Review Article on Looking for Chimeras in NSCLC: Widen Therapeutic Options Targeting Oncogenic Fusions

Resistance to anaplastic lymphoma kinase inhibitors: knowing the enemy is half the battle won

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Abstract: Anaplastic lymphoma kinase (*ALK*) translocations are responsible of neoplastic transformation in a limited subset of non-small cell lung cancer (NSCLC) patients. In recent years outcomes of these patients improved due to the development and clinical availability of specific and extremely active targeted therapies [i.e., next-generation Tyrosine Kinase Inhibitors (TKI)]: ALK+ patients are now reaching impressive results when treated with more potent inhibitors upfront with an average median progression-free survival (mPFS) around 35 months. However, under drug pressure, cancer cells develop resistance and patients eventually progress. Multiple mechanisms of intrinsic or acquired resistance have been extensively characterized. Less potent ALK inhibitors (ALKi)—like crizotinib—usually tend to induce a large spectrum of secondary intra-kinase mutations; however, these alterations may be observed also after sequential administration of multiple ALKi. Noteworthy, neoplastic cells may evade ALK targeting through a myriad of different mechanisms involving cell-stroma interaction, activation of parallel signaling pathways, intracellular downstream adaptation and histological reshaping, as relevant molecular events. Often these phenomena are restricted to a limited number of cases or even can be patient-specific, thus hindering the development of therapeutic strategies largely applicable. Consequently, the recognition of specific resistance mechanisms seldom translates in clinical opportunities. Management of ALK+ patients is drastically changed and deciphering the molecular biology underlying this disease during treatment is of paramount relevance. The bedrock of resistance to TKI is that, after the diagnosis, we face with a different disease that needs to be re-characterized through tissue or/and liquid biopsies. Understanding molecular pathways driving the resistant phenotype will give us the chance to know what we are dealing with and, rather than choose an empirical approach, will help us to properly define the best targeted treatment for these patients.

Keywords: Anaplastic lymphoma kinase (ALK); resistance; anaplastic lymphoma kinase inhibitors (ALK inhibitors); acquired mutations; clonal evolution

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Introduction

Efficacy outcomes of *ALK*-translocated NSCLC patients have been strikingly improved with the advent of new generation drugs which inhibit more efficiently aberrant activity of chimeric ALK proteins (1,2). Multiple ALKi are currently available in the clinical setting, nevertheless several open questions arise concerning the correct administration sequence (i.e., stepwise or more potent inhibitor upfront), since TKI may deeply influence the natural history of the tumor and, consequently, patients' long-term outcomes (3,4).

Table 1 The most frequent on-target mutations to ALKi: resistance and sensitivity

ALK TKI	Resistance mutations occurrence after TKI	Resistance mutations sensitivity to TKI
Crizotinib	L1196M, G1269A, C1156Y/T, L1152P/R, I1151Tins, F1174C/L/V, I1171T/N/S, G1202R, S1206Y	L1198F, E1210K
Ceritinib	G1202R, F1174L/C, C1156Y, L1196M, V1180L, G1202del, D1203N	I1171T/N, C1156Y, L1196M, S1206C/Y, G1269A/S, D1203N
Alectinib	G1202R, I1171T/N/S, V1180L, L1196M, S1206Y, E1210K	L1152P/R, C1156Y/T, F1174C/L/V, L1196M, L1198F, S1206C/Y, G1269A/S
Brigatinib	G1202R, E1210K + D1203N, E1210K + S1206Y/C	I1151Tins, L1152P/R, C1156Y/T, I1171T/N/S, F1174C/L/V, L1196M, G1269A/S
Ensartinib	G1202R, G1269A	G1123S, L1198F
Lorlatinib	L1198F + C1156Y, G1202R + L1196M, E1210K + D1203N + G1269A, I1171N + L1196F, L1196M + D1203N	I1151Tins, L1152P/R, C1156Y/T, I1171T/N/S, F1174C/L/V, L1196M, G1202R/del, S1206C/Y, E1210K, G1269A/S +/– C1156Y

The frequency of secondary on-target mutations acquired after crizotinib treatment is around 20–30%. Their occurrence after 2nd generation inhibitors is detectable, instead, in the 50–70% of patients, with G1202R as the most frequent event. Around 13% of patients who received a first- and second-generation inhibitors and the 55% treated with lorlatinib develop complex compound mutations. The sensitivity of on-target mutations and their coverage, which varies among different TKIs, is reported, TKI, tyrosine kinase inhibitors.

