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In vitro and *in vivo* characterization of resistance to lorlatinib treatment in ALK-mutated cancers

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To my mother,

who always put me first

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Chapter I

Introduction

1. Tyrosine Kinases

The human protein kinase (PK) family of proteins, comprises 518 enzymes that modify other proteins by catalyzing the transfer of the γ-phosphate of ATP to specific amino acids of a target protein[1]. There are two subclasses of PKs: tyrosine kinases (TKs) and serine-threonine kinases (STKs), based on their catalytic specificity. STKs phosphorylate on serine or threonine residues a variety of substrates including transcription factors, regulators of cell cycle, cytoplasmic and nuclear effector molecules[2]. Protein kinase A (PKA), protein kinase B/Akt, mitogen activated protein kinases (MAPK) belong to the STKs subclass. TKs catalyze the phosphorylation of tyrosine residues and play a very important role in cellular communication by transmitting activating signals from the cell surface or specialized cellular structures to cytoplasmic proteins and cell nucleus[3]. This in turn leads to the regulation of various cell processes such as cell proliferation, migration, differentiation and survival.

TKs can be further subclassified into receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (NTRKs). RTKs contain an extracellular ligand-binding domain (ECD), an intracellular catalytic domain or tyrosine kinase domain (TKD), a transmembrane domain (TMD) that connects the intracellular and extracellular domains and a juxtamembrane domain (JMD). While the ECD conveys the ligand

specificity, the kinase activity of RTKs is mediated by the intracellular TKD[4]. Binding of the ligand to the ECD, followed by dimerization of the receptor, leads to the *trans*-phosphorylation of the kinase domain in RTKs whereas activation mechanism of NRTKs is a complex process involving heterologous protein-protein interaction for transphosphorylation[5]. Some well characterized and intensively studied examples of RTKs include platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), epidermal growth factor receptor (EGFR), and insulin receptor (IR). NRTKs are cytoplasmic proteins that contain the intracellular kinase domain but lack the extracellular and transmembrane domains. NRTKs show considerable structural variability among its subfamily members. NTRKs also contain several additional signaling or protein-protein interaction domains such as Src homology 2 (SH2), SH3 and pleckstrin homology (PH) domain[6]. Some examples of NTRKs include Src family, focal adhesion kinase (FAK), Abelson (Abl) kinase family of proteins[7]. Although, many PKs are significantly important for various cellular functions, the focus of this thesis is mainly on RTKs; therefore, only RTKs are discussed herein after.

1.1 Regulation of receptor tyrosine kinases under physiological conditions

Given the pivotal role RTKs play in all aspects of cellular function, they are tightly regulated by different mechanisms[8]. The first and most obvious regulation is performed by the tyrosine phosphatases

that can remove the phosphate group from phosphorylated tyrosine residues. But, there are several other mechanisms in place and one such mechanism is autoinhibition of the catalytic activity of the kinase because of a *cis* inhibition/*trans* activation mechanism[1]. Binding of a growth factor ligand to the extracellular regions of the RTKs results into a conformational change in the form of dimerization and/or oligomerization of the receptor. For most of the RTKs, the conformational change in the receptor alone results in trans-autophosphorylation, relieving the kinase from its cis-autoinhibition. However, receptor dimerization is not always enough on its own for the kinase activation. Additional ligand-induced conformational changes occur, juxtaposing catalytic domains in the correct configuration for *trans*-phosphorylation between receptor subunits[9,10].

For some RTKs, other regions of the protein have also been shown to exert autoinhibitory effects. Shewchuk et al reported that the carboxy-terminal tail of Tek 2 kinase can partially block access to the substrate tyrosine-binding site[11]. Therefore, for the full activation of the Tek 2 kinase the carboxy-terminal tail must also undergo a conformational change upon protein activation, to expose the substrate binding sites for tyrosine residues phosphorylation and subsequent signaling. In some other RTKs such as PDGFR, ephrin receptor (EphR) and IR, autophosphorylation of tyrosine residues of the JMD is required to overcome autoinhibition[12-14]. Autophosphorylation of the tyrosine residues in RTKs not only relieves the kinase from an inhibitory conformation but also creates binding sites for numerous SH2-containing signaling molecules, such as Src kinases, and phosphatidylinositol-3-kinases (PI3Ks).

The general kinase domain fold is rather conserved throughout the entire kinome, with minor differences among different subclasses: a bilobate structure, with a central cleft where ATP is accommodated; within the C-terminal lobe, a flexible activation loop assures conformational regulation of the catalytic activity. Structural features are discussed more in detail in the next sections, referred to the anaplastic lymphoma kinase.

