]. Concerning ALK-dominant mechanisms, seven different secondary mutations in the ALK kinase domain have been identified in approximately 30% of ALK-positive patients with crizotinib resistance: L1196M, C1156Y, 1151Tins, L1152R, G1202R, S1206Y and G1269A. The L1196M substitution is notable because it involves the ALK gatekeeper residue, analogous to T790M in EGFR, and likely causes resistance by steric interference with crizotinib binding. ALK gene amplification with or without concurrent ALK mutation is another cause of drug resistance in about 7–18% of patients [50–52]. In ALK nondominant group, resistance can also develop through reactivation of downstream signaling pathways via bypass tracts, including emergence of alternate of EGFR of KRAS mutations, and other not yet clear (probably related to KIT, EGFR or HER-2 variants) [50–52].

In order to overcome the resistance, it is important to differentiate patients that preserve ALK dominance (secondary mutations and amplifications) versus those that have diminished ALK dominance (separate or second oncogenic drivers). In the first case, a second-generation ALK inhibitors may represent a promising treatment approach.

Among these, ceritinib (LDK378) is an orally novel ALK inhibitor more potent than crizotinib in enzymatic and cell-based assay and crizotinib-resistant animal models. In a dose-escalation Phase I study single arm, the maximum tolerating dose (MTD), safety, pharmacokinetics and preliminary antitumor activity of LDK378 have been investigated in ALK-positive solid tumors of any type. After MTD determination (750 mg once daily in 39 patients) [53], 255 patients from 11 countries were enrolled to expansion groups: ALK inhibitor (ALKi) pretreated NSCLC (163 patients), ALKi naïve NSCLC (83 patients) and non-NSCLC disease (9 patients) [54]. Out of 246 ALK-positive NSCLC patients, 67.5% had received ≥2 anticancer therapies: ORR was ≥60% in each subgroup of patients with 0, 1, 2 and 3 prior anticancer regimens. Among 163 ALKi-pretreated patients, 91% had progressive disease during prior ALK therapy (≤2 weeks from last dose) and 77% had received
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Alectinib (CH5424802) was a selective ALK inhibitor, tenfold more potent than crizotinib, and effective against most of the mutations of the ALK domain. In a multicenter, single-arm, open-label Phase I/II study, 70 ALK-positive NSCLC Japanese patients received alectinib orally two-times a day by dose escalation. In the Phase I study, 24 patients were treated with 20–300 mg twice daily. Even with a dose of 300 mg twice a day, any dose-limiting toxicity (DLT) was reported, and so it was recommended for the treatment of 46 patients in the Phase II of this study. Alectinib reported an ORR of 93.5% (43/46), including 2 CR and 41 PR. Grade 3 treatment-related AEs were reported in 12 (26%) patients, serious side effects occurred in 5 patients (11%), including decreased neutrophils and increased blood creatine phosphokinase. Median PFS has not been reached yet, since 40 of the 46 patients in the Phase II portion remain on treatment.

Alectinib showed a significant clinical activity also in 47 ALK-positive NSCLC patients who are refractory to crizotinib. In this Phase I trial, alectinib was well tolerated, with any treatment-related dose reductions up to 600 mg two-times a day, but two DLTs (grade 3 headache and neutropenia) were reported in the 900 mg two-times a day (highest tested dose). Alectinib at oral dose of 600 mg two-times a day was chosen as recommended Phase II dose. Most common AEs (≥15%) were fatigue, myalgia, peripheral edema, blood CPK increased and nausea; grade 3–4 AEs included γ-glutamyltranspeptidase increased (4%), neutropenia (4%), hyphosphatemia (4%), hyperglycemia, syncope, acute renal failure and pericardial effusion (2% each). In terms of activity, ORR was 54.5% across all cohorts (all PR), with a median duration on treatment greater than 4 months. Preliminary clinical data indicated that alectinib was active against CNS metastases. Lipid-soluble solutes can freely diffuse through the capillary endothelial membrane and may passively cross the blood–brain barrier. However, this barrier is reinforced by a high concentration of Pgp, drug-efflux-transporter protein. While crizotinib is a good P-gp substrate, alectinib is not. Out of 21 enrolled patients with baseline CNS lesions, nine had a measurable lesion, and none of these received prior radiation within 4 weeks from the first dose of alectinib: five out of nine achieved a CNS PR (≥30% reduction in sum of largest dimension), two out of nine had CNS SD and two out of nine had CNS progression. So, Phase II–III studies of alectinib in ALK rearranged NSCLC patients have been activated (Table 1).