Similarly to other oncogene-addicted NSCLC, ALK+ patients become sooner or later resistant to targeted therapy, showing clinically progressive disease with a wide range of aggressiveness (5-7). Multiple resistance mechanisms to ALK inhibition have been reported in the past years, even though they can be categorized in "ALK-dependent" or "on-target", mainly due to intra-kinase domain mutations or *ALK* gene copy gain, and "ALK-independent" or "off-target", such as by-pass signaling pathways activation, drug efflux mechanisms or histological transition (8-10). To recognize the specific molecular mechanism underpinning cancer progression is of paramount relevance, since it allows physicians to properly change the treatment, targeting that resistance mechanism at the best (11).

New high-throughput technologies [i.e., next-generation sequencing (NGS)], capable to interrogate tissue and plasma samples thoroughly and rapidly, allow the investigation of changes in the molecular landscape at the time of disease progression (12,13). Even sometimes hard to be accomplished due to the limited accessibility to site of disease progression for re-biopsy (e.g., central nervous system) or technological limitations (poor access to interventional radiology or sequencing platforms), ALK-translocated patients should be re-characterized along their disease story. The elucidation of biological mechanisms of resistance, not exclusively relevant for clinical decisions, will improve our knowledge on this patients' subset providing physicians with tools to forecast clonal evolution and rapidly adjust therapeutic strategies.

On-target mechanisms of resistance

ALK mutations

A broad spectrum of mutations within the ALK enzymatic domain has been documented, similarly to what previously observed in patients with Philadelphia+ Chronic Myelogenous Leukemia receiving target therapies (i.e., imatinib, dasatinib, etc.) (14). The explanation why mutational landscape of ALK+ NSCLC is notably different from epidermal growth factor receptor (EGFR)+ patients, who develop T790M mutations as main mechanism of resistance to first- and second-generation TKI, may rely into different tumor biology (i.e., genomic instability in fusion-positive tumors and different oncogenic dependency) and distinct TKI properties (i.e., binding modalities, inhibiting potency) (15,16). Thus, ALKi of different generation, with different profiles of activity, generate different mutation profiles within ALK tyrosine-kinase domain (8) (*Table 1*).

First generation ALKi (crizotinib)

The reported frequency of secondary mutations acquired following treatment with crizotinib is around 20–30%, a lower proportion of cases compared to second-generation

TKIs (8). Data from a large dataset of tumor samples assessed at the time of progression identified different mutations, even if two of them appear in the majority of the cases: L1196M (7%) and G1269A (4%) (8); both of them, the first as a classical gatekeeper mutation and the second one lying in the ATP-binding pocket, alter 3D conformation and hinder TKI binding (17-19). Other mutations (C1156Y/T, L1152P/R, I1151Tins, F1174C/L/V) are supposed to enhance the kinase activity, being localized at the C-terminus or N-terminus of the αC-helix domain (17,20-22). I1171T/N/S, G1202R, S1206Y, E1210K, G1269A mutations also interfere with the binding of TKI determining a conformational alteration of the αC-helix or a steric hindrance (8,23-25). Other rare variants have been identified in individual cases, such as G1128A located in the P-loop and conferring an increased enzymatic activity (26).