Although kinase activity is tightly controlled, this regulation is not always maintained, owing to various genetic abnormalities, and many times lead to aberrant activity of kinases. The irregularity in the activity of the kinase could either be a constitutive active state of the enzyme or an under-activation state of the enzyme. Deregulated kinases have been implicated in several diseases such as type II diabetes, anemias, immune suppression and neuropathies due to less active TKs[14-16]. But, what has gathered more attention is the frequent involvement of TKs in carcinogenesis and metastases of various types of cancer[17].

1.2 Failure of regulation of receptor tyrosine kinases and oncogenesis

There are different mechanisms by which RTKs can acquire transformation functions, however the eventual oncogenic transformation is the result of failure of one or more of the auto-regulatory mechanisms that control the catalytic activity of TKs. Perturbations of the TKs regulation mechanisms lead to a constitutively

activated TK which based on its role, in turn, can lead to uncontrolled cell proliferation, promotion of angiogenesis and cell motility or deactivation of the apoptotic pathways[16]. Advancements in our understanding of the role of RTKs in tumorigenesis come from the molecular analyses of differences between RTKs and their oncogenic counterparts. Not being under the autoregulatory control is the primary difference between kinases and oncogenic kinases. To escape the governing control of their activity, kinases mostly take advantage of genomic instability. In recent years, it has been realized that cancer is an evolutionary process that takes place over time accumulating a number of genomic alterations. Tyrosine kinases follow the same path to become oncogenic by mostly acquiring genomic alterations in the form of re-arrangements, point mutations, and gene amplifications.

Genomic rearrangements such as chromosomal translocations most likely occur as a result of DNA damage, in particular because of double strand breaks, and repair via the non-homologous end-joining pathway, however the detailed mechanism is unknown. Chromosomal translocations lead to gene fusion between two otherwise separated genes which in turn can lead to the expression of a chimeric fusion protein. Several tyrosine kinase fusions have been identified in solid and hematopoietic malignancies[18]. Since, the N-terminus of the kinase plays an inhibitory role in its activation by limiting the activation only upon ligand-binding, the kinase fusion partner genes replace the N-terminus and keep only the C-terminus with kinase activity in the chimeric oncoproteins. Because of the elimination of the extracellular domain, the expression of fusion kinase is controlled by the promoter of the partner gene. And, given that almost always, the partner genes

are under a strong and/or regularly active promoter, the fusion leads to an aberrant expression of the kinase. Examples of deregulated kinases, due to translocations include ABL, PDGFR, FGFR, fms related tyrosine kinase 3 (FLT3), anaplastic lymphoma kinase (ALK).

Another frequently found genomic alteration of RTKs is somatic mutations in the genes encoding them. However, not every mutation acquired by the kinases confers an advantage to the cells in terms of growth and survival. Usually mutations occurring in the evolutionary conserved part of the kinase structure such as the DFG motif of the activation loop and the ATP-binding pocket of the kinases can confer a selective advantage to the cells because of an aberrant downstream signal transduction. Such mutations are defined as 'driver mutations'. Numerous studies have identified mutations in the tyrosine kinase domain of various RTKs (in *in vitro* as well as in patients) which hyperactivate the kinase as a result of an altered catalytic activity and substrate binding[19]. Mutations can also occur in other regions of the RTK structure such as in the ECD, TMD and JMD, although these mutations adopt alternative activating mechanisms compared to mutations within the TKD. For example, mutations in the ECD of EGFR in glioblastoma (GBM) adopt inactive conformations as compared to active conformations acquired by point mutations in TKD of EGFR in lung cancer, and are better targeted by inhibitors that can only be accommodated by the inactive conformation of the EGFR catalytic domain[20,21]. Since JMD of the RTKs negatively regulate their kinase activity, mutations in the JMD relieves the internal autoinhibition and leads to hyperactivation of the kinase. Somatic mutations in the TMD of RTKs have also been documented. For

example, in the oncogenic rat homologue of ErbB2, NeuT; the Val664Glu substitution was found to be responsible for the overactivation of the receptor[20]. Similarly, an activating Gly382Arg mutation in the TM domain of FGFR3 has been reported in bladder cancer[22]. Mutations in the TMD can augment receptor dimerization, enhance receptor phosphorylation, and eventually lead to cell transformation.

Gene amplification is one other mechanism that have been implicated in many types of cancers to abrogate physiological activity of RTKs and an uncontrolled activation of the kinases. Genomic amplification of RTK results in elevated level of the local kinase receptor concentrations which in turn increase the downstream signaling. There are also other genomic alterations that can culminate in RTK overexpression, e.g. transcriptional/translational enhancements, oncogenic viruses, deregulation of regulatory checkpoints.