AP26113 is a novel, orally active TKI that potently inhibits mutant-activated forms of ALK positive and EGF R mutated (not native), and TKI-resistant forms including L1196M (ALK) and T790M (EGFR). A Phase I/II single arm, multicenter study enrolled 114 patients with advanced solid tumors, including 106 NSCLC: 65 patients in the dose-escalation Phase I (30–300 mg q.d.) and 49 patients in Phase II (180 mg q.d.) [59,60]. The most common AEs (≥20%) were nausea (38%), diarrhea (31%), fatigue (31%), cough (23%) and headache (20%), which were generally grade 1/2 in severity. Early onset of pulmonary symptoms (dyspnea with hypoxia and/or findings on imaging) was observed in 13% (6/45) at 180 mg once a day, but not at 90 mg once a day or in the lead-in dose cohort (initiated at 90 mg q.d., escalated to 180 mg q.d.)
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after 1 week). The expansion Phase II trial is assessing the antitumor activity of AP26113 in four different cohorts of patients: crizotinib-naïve or -resistant ALK-positive NSCLC (including patients with active brain metastases); *EGFR* T790M mutated and *EGFR* TKI-resistant NSCLC; other tumors with abnormalities in ALK or other AP26113 targets. Preliminary data reported a promising antitumor activity among 38 evaluable patients with crizotinib-resistant ALK-positive NSCLC (not confirmed ORR: 63%, with 23 PR and 1 CR), including patients with brain metastases (6/10 patients showed a brain response). Duration of response was 1.6–14.7 months (ongoing). Among 42 evaluable patients with ALK-positive NSCLC, median PFS was 47 weeks [59,60]. A randomized Phase II trial (NCT02094573) evaluating 90 mg once a day versus 90 mg once a day escalating to 180 mg once a day in crizotinib-resistant ALK+ NSCLC is ongoing (Table 1). Finally, X-396 is a novel, potent ALK-TKI with significant antitumor activity in both ALK TKI-naïve and crizotinib-resistant models of ALK fusion-positive NSCLC. In this multicenter dose-escalation Phase I study, 30 patients with advanced solid tumors (21 NSCLC patients, 13 ALK positive: 3 crizotinib naïve and 10 crizotinib resistant) received X-396 at doses 25–250 mg on a continuous 28-day schedule. X-396 is generally well tolerated at doses up to 250 mg daily. The most common AEs included rash (36%, G1-G3), fatigue (30%, G1-CR), nausea (27%, G1), vomiting (27%, G1) and edema (20%, G1-G2); grade 3–4 AEs were rash (two patients) and edema (one patient). To date, 18 patients are evaluable for response; SD is 28% and PR 28%. Notably, among eight evaluable ALK+ NSCLC cases, X-396 induced responses in both crizotinib-naïve and crizotinib-resistant (Table 1) [61].

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**Table 1. Second-generation anaplastic lymphoma kinase inhibitors: ongoing clinical trials.**

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Heat-shock protein 90 inhibitors

Heat-shock protein 90 inhibitors (Hsp90) may provide another targeted treatment option beyond direct ALK inhibition. Heat shock proteins function as part of normal cellular stress responses, preserving cells from lethal damage and their increased expression in cancers contributes to tumor growth, metastasis and a worse prognosis. HSP90 inhibitors bind in the ATP-binding pocket of the enzyme, and prevent it from regulating the activation and stability of its client proteins, including ALK. Thus, inhibition of HSP90 resulted in reduction of the expression of EML4-ALK through proteasome-mediated degradation [62]. However, only retrospective analyses in ALK-positive NSCLC are reported in the literature, with promising results [63–65]. To date, these clinical trials have tested Hsp90 inhibitors (ganetespib, IPI-504 and AUY922) in NSCLC patients with heterogeneous molecular subtypes, assessing common genetic aberrations, such as KRAS and EGFR mutations as well as ALK rearrangements. In the subset analysis of ALK-positive NSCLC patients (most of whom were crizotinib-naïve), Hsp90 inhibitors have shown promising results.