Second generation ALKi (ceritinib, alectinib, brigatinib, ensartinib)

The occurrence of acquired mutations following second-generation ALKi increases till 50–70% of patients with G1202R as the most frequent event (35–60% of all mutated samples) (8). In post-ceritinib tissue biopsies secondary mutations were detected in the 56% of the cases—with 17% of double mutations—mainly represented by G1202R (21%), F1174 C/L (17%) and, to a less extent, C1156Y (8%). Moreover, G1202del has been identified (8%) and functional studies demonstrated its partial sensitivity to crizotinib and moderate resistance to second-generation agents, differently from G1202R that confers high level of resistance to first and second generation TKIs (8). At the post-alectinib relapse, acquired mutations have been identified in 53% of the patients, again with G1202R as the most frequent event (29%); other mutations identified are: I1171T/S (12%), V11180L (6%), L1196M (6%) (8,22,24,25). Even though a limited number of samples from patients treated with brigatinib have been analyzed at relapse, the majority reported G1202R (43%) and then E1210K (29%), D1203N (14%), S1206Y/C (14%) (8). Of note, most patients had previously received crizotinib as first ALKi. Compared to other ALKi, ensartinib seems to be the most active inhibitor against G1123S and L1198F mutations, but less potent against G1202R and G1269A. At disease progression two mutations emerged: E1210K and, less relevant, S1206F. Longitudinal changes in ALK mutations were identified during the treatment with ensartinib, showing the relevant role of plasma analyses to track disease evolution (13).

Around 13% of patients who received a first- and second-generation ALKi developed two or more mutations, supporting the concept of progressive multistep genetic complexity. Sequential analyses of mutational profiles help to clarify that resistance is "private" for each ALKi, like F1174V that confers sensitivity and I1171 resistance to alectinib, differently from what has been observed for ceritinib (23,27). In the work by Yoda *et al.*, a single patient, thoroughly analyzed along clonal evolution, developed a E1210K mutation post-crizotinib and two additional different post-brigatinib mutations; this denotes the emergency of two different clones (E1210K + S1206C and E1210K + D1203N) suggesting how TKI administration influences tumor evolution and response to more potent inhibitors (8,9).

Third generation ALKi (lorlatinib) and emergence of compound mutations

The mutational landscape of acquired resistance to lorlatinib has been extensively described, analyzing patients' samples after different ALKi sequences. Initially, experimental models of acquired resistance have been generated through ENU mutagenesis assays of Ba/F3 cell lines harboring EML4-ALK *wt* or *mut* (G1202R) and subsequently these data were validated in a cohort of 20 tissue biopsies of patients progressing on lorlatinib (9).

Almost no patients with primary intrinsic resistance to lorlatinib carried any ALK mutations, suggesting the presence of ALK-independent mechanisms of resistance. On the other hand, 55% of patients with acquired (after initial response) resistance developed compound mutations (9). Consistently with pre-clinical results, patients' samples showed double or triple mutations due to stepwise accumulation of resistance mechanisms. Inversely, two post-lorlatinib samples lose initially detected mutations. Studying the clonal evolution of three different patients, investigators identified specific pattern of compound mutations, such as: ALK-G1202R/L1196M, ALK-E1210K/D1203N/G1269A, ALK-I1171N/L1198F (9). Another recent study identified acquired compound mutations with different degrees of resistance to lorlatinib: F1174L/G1202R, L1196M/D1203N and C1156Y/G1269A. To what extent such patterns of resistance are sensitive to different ALKi is currently a field of research. It has been showed that, among others, the C1156Y/G1269A mutation maintains responsiveness to lorlatinib, thus indicating the presence of a concomitant off-target mechanism of resistance. On

the other hand, the L1196M/D1203N secondary mutation confers a potent lorlatinib resistance, comparable to the L1196M/G1202R mutation that confers primary resistance to third generation ALKi. Conversely, the F1174L/G1202R mutation induces a slight increase in resistance to lorlatinib compared to the single G1202R mutation, which can be overcome by elevated compound concentration *in vitro* (28).

Taking into account the prevalence of G1202R mutation as mechanism of resistance after second-generation TKI, it could be speculated that the double compound mutation ALK-G1202R/L1196M (identified both through mutagenesis assays and in patient samples) could be the most frequent acquired mutation in post-lorlatinib patients in the clinical setting. Frighteningly, this genotype (comparable to other G1202-coupled mutations) is resistant to all commercially available ALKi, strongly narrowing treatment options of these patients. However, changeable scenarios may emerge such as the case of a patient treated with the sequence of crizotinib-ceritinib-lorlatinib, who developed a post-lorlatinib L1198F mutation, along with the post-crizotinib C1156Y, and surprisingly responded to crizotinib; this double compound mutation C1156Y/L1198F re-sensitizes patient's tumor to first-generation ALKi (29). Also the I1171S/G1269A mutation, identified in a liver lesion at progression to lorlatinib, turned out to be sensitive to ceritinib and brigatinib and the patient responded to treatment with ceritinib (30).