2. Anaplastic Lymphoma Kinase (ALK)

The ALK protein is encoded by the *ALK* gene located on the 2p23 chromosomal region. It was first identified in anaplastic large cell lymphoma (ALCL) samples as part of a chromosomal translocation t(2;5)(p23:q35)[23,24]. The chromosomal translocation causes the N-terminal part of nucleophosmin (*NPM*) gene present on chromosome 5 to fuse with the kinase domain of *ALK* gene and results in the formation of an oncogenic protein, NPM-ALK. ALK belongs to the insulin

receptor superfamily and is similar to the leukocyte tyrosine kinase (LTK) based on an overall homology[25,26]. Full-length human ALK comprises 1620 amino acids (aa) with a protein size of approximately 180 kDa of the unmodified protein. Although, a larger protein of size ~200 kDa can also be detected as a result of ALK post-translational modifications such as N-linked glycosylations.

2.1 Structure of ALK

Like the other members of the RTKs family, ALK is composed of an extracellular ligand-binding domain (1020 aa), a transmembrane-spanning region (21 aa) and an intracellular domain (561 aa)[27] (Figure 1).

Additionally, the ECD of ALK carry a distinctive short signal peptide (18 aa). The ECD was further characterized by Palmer et al and reported to contain an LDLa domain, a MAM (meprins, A-5 protein and receptor protein tyrosine phosphatase mu) domain and a glycine-rich region[28]. Since the MAM domain found in transmembrane proteins such as the meprins and receptor protein-tyrosine phosphatases have been insinuated to function in cell/cell interactions, the MAM domain of ALK is thought to play a similar role. Even though the function of MAM domain of mammalian ALK is not quite clear, its importance could be drawn from the studies conducted on *Drosophila* ALK (dALK) where a point mutation substituting a highly conserved aspartic acid residue in the MAM domain to an arginine, rendered the dALK inactive[29]. On the other hand, the intracellular domain of ALK is

composed of a juxtamembrane region, a tyrosine kinase domain and a carboxy terminal tail.

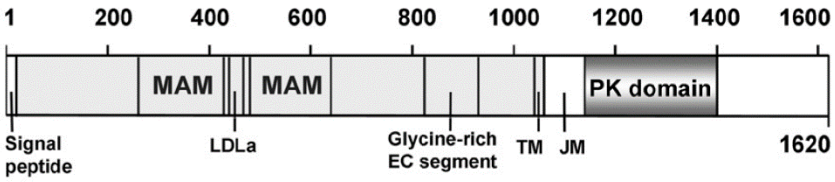

Figure 1. *Simple schematic representation of ALK structural domains*. (adapted from Roskoski R Jr. Anaplastic lymphoma kinase (ALK): structure, oncogenic activation, and pharmacological inhibition. Pharmacol Res. 2013 Feb;68(1):68-94)

2.2 ALK protein kinase domain structure

The kinase domain structure of ALK is similar to other tyrosine kinases in terms of architecture and topology. It is composed of two lobes: a smaller amino-terminal lobe and a large carboxy terminal lobe, which are connected by a loop named as hinge region (Figure 2). The hinge region is flexible and confers conformational plasticity to the whole kinase domain. The space between the N- and C-terminal lobes and the hinge region defines the binding site for ATP and ATP competitive inhibitors. The small N-terminal lobe typically consists of five-stranded antiparallel β-sheets (β1- β5) and an important regulatory major αC-helix that can exist in active or inactive positions. β1 and β2 strands of the N-terminal β-sheet are connected by a glycine-rich P-

loop. P-loop contains a GxGxxG motif which interacts with ATP by positioning the β- and γ-phosphates of ATP[30]. Another residue of N-lobe, a conserved valine (V1130) also interacts with ATP by making a hydrophobic contact with the adenine group of ATP. The β3-strand contains an Ala-Xxx-Lys sequence that can form a salt bridge with the αC-helix (residues 1158-1173), whose orientation is pivotal for kinase activation.