In a recent Phase II trial of ganetespib (STA-9090) monotherapy (at a dose of 200 mg/m2 weekly for 3 weeks with 1-week rest) conducted in patients with advanced NSCLC, eight patients (8%) were identified as harboring ALK gene rearrangements, all crizotinib naïve. Out of these patients, four achieved an objective PR, three showed SD and one experienced PD at 16 weeks. The median PFS observed in four patients with PR was 8.1 months, significantly better than for patients without ALK rearrangement [63]. In addition, the responses were durable, lasting an average of approximately 1 year. Finally, seven of eight patients (88%) experienced disease control. Based on earlier results, the CHIARA trial (CHaperone Inhibition in ALK Rearranged lung CAncer) was initiated to evaluate ganetespib monotherapy in up to 110 patients with advanced ALK rearranged NSCLC and who have not been previously treated with a direct ALK inhibitor (i.e., crizotinib, NCT01562015). A Phase I/II study is evaluating the combination of crizotinib and ganetespib in previously treated ALK-positive NSCLC patients not pretreated with any specific inhibitor, with the primary endpoint to define the MTD to be investigated in the subsequent Phase II trial (NCT01579994).

Retaspimycin hydrochloride (IPI-504) is the first potent Hsp90 inhibitor with a demonstrated clinical activity in NSCLC ALK rearranged patients in a Phase II trial, enrolling 76 patients heavily pretreated (including a line with EGFR-TKI). Among these, two of three ALK-positive patients treated with IPI-504 (at the starting dose of 400 mg/m2 on days 1, 4, 8 and 11 of a 21-day cycle and then at the dose of 225 mg/m2 due to hepatotoxicities observed at the highest dose in another trial) had a PR and the third patient had a durable SD (7.2 months). Fatigue, nausea and diarrhea were the most common grade 1 and 2 AEs, while grade ≥3 hepatotoxicities were observed in nine patients (11.8%) [64].

Last, AUY922 was tested at a weekly dose of 70 mg/m2 in 121 previously treated patients, including ALK-positive or EGFR-mutated NSCLC [65]. In this Phase II trial, among 22 ALK-positive patients, seven objective responses (32%) were noted, three among 14 crizotinib-resistant NSCLC patients; disease control rate for the whole group was 59% (100% in the crizotinib-naive group and 36% in the crizotinib-resistant group). The eye disorders (77%), diarrhea (74%) and nausea (46%) were the most common grade 1–2 AEs reported. To date, two ongoing trials are investigating AUY922 alone in a Phase II (primary endpoint: ORR; NCT01752400) and in combination with LDK378 in a Phase Ib (primary endpoint DLT; NCT01772797), both in ALK-positive patients, resistant to an ALK-TKI therapy.

ROS1-rearrangement & crizotinib

ROS1 is a receptor tyrosine kinase with homology to the ALK and insulin receptors, and signals via various pathways, including AKT1, MAPK, IRS1 and PLCG2 [66]. ROS1 rearrangements have long been associated with transformation in glioblastoma, and recently were identified as potential driver mutations in NSCLC, other than ALK. Chromosomal rearrangements involving the ROS1 gene lead to constitutive kinase activity and are associated with sensitivity in vitro to TKIs. Approximately 1–2% of patients with NSCLC harbor ROS1 rearrangements. ROS1 rearrangement defines a molecular subset of NSCLC with distinct clinical characteristics that are similar to those observed in patients with ALK-rearranged NSCLC: these patients tend to be younger (median age 49.5) never-smokers, with a histological diagnosis of adenocarcinoma and seem to be mutually exclusive with other gene alterations [66]. The multtargeted ALK/MET/ROS1 inhibitor crizotinib shows clinical activity in ROS1-rearranged (detected by break-apart FISH) NSCLC pretreated patients, enrolled in an expansion cohort of Phase I (PROFILE 1001). In April 2013, 42 patients were enrolled and only 36 patients were evaluable for response (two patients were subsequently confirmed negative for the ROS1 rearrangement). The response rate observed in ROS1-positive NSCLC was similar to that reported in ALK-positive NSCLC (ORR: 61%, DCT: 81% and 67% at 8 and 16 weeks, respectively). The AE profile of crizotinib in patients with ROS1-positive NSCLC was similar to that established in patients.
with ALK-positive NSCLC, with AEs being generally tolerable [67]. Mechanisms of resistance to crizotinib in ROS1-positive disease continue to be investigated.