ALK amplification

ALK gene copy number gain has been observed after exposure to crizotinib with a frequency of 7–18%, occasionally coupled with an ALK kinase mutation (19,21). In a cell line model, the association of amplification and mutation conferred high degree of resistance, while the copy number gain alone was not sufficient to generate a resistant phenotype to intermediate doses of crizotinib (18). However, this biological event was not reported as resistance mechanism after more potent ALKi, thus it might represent a non-clinically relevant phenomenon.

Off-target mechanisms of resistance

If intra-kinase domain mutations justify ~30% and ~50% of resistances following first and second/third generation TKI respectively, other resistance mechanisms, which are relevant in remaining cases, need to be investigated. If patients with ALK-independent mechanisms of resistance post-crizotinib still remain sensitive anyhow to ALK inhibition, due to a lower inhibiting potency of first-generation TKI, ALK-Independence post-second/third generation ALKi seem to no longer respond to ALK inhibition, suggesting how the presence of ALK secondary mutations predict a certain degree of ALK-dependency and, therefore, response to more potent ALKi (23,31,32).

Even if through different mechanisms (genetic mutations, by-pass kinase signaling, gene amplification, etc.), the result is a constitutive activation of intra-cellular downstream signaling pathways, such as RAS-MAPK axis, whose re-activation represents a crucial event driving resistance in ALK positive tumors (33) (*Figure 1*).

Co-occurring mutations

NGS approaches let the systematic screening of co-occurring genetic mutations as putative mechanisms of resistance to targeted therapy. Non-*ALK* mutations, in at least one different gene, have been identified in more than half of the cases (56%) of a post-second generation TKI cohort and in a large part of post-lorlatinib samples (8,9). The most frequently disrupted gene was *TP53*, even though, due to the low amount of pre-therapy samples to be matched, it is not clear if these mutations may pre-exist the development of resistance. Among other genes, rare private alterations at low frequency were identified in *BRAF*, *FGFR2*, *MET*, *NRAS* and *PIK3CA* genes (8,34). Among the others, mutations of *POLE* gene that encodes the catalytic subunit of DNA polymerase epsilon, permissive of the accumulation of a high number of somatic mutations, have been detected in post-crizotinib specimens (34). In a post-ceritinib patient-derived cell line a MAP2K1-K57N mutation was identified and it conferred sensitivity to the dual blockade of ALKi plus selumetinib (MEKi) (35). In the post-lorlatinib samples analyzed by Soda *et al.*, several mutated genes identified may be causative of resistance, such as *MAP3K1* disruption or *NRAS*-G12D activation (9).

Overall, these point mutations occur in a patient-private fashion, since appear as isolated event. Only *TP53* mutations have been identified as relevant co-occurring mutations and, recently, their role as prognostic/predictive biomarker has been validated when tested baseline, rather than as a resistance-inducing event (8,34,36). Indeed, Christopoulos *et al.* recently highlighted the relevant clinical meaning of baseline *TP53* mutational status in ALK+ NSCLC, showing that it significantly correlates with worse progression-free