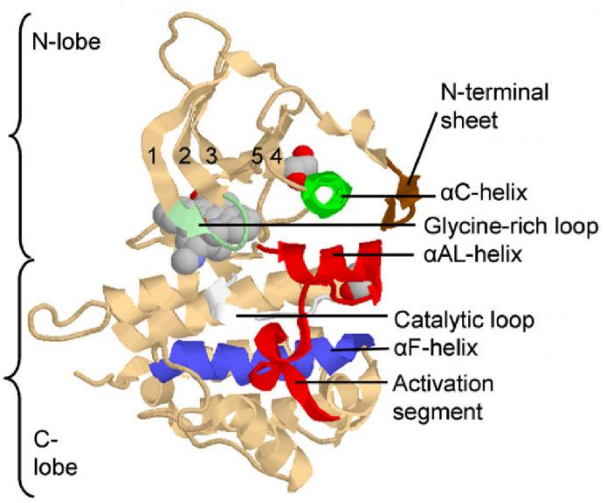

Figure 2. *Ribbon diagram of human ALK kinase domain showing the two lobes and different α-helix and β-sheet structures. Staurosporine, inhibitor of many kinases is shown occupying the ATP-binding site behind the glycine-rich loop while glycerol is shown behind the αC-helix.*

(adapted from Roskoski R Jr. Anaplastic lymphoma kinase (ALK): structure, oncogenic activation, and pharmacological inhibition. Pharmacol Res. 2013 Feb;68(1):68-94)

In contrast to the N-terminal lobe, the larger C-terminal lobe is predominantly comprised of α-helices. The C-terminal lobe contains six α-C helices (αD- αI) and two short β-sheets. Both, the activation loop as well as the catalytic loop, are located within the C-terminal lobe. The activation loop (A-loop), which is made of residues 1270-1299, is the most important regulatory segment of ALK kinase. A-loop is imperative for the activation of the kinase, hence named A-loop. In most kinases, A-loop starts with a DFG (Asp-Phe-Gly) motif and ends with an APE (Ala-Pro-Glu) motif. While the A-loop of ALK begins with the DFG motif, it ends with a PPE (Pro-Pro-Glu) motif. The A-loop of ALK contains a conserved a YxxxYY motif. Autophosphorylation of the three tyrosine residues of this motif are pivotal for kinase activation. Unlike its family member, IR, the order of tyrosine residue phosphorylation in ALK is different. In ALK, the 1278 tyrosine residue (Y1278) is phosphorylated first, followed by the phosphorylation of the second and third tyrosine residues (Y1282, Y1283) as compared to IR, where A-loop tyrosines are autophosphorylated in the order, second, first, and then third[31]. The catalytic loop (1247–1254 aa) of ALK is situated between αE and the first strand of the two-stranded β-sheet and has a conserved His-Arg-Glu (HRD) component, generally known as the catalytic triad of protein kinases. The histidine residue of HRD allows the correct orientation of P-site on the substrate.

2.3 Activation of ALK catalytic domain

As mentioned earlier, binding of the ligand to the extracellular domain of RTKs including ALK activates the catalytic activity of the protein by inducing receptor dimerization. Receptor dimerization helps in the transphosphorylation of tyrosine residues of the A-loop. In the inactive state of the kinase, the positioning of A-loop between the N- and C-lobe prevents both, ATP and substrate, from binding to its pocket. Phosphorylation of all the three tyrosine residues of the YxxxYY motif (triphosphorylated state) in the A-loop changes its conformation such that the binding pocket of the kinase domain becomes available for ATP or substrate. It is also worth noting that only the triphosphorylated form of the kinase is fully active. Additional further conformational changes help in the recruitment and activation of downstream target proteins thereby initiating the cellular signal transduction.

2.4 ALK signaling

The transforming activity of oncogenic ALK is arbitrated to the cells by its interactions with the downstream molecules that substantiate the signaling cascade process. Most of our knowledge about ALK signaling networks come from oncogenic ALK that is constitutively active because of different genetic abnormalities, such as *ALK* translocations (resulting in ALK fusion proteins), activating point mutations in *ALK* as well *ALK* amplifications, found in different disease

settings. However, it is also important to note that some of the downstream targets as well as interacting proteins known to be part of the ALK signaling might be associated only with oncogenic ALK, given that the oncogenic ALK activity is driven by either the fusion partners or other genetic aberrations.

ALK fusion proteins signal through several pathways but broadly they can be divided into two categories; proliferation and survival pathways. Among the different effector proteins phosphorylated by ALK kinase activity, the most important signaling is mediated by the activation of JAK/STAT, PI3K/AKT, RAS/ERK and PLC-γ pathways[32] (Figure 3).

The RAS/ERK, and PLC-γ pathways are essential mostly for ALK mediated cellular proliferation, while cell survival and phenotypic changes are signaled through the JAK/STAT and PI3K/AKT pathways[32]. Despite their categorization, these pathways are interconnected and can also overlap in physiological conditions. A large number of the ALK signaling information is derived from ALCL (discussed later) disease where ALK was first discovered in the form of NPM-ALK fusion protein, however the same signaling networks are applicable in other ALK related diseases.