Recently, an acquired mutation for crizotinib resistance was described in a cancer driven by an oncogenic ROS1 fusion: glycine-to-arginine substitution at codon 2032 in the ROS1 kinase domain (G2032R) [68]. Unlike the classic gatekeeper mutations for drug resistance previously reported in ALK, the G2032R ROS1 mutation is located in the solvent front of the kinase domain and is analogous to the G1202R ALK mutation identified in crizotinib-resistant ALK rearranged lung cancers [50]. Whereas the L1196M ALK gatekeeper mutant may still be sensitive to newer ALK inhibitors, such as CH5424802, G1202R ALK confers high-level resistance to crizotinib and to all the next-generation ALK inhibitors that were examined. In light of these observations, it may be necessary to identify novel compounds that specifically target the G1202R ALK or G2032R ROS1 mutant, to overcome the development of crizotinib resistance in these cancers [68].

**Future perspective**

Better understanding of multiple molecular mechanisms underlying the development, progression and prognosis of lung cancer has a key role to address the appropriate strategy of treatment.

Different molecular aberrations have been identified in NSCLC, with subsequent development of targeted therapies, such as gefitinib and erlotinib in patients harboring activating mutation of EGFR. Recently, another potential therapeutic drug was added to tailored strategy: crizotinib. After a rapid clinical development period, crizotinib was approved first by FDA, and after by EMA as the first NSCLC personalized therapy in which treatment is determined by clinically validated ALK testing. Although kinase-directed therapies have reshaped treatment approaches in oncogene-driven NSCLC, these therapies have been universally limited by the development of resistance. So, repeat biopsies should be mandatory, when possible, for discovering clinically relevant resistance mechanisms, including secondary mutations within the target, activation of alternative signaling pathways and in the case of EGFR-mutated lung cancer, small-cell transformation. Elucidating these mechanisms has helped to guide the development of new treatment strategies designed to overcome resistance. To date, some questions remain unclear. First, data reported on the potential relationship between ALK positivity and sensitivity to pemetrexed are disappointing and further prospective data are needed to clarify it. Second, the promising results of alectinib in crizotinib-naïve raise doubt on the role of crizotinib as the best first-line treatment, and randomized Phase III trial of direct comparison (crizotinib vs alectinib) is planned. Finally, further studies must better clarify the promising activity of alectinib in brain metastasis ALK-positive NSCLC patients, progressing on crizotinib, likely due in part to low penetration of crizotinib into the CNS. In conclusion, targeted therapy has already been a reality for many patients and it is certain that several components will soon follow to become valid options in the therapeutic arsenal of oncologists.

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**Executive summary**

- EML4-ALK translocation is found approximately in 2–5% of all cases of non-small-cell lung cancer (NSCLC). The ALK fusion genes appear to be more common in younger patients, patients who were never or light smokers, and patients with adenocarcinoma with solid pattern and signet-ring cells.
- FISH analysis is considered the gold standard for diagnosing ALK-positive NSCLC.
- Crizotinib, a selective small-molecule competitive inhibitor of ALK with additional MET, ROS1 and RON kinase inhibitory activity, was approved by FDA as the first NSCLC personalized therapy in which treatment is determined by clinically validated ALK testing.
- Despite the remarkable initial sensitivity, the long-term effectiveness of crizotinib is universally limited by the development of resistance, generally within one year: second-generation ALK inhibitors may represent a promising treatment approach in order to overcome it, such as LDK378, alectinib, and AP26113.
- ROS1 rearrangement defines a molecular subset of NSCLC with distinct clinical characteristics that are similar to those observed in patients with ALK-rearranged NSCLC and it has been identified as potential driver mutations in NSCLC, other than ALK.
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Clinical Trial Outcomes  Sgambato, Casaluce, Rossi et al.


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