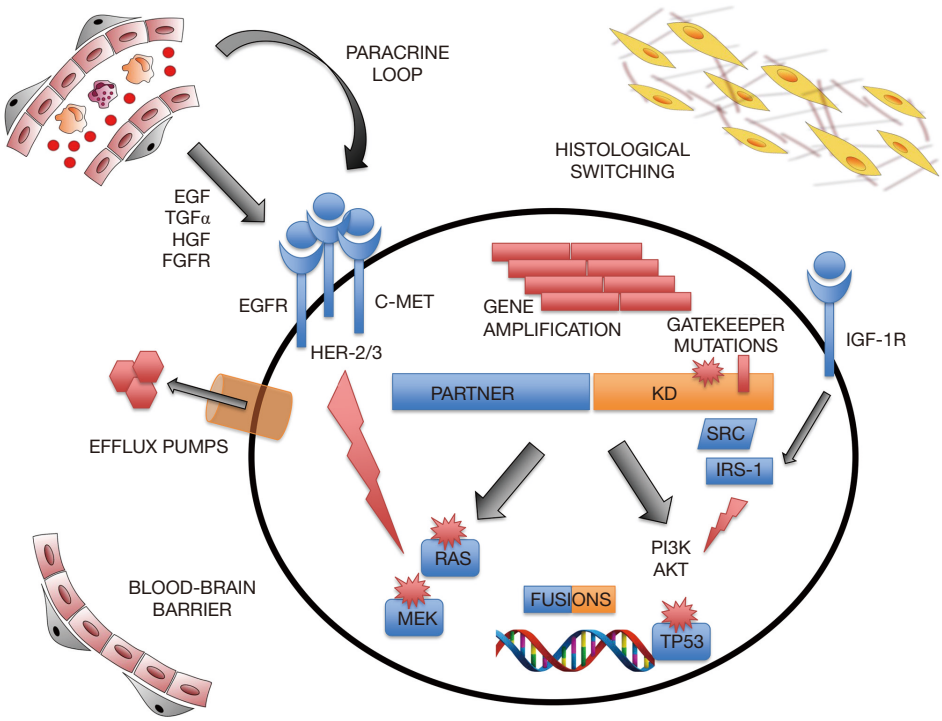

Figure 1 Mechanisms of intrinsic and acquired resistance to ALKi. The most frequent are: development of second mutations in the Kinase Domain (KD) at gatekeeper sites; copy number gain; activation of alternative oncogenic pathways via compensatory "by-pass" routes by receptor tyrosine kinases signalling (i.e., EGFR, HER-2, HER-3, c-MET); acquisition of other somatic mutations or kinase translocations. Alternative resistance may also be due to histological transformation or reduced drug delivery (impaired penetrance through the blood-brain barrier), which represents a source of primary resistance.

survival (PFS) and a shorter overall survival (OS), especially when associated with ELM4-ALK V3 variant. Moreover, the identification of *TP53* mutations by liquid biopsies at progression resulted to be associated with a more aggressive disease (37,38).

By-pass kinases and downstream signaling pathways activation

Concomitant activation of other kinases, both on the cell surface and in the cytoplasmic compartment, is frequently the crucial by-pass signaling track that confers resistance to conventional ALK inhibition. Human epidermal growth factor receptor family (*EGFR*, *HER2/HER3*, *HER4*) activation is one of the first and more relevant mechanisms identified (20,21,39). EGFR up-regulation—in absence of mutations—has been identified both in post-crizotinib cell lines and in resistant patients' samples, sometimes induced by paracrine stimuli, like NRG-1 upregulation (40-42). This event might pre-exist in therapy-naive tumor cells or appear during ALK targeting, rising and falling along treatments in a reversible manner (43,44). A recent study demonstrated that moderate resistance to alectinib in patients with leptomeningeal carcinomatosis (LMC) could be due to amphiregulin (AREG)-triggered EGFR activation, based on the important AREG overexpression found in resistant LMC cells *in vitro*. Moreover, in this model, the combined use of alectinib and EGFR-TKI, including the third-generation inhibitor osimertinib, significantly led to the control of disease progression at the central nervous system (45). Similarly, Redaelli *et al.* have identified EGFR phosphorylation as an escape mechanism in lorlatinib-resistant EML4-ALK cell lines, which can be controlled by combination treatment (e.g., lorlatinib plus erlotinib); thus highlighting the role of this adaptive mechanism also during third generation ALK inhibition (46).