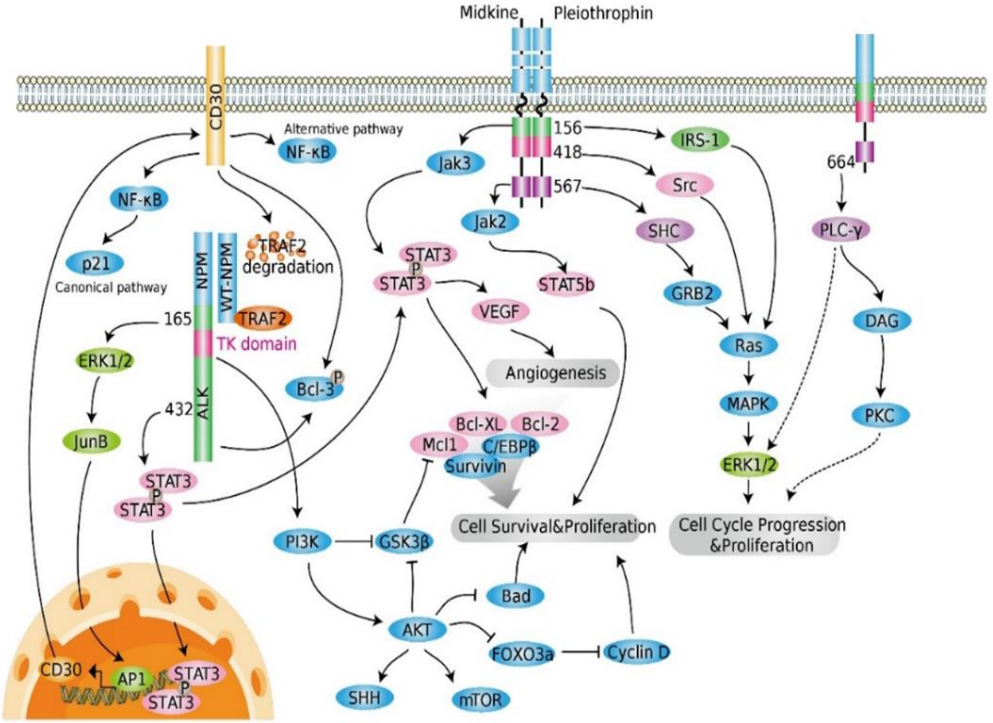

Figure 3. *Anaplastic lymphoma kinase signaling. ALK fusion proteins (e.g. NPM-ALK) lead to the concomitant activation of multiple downstream signaling pathways.*

(adapted from Chiarle R, Voena C, Ambrogio C, Piva R, Inghirami G. The anaplastic lymphoma kinase in the pathogenesis of cancer. Nat Rev Cancer. 2008 Jan;8(1):11-23)

2.4.1 RAS/ERK pathway

Physiological activation of the extracellular-regulated kinase (ERK) pathway is mainly triggered by several stimuli, such as growth factors, serum and phorbol esters as well as by some cytokines and osmotic stress to a lesser degree. Activation of the ERK pathway eventually culminates in impacting a number of cellular functions, from proliferation and survival, to migration and cell division. Activation of ERK1 and 2 involves activation of its upstream mitogen-activated protein kinase (MAPK) signaling pathway, which includes MAPK kinase kinases (MAPKKK) such as A-Raf, B-Raf and c-Raf and the MAPK kinases (MAPKK) MEK1 and MEK2, which directly activate ERK1 and ERK2[33].

RAS/ERK pathway was shown to be the main pathway responsible for ALCL cell proliferation. The authors demonstrated that NPM-ALK serves as a docking molecule for various downstream adaptors, scaffolding molecules with SH2 or phosphotyrosine binding domains that can bind to the autophosphorylated NPM-ALK tyrosine residues and in turn activate the RAS/ERK pathway. In particular, SH2 domain-containing transforming protein (SHC), insulin receptor substrate 1 (IRS1) and growth factor receptor-bound protein 2 (Grb2) were identified to form a complex with NPM-ALK. SHC and IRS-1 bind to the phosphorylated tyrosines (Tyr567 and Tyr152–156, respectively) of NPM-ALK. Despite the evidence of their direct binding to NPM-ALK, SHC and IRS1 appear to be not essential in NPM-ALK-mediated transformation as the NPM-ALK mutants defective in the

binding and phosphorylation sites (thereby not being able to bind to IRS1 and SHC) could still transform NIH3T3 cells [34].

On the other hand, Grb2 binds to different regions of NPM-ALK. SH3 domain of Grb2 mainly binds to a proline-rich region, (Pro 415–417 of NPM-ALK), and lead to the activation of RAS/MAPK pathway and eventually regulate ALCL cell growth[35]. Further evidence of the interaction of NPM-ALK with ERK pathway was provided by Marzec et al. The group reported that NPM-ALK activates the mammalian target of rapamycin (mTOR) through activation of the MAPK pathway[36]. mTOR is a serine/threonine kinase that forms the core component to join two distinct complexes, mTORC1 and mTORC2. The two complexes regulate cellular growth, proliferation and survival. mTORC1 can directly activate p70S6 kinase 1 (p70S6K1) which in turn phosphorylates S6 protein of the 40S ribosomal subunit (S6rp) at multiple sites. mTORC1 can also phosphorylate the eukaryotic initiation factor 4E (eIF4E) binding protein (4E-BP1). Phosphorylation of 4E-BP1 dissociates it from eIF4E leading to the inactivation of eIF4E. Targeting mTOR pathway in ALK-positive (ALK+) ALCL cells lead to cell cycle arrest and cell death[37]. These findings point towards the role of RAS/ERK pathway in NPM-ALK mediated transformation.

2.4.2 PLC-γ pathway

PLC-γ belongs to the family of phosphoinositide-specific phospholipase C (PLC) enzymes which are important for

transmembrane signaling. Many external stimuli such as hormones, growth factors, and neurotransmitters can induce the activation of PLC. Upon activation, PLCs catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into secondary messengers: inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). DAG and IP3 can then activate serine-threonine protein kinase C (PKC) and release stored calcium from intracellular compartments, respectively. Different members of the PLC family can be phosphorylated at different sites. PLC-γ, in particular, is activated as a result of the interaction between its SH2 domain and one or more autophosphorylation sites of the activated RTKs.

Phosphorylation and activation of PLC-γ was shown to be associated with NPM-ALK. The authors of the study also identified, Y664, corresponding to Y1604 of human full-length ALK, in NPM-ALK which facilitates the binding of PLC-γ. The authors concluded that PLC-γ signaling is needed by NPM-ALK to mediate its mitogenic signals based on the findings that single Y664F mutants resulted in the loss of NPM-ALK mediated transformation of lymphocytes[38]. Interestingly, Y664 is present only in the human ALK but not in the mouse gene insinuating a unique function in human ALK-mediated transformation.

Another study found that monoclonal antibody (mAb)-induced ALK activation recruits PLC-γ in neuroblastoma cell lines, further substantiating the involvement of PLC-γ pathway in ALK-mediated signaling[39].

2.4.3 JAK/STAT pathway

Another major pathway that has been implicated to interact with oncogenic ALK is the JAK/STAT pathway. Signal transducers and activators of transcription (STAT) are a family of transcription factors. STATs were first characterized for their role in cytokine signaling. STAT proteins contain SH2-domain through which they can interact with receptor phosphotyrosine residues. Phosphorylation of STATs leads to their dimerization and subsequent accumulation in the nucleus. Activation of STAT proteins is tightly regulated by several activating or suppressing proteins, such as MAPK or suppressors of cytokine signaling. The main regulators of STAT activation are proteins belonging to the Janus Kinase (JAK) family. JAK proteins can specifically bind to the intracellular region of cytokine receptors that lack intrinsic tyrosine kinase activity and catalyze phosphorylation of the receptor as well as of themselves. In the context of STAT activation, JAK-mediated phosphorylation of the cytokine receptors creates docking sites for STATs. STAT proteins are recruited to these docking sites and phosphorylated, which in turn leads to their activation[40].

Out of the seven members of the STAT family, only the role of STAT3 and STAT5 have been studied in oncogenic ALK settings. Increased expression of phospho-STAT3 (p-STAT3) has been reported in ALK+ lymphomas[41-43]. In fact, STAT3 has emerged as the main transducer of NPM/ALK oncogenic signaling in ALCL[32]. Activation of STAT3 regulates cell survival by promoting the transcription of anti-apoptotic proteins such as Bcl-2, Bcl-xl, survivin and Mcl-1. It can also modulate cellular proliferation by elevating the transcription of other

transcription factors like c-myc and the G1 checkpoint controller; cyclin D1[41]. Another STAT3 target, C/EBPβ, a transcription factor, involved in lymphomagenesis, is transcriptionally induced by NPM-ALK[44], and has been shown to play a role in NPM-ALK-mediated tumorigenicity[45]. In addition, protein phosphatase 2A, a STAT3-interacting protein essential for sustained STAT3 phosphorylation, is overexpressed in ALK-positive ALCL[43].