Co-stimulation and transactivation of EGFR/MAPK pathway may be sustained also by different players, such

as P2Y purinergic receptors that, when upregulated, boost signaling by PKC activation, a mechanism itself capable to induce resistance to ALK inhibition (42). Moreover, Lovly *et al.* identified a synergistic effect when co-targeting ALK and insulin-like growth factor 1 (IGF-1). IRS-1 was identified as a central adaptor protein for IGF1-R and ALK signaling and inhibition of the IGF1R/IRS1 pathway sensitizes tumor cells to ALK targeting (47). A drug-screening assay, through patient-derived and cell line models, highlighted a relevant synergistic effect of saracatinib (a dual Src and Bcr-Abl inhibitor) and ALKi, pinpointing the cause of resistance in SRC kinase upregulation. In fact, the phosphorylation of SRC substrates was increased after both first and second generation TKI (35,48).

More recently, other molecular changes have been identified. Recondo *et al.* reported a case of *NF2* splicing site mutation that was detected at the progression to crizotinib, subsequently responsive to lorlatinib. At the time of progression to this latter agent, more disrupting events in *NF2*—a well-known tumor suppressor gene—appeared, causing a stronger bypass activation of the PI3K-AKT-mTOR pathway. Interestingly, cell-lines were sensitive to mTOR inhibition *in vitro* and *in vivo* with potential clinical implications (28). Activation of *YAP* (Yes-associated protein), a major downstream effector of the Hippo pathway, has been recognized as involved in resistance to ALK. YAP overexpression, indeed, hinders the response to alectinib in pre-clinical models and in tumor biopsies of crizotinib-resistant patients. These results, even if still premature, can provide the basis for YAP pathway targeting to overcome ALK-TKI resistance (49,50).

The RAS-MAPK dependency of ALK-rearranged cancers has been clearly elucidated and, being a crucial node where multiple molecular pathways converge, represent a solid pre-clinical evidence for upfront multi agent therapy approach, as emerging also in other types of oncogene addiction (i.e., TRK+ tumors) (33,51).

Gene amplification and translocations

Rather than ALK, different studies reported the occurrence of other genes' amplification as causative of resistance phenotype. *KIT* amplification has been identified in one patient progressed on crizotinib; it has been shown that the interaction with stromal paracrine SCF ligand is crucial to confer this phenotype and dual ALK/KIT inhibition might overcome this constraint (21).

Whether alectinib is superior to crizotinib as first line therapy has been demonstrated in the clinical setting (1). However, some investigators claim for the dual ALK/MET inhibitory activity of crizotinib that is not observed with alectinib (52). Initially, anecdotic case reports showed MET amplification as potential cause of massive progression after alectinib administration (53). Nevertheless, *HGF/MET* amplifications have been found also in crizotinib-progressing samples as proper on-target escape mechanism (34). In sequential analyses of circulating tumor cells derived from a patient who progressed on crizotinib and become primary resistant to ceritinib and alectinib high level of MET amplification was detected; gene amplification developed after crizotinib administration and was confirmed in the liver biopsy at the time of clinical progression and claimed as the causative mechanism of resistance (54). More recently *MET* gene amplification has been confirmed as relevant mechanism after second and third generation ALKi. Dagogo-Jack *et al.* identified this event in the 12% and 22% of patients' samples at progression after second generation or lorlatinib, respectively, therefore highlighting how the probability increases after more potent TKI treatment upfront; interestingly, proper dual inhibition of ALK plus MET, co-administering lorlatinib and crizotinib at low doses, seems a potential strategy to overcome resistance status (55). Similarly, other genes amplification, such as *MYC* gene copy number gain, may cause resistance to classical ALK inhibition, though these events seldom occur (56).

Genomic instability is the bedrock of acquired resistance mechanisms. An emerging molecular event, observed also in post-osimertinib EGFR+ patients, is the detection of fusion-driven clones (57). Although rarely reported, new gene translocations may restrain ALK+ NSCLC response to TKI. In the above reported paper, MET rearrangements were documented in two cases (one paired to MET amplification) (55). Moreover, a single case report shed light on the potential role of co-occurring NRG1 fusions as potential mechanism of resistance (58).

Phenotypic changes and stem-cell like properties

Among mechanisms of acquired resistance to ALK inhibitors, histological transformation such as epithelial-mesenchymal transition (EMT), switch to small cell (SCLC) or squamous cell lung cancer should be included. Notably, approximately 3–10% of EGFR+ lung adenocarcinoma, under the selective pressure of EGFR TKIs, undergoes

lineage change into SCLC or other neuroendocrine tumors, acquiring *TP53* and *RB1* mutations (59,60). Multiple clinical reports showed transformation to SCLC even in ALK+ NSCLC at progression on different generations of ALKi. In all the cases, ALK rearrangement was maintained on re-biopsy, suggesting a real histological switch, and not the outgrowth of a baseline pretreatment SCLC clone. Of note, histological switching may appear associated at the same time with acquisition of resistant mutations (e.g., L1196M or G1202R) (61-63). Similarly, transformation to squamous-cell histology has been claimed as causative of ALKi resistance; even though a rare event, morphological and immunohistochemical transformation (i.e., loss of TTF1 and gain of p40 positivity) were reported in the same primary site of initial adenocarcinoma diagnosis (64-66).

In post-ceritinib samples, analyzed by Gainor *et al.*, 42% of the cases expressed immunohistochemical markers of mesenchymal differentiation (i.e., vimentin) and loose E-cadherin, acquiring spindle cell features; of note, 3 of the 5 cases reported also an ALK intra-kinase mutation, meaning that EMT might be one of the mechanisms hindering ALKi sensitivity in these patients (8). Another study suggested the co-existence of L1196M mutation and EMT in a crizotinib-resistant tissue sample, and the administration of HDAC inhibitors seemed to revert the transition both *in vitro* and *in vivo* models (67). Further on, Recondo *et al.* expanded two cell lines derived from patients with EMT during lorlatinib treatment: one associated with C1156Y/G1269A compound mutation, the other apparently without any intra-ALK mutations. Both models, expressing EMT features (high levels of vimentin, ZEB1, FGFR1), showed increased SRC pathway activation, demonstrating that its inhibition could in part re-establish E-cadherin expression. In this context, it is of interest that *in vitro* fibroblast growth factor receptor (FGFR) inhibitors sensitized ALK-rearranged EMT+ cell lines to lorlatinib, spurring the investigation of combination treatments for patients with phenotypic changes (28).

Molecular signaling underling these phenotypic transformations have not been extensively understood in ALK+ disease. Preclinical studies investigated cell line models matching the resistant EMT phenotype with the emergence of a stemness trait of ALK-translocated cells (68). Nakamichi *et al.* generated H2228 resistant cell lines to first- and second-generation ALKi and identified the acquisition of EMT and stem-cell like (CSC) features in a drug-tolerant cell subpopulation that had an augmented signaling of ALK and HSP90 pathways. Authors stated that co-targeting these molecules, rather than solely ALK inhibition, might prevent or delay the phenomenon of drug tolerance, due to the inhibitory effect on the associated proteins, clients of the chaperone machine (69,70).

Intrinsic primary resistance

Intrinsic resistance to ALK inhibition has been reported after first- and second/third-generation TKI, when the best-obtained clinical response is disease progression. Around 5–7% of post-crizotinib, 9% of post-ceritinib and 25% of post-lorlatinib cases demonstrated unresponsiveness to treatments and no specific ALK mutations have been identified (8). Multiple factors may underline primary resistance to ALK targeting. First of all, anyone of the above-described mechanisms existing *de novo* in tumor cells might impinge ALK dependency. If ALK mutations have not been claimed to be causative of this phenomenon, other co-existing by-pass signaling tracks may be taken into consideration. Receptor tyrosine kinases-dependent pathways (e.g., HER members), among others, may represent the co-stimulatory signaling able to re-shape the ALK addiction and may constitute the cause of primary resistance. This concept is indirectly supported by the evidence that, targeting SHP2, a non-receptor protein tyrosine phosphatase on which multiple signals converge, may revert resistance in absence of ALK mutations (71).