Moreover, the JAK2-STAT5 pathway is also involved in NPM-ALK-mediated proliferation. Direct binding of JAK2 to NPM-ALK aids in NPM-ALK driven proliferation and survival through STAT5B signaling[46]. In contrast, STAT5A is methylated and consequently silenced by NPM-ALK in ALK+ cell lines. In line with this, induced re-expression of STAT5A resulted in the downregulation of NPM-ALK and a reduction in STAT3 phosphorylation. Together these findings underlie the oncosuppressive role of STAT5A[47].

Furthermore, SH2 domain-containing phosphatase 1 (Shp1), a negative regulator of JAK/STAT pathway is found silenced in ALK+ ALCL as a result of DNA methylation[48]. Restoring the expression of Shp1 in NPM-ALK-expressing cell lines results in the dephosphorylation of JAK/STAT pathway and ultimately to cell cycle arrest[49].

2.4.4 PI3K/AKT pathway

Phosphatidylinositol 3-kinase (PI3K) proteins are a family of lipid kinases that phosphorylate phosphatidylinositol and

phosphoinositides. PI3Ks are classified into three classes (I-III), based on their structure and substrate specificity. PI3Ks are heterodimeric proteins with one catalytic and one regulatory subunit. Out of the three PI3K classes, class IA PI3Ks are the most frequently implicated in human cancers. Class IA PI3Ks contain a p110 catalytic subunit and a p85 regulatory subunit, corresponding to the molecular size of their respective proteins. In the absence of RTK activation, the regulatory p85 subunit inhibits the activity of p110. Upon ligand binding and the subsequent RTK phosphorylation, p85 binds to the activated RTK freeing up the p110 catalytic subunit resulting in its localization to the plasma membrane. The catalytically active p110 subunit present in the plasma membrane, catalyzes the phosphorylation of PIP2 to phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 can be recognized by the PH domain of AKT and phosphoinositide-dependent protein kinase 1 (PDK1), that are subsequently recruited to the membrane. Binding of PIP2 to the PH domain of AKT leads to its phosphorylation at Thr308 in the catalytic domain by PDK1 and at Ser473 in the C-terminal hydrophobic motif by the mTORC2. Phosphorylation and subsequent activation of AKT in turn leads to the transcription of several target genes involved in survival and proliferation[50]. AKT signaling promotes ALCL cellular proliferation by preventing cell cycle arrest via proteasomal degradation of the cyclin-dependent kinase inhibitor p27[51]. Activated AKT can also hyperphosphorylate the transcriptional activator FOXO3a, rendering it incapable of moving to the nucleus. Nuclear exclusion of FOXO3a leads to downregulation of the pro-apoptotic Bim-1 and cell cycle arrest-related p27kip1 and up-

regulation of cyclin D2, involved in the transition between G1and S phase[52].

ALK fusion proteins interact directly or indirectly with PI3Ks. The interaction leads to the activation of AKT and subsequent downstream signaling. PI3K/AKT pathway plays a crucial role in ALK-mediated transformation, as the dominant negative PI3K or AKT mutants inhibited proliferation and clonogenic properties of NPM-ALK+ Ba/F3 murine cells[50]. Further evidence of the involvement of PI3K/AKT pathway in ALK-mediated oncogenesis comes from the findings by Gu et al, who found that forced expression of ALK hyperphosphorylates FOXO3a which in turn up-regulate cyclin D2 and down-regulate Bim-1 and p27[50]. Moreover, McDonnell et al recently identified GSK3β as a signaling mediator of NPM-ALK using a mass spectrometry-based phosphoproteomic screen[53]. The group showed that the tyrosine kinase activity of ALK regulates phosphorylation of GSK3β which is mediated by PI3K-AKT pathway. PI3K/AKT signaling pathway controls the phosphorylation levels of GSK3β by upregulating the expression of MCL-1 and Cdc25A which provides the cells with growth advantage and protection from apoptosis[53]. Additionally, pharmacological block of PI3K/AKT signaling, using the inhibitor wortmannin, diminished growth and induced apoptosis in the Ba/F3 cells expressing human NPM-ALK[54]. All the aforementioned findings show that PI3K/AKT pathway is important for the antiapoptotic signaling regulation and is critical for cell transformation mediated by NPM-ALK.