Multiple studies investigated whether ALK fusion partner and kinase variant play a role in determining a different degree of response to TKI. Partner genes fused to tyrosine kinase domain play critical roles in the oncogenic transformation, providing oligo-dimerization domain, recruiting scaffold proteins and dictating subcellular localization of the chimera; since a wide number of different partners has been described, a certain degree of intrinsic resistance to therapies may be properly justified (72). Moreover, specific variant (V3 > V1) of the classical EML4-ALK fusion protein seems to confer a more aggressive tumor behavior, with shorter TKI response, high-metastatic spread and reduced PFS/OS. Nevertheless, these retrospective evidences are not supported by data coming from large phase III trials (e.g., ALEX trial) (37,73).

A potential mechanism of intrinsic resistance relies on the augmented capability of the neoplastic cells to eliminate the drug accumulated in the cytoplasm, thus not reaching a proper concentration. This is why crizotinib, being substrate of ATP binding cassette drug efflux transporters, has a low penetration in the cerebrospinal fluid (CSF) not

controlling, therefore, disease localization in the central nervous system (CNS) (74). More potent, next-generation TKI have been also developed to overcome this limitation and CNS disease control by these agents is definitively better, considering their capacity of delay brain metastases occurrence and produce more profound intra-cranial objective responses (75).

Lastly, the genomic complexity of cancers bearing translocations should always be taken into account. A major issue is represented by the correct detection of the genomic aberrations and false-positive cases may be a possible cause of the absence of response to TKI. Rosenbaum *et al.* have recently characterized the presence of "non-productive" rearrangements that are detected by FISH (gold-standard technique) but totally lacking the expression of chimeric protein (76). The harmonization of different diagnostic assays, including NGS, is therefore mandatory and will help refining the definition of ALK+ patients prone to receive TKI.

Conclusions

The Darwinian evolution of cancer cells under drug pressure is an inexhaustible source of resistance mechanisms underpinning disease progression; identification and characterization of these molecular transformations has to be at the crossroads of both preclinical and clinical research. Tumor heterogeneity preexists drug exposure and, multiplying during treatment, may facilitate the selection of cancer clones capable to evade TKI blocking (77). The pre-treatment landscape cooperates in generating the genomic complexity: the more instable is the cancer genome, the higher are the probabilities of acquire additional driver events. Within the molecular portrait of oncogene-positive lung adenocarcinoma, such as ALK-driven tumors, the inactivation of SETD2, a gene involved in the recruitment of DNA repair machinery, frequently occurs (78).

Resistance and disease progression may be due also to incapacity of drug delivery in sanctuary site, such as CNS. This had led to the development of more potent ALK inhibitors, giving the opportunity of sequential TKI administration. However, this strategy may contribute to the emergence of highly resistant mutations (i.e., compound resistance mutations after lorlatinib). The systematic analysis of molecular changes observed at the time of disease progression following upfront next-generation ALKi is matter of research and will be fully clarified in the next years. Other strategies have been investigating how delay or circumvent the emergence of resistance. Intermitting dose or alternate dose schedules in order to relieve drug pressure, administration upfront of dirty pan-inhibitory TKI capable of multi-targeting or early drug combination to maintain and restore drug sensitivity within drug-tolerant cells (70,79,80), are all strategies under investigation. However clinical applicability is not always an easy process and the accessibility into clinical trials for progressing patients remains a stronghold.

The knowledge of the molecular aspects surrounding ALK+ tumors is complex and, often, only marginal information is gained, not representative of the overall adaptive tumor plasticity. Nevertheless, the introduction of novel technologies and the possibility to perform extensive genomic analyses even on liquid specimens (e.g., blood, CSF) is a unique opportunity to speed up the research in this exciting field. The development and application of liquid biopsies on blood specimens—whose discussion is out of the scope of this review—has become a real milestone in the clinical management of oncogene-addicted patients. However, as previously discussed, the emergence of non-oncogene-driven resistance, such as phenotypic changes, underscores the indubitable role of tissue biopsy, when feasible. Differently from other scenarios (e.g., EGFR+ lung cancer, c-KIT+ gastrointestinal stromal tumors), ALK-rearranged NSCLC harbor specific features. The deep understanding of this disease both at baseline and after progression on different drugs is of paramount relevance to increase patients' survival. At the same time, such wealth of knowledge should be translated to other rare rearrangement-driven tumors.

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