There are a number of other interesting but not well characterized oncogenic ALK target proteins including but not limited

to nuclear interacting partner of ALK (NIPA), the small GTPases Rac1 and cell division control protein 42 (CDC42), SHP1 (also known as PTPN6) and SHP2 (also known as PTPN11). Upregulation of GTPases has been associated with higher metastatic activity and poor prognosis in bladder cancers[55,56]. Rho family of GTPases act as molecular switches in regulating various signal transduction pathways. Some members of the Rho GTPases family such as Rac1 and Cdc42 have been studied in much details for their role in modulation of cytoskeleton dynamics especially in the context of ALCL. While Cdc42 is essential for cell proliferation and survival, Rac1 seems to be crucial for cell migration in ALK-transformed cells[32,57]. In line with these findings, inhibition of Cdc42 and Rac1 activity have been demonstrated to induce apoptosis *in vitro* and abrogate NPM-ALK mediated disease progression and metastasis *in vivo*[32,58]. Moreover, it was recently shown that targeting both GTPases, Rac1 and Cdc42 could prevent NPM-ALK lymphoma dissemination *in vivo*[59].

Several reports have also demonstrated that microRNAs are also involved in ALK signaling, for example, miR-135b, miR-29a and miR-16 are reported to be downstream of NPM-ALK, while miR-96-mediates regulation of ALK expression being upstream of NPM-ALK[60]. Overall, it is clear that oncogenic ALK proteins interact with a plethora of other proteins to achieve neoplastic transformation.

2.5 Physiological role of ALK

The exact physiological role of mammalian ALK is still poorly understood but several studies on different animal models have helped gather some information about ALK functions in development. ALK presumably plays an important role in development and nervous function by regulating cell proliferation, survival and differentiation. In fruit fly *Drosophila melanogaster*, dALK signaling is critical for embryonic development as it is involved in differentiation of mesenchymal cells, development of the visual system[61], maturation of the neuromuscular junction[62] and in the regulation of body size, learning and memory[63]. Activation of dALK is achieved upon binding of its ligand; Jelly Belly (Jeb), which consequently activates the downstream Ras-MAPK signaling[64]. However, the mammalian ALK receptor is unable to bind to the Jeb ligand[65], indicating an evolutionary divergence between mammalian and *D. melanogaster* ALK proteins.

On the other hand, in *Caenorhabditis elegans*, the nematode homolog of ALK, suppressor of constitutive dauer formation 2 (SCD-2), is not essential for nematode development but is required for the integration of sensory inputs and development of neuromuscular junctions[66]. SCD-2 is activated by the hesitation behavior (HEN-1) ligand. SCD-2 signaling regulates the dauer response to environmental stress.

In zebrafish, two ALK family members, DrLTK and DrALK, have been identified, which are expressed in neural crest cells (NCCs) and iridophores, a type of pigment cell widely found in anamniote

vertebrates. Mutations in DrLTK leads to the formation of zebrafish shady (shd) mutants, that lack iridophores and a subset of NCCs as a result of apoptosis while, DrALK mutations can impair neurogenesis of zebrafish[67-69]. Till date, no ligands have been reported for ALK in zebrafish. A number of FAM159 (AUG)-like ligands found in zebrafish are suspected to activate zebrafish ALK, although they are yet to be biologically validated.

Mouse and human ALK proteins are 87% similar, with only 4 differing amino acids in the kinase domains. In fact, speculations of ALK role in the development and function of mammalian nervous system arise from ALK expression patterns throughout the nervous system during mouse embryogenesis[25,70,71]. Similar pattern of ALK expression is found in the developing central nervous system (CNS) of chicken and rat with ALK localization characterized to a subset of spinal motor neurons, the sympathetic ganglia and dorsal root ganglia (DRG)[25]. However, ALK mRNA and protein expression diminishes over time from all tissues gradually after birth, ultimately reaching its minimum at three weeks of age and from there on maintained at low levels throughout the adult life of the animal[25]. Consistent to these findings are the observations of weak ALK antibody signals in the CNS from immunohistochemical studies of adult human tissues[72]. Additionally, different ALK mRNA transcripts have also been identified in other adult human tissues such as testis, small intestine, colon, prostate and brain, advocating that differential splicing of ALK may occur in different tissues[23]. Although, the biological function of ALK is not clear in the tissues other than brain.

The role of ALK does not seem critical for adult mice as ALK deficient mice are viable and fertile without any apparent variations[73,74]. However, loss of ALK signaling affects the number of neurons, regeneration of myelinated axons and leads to an increased number of progenitor cells within the hippocampus[73,75].

Various proteins, such as pleiotrophin (PTN), midkine (MK), osteoblast-specific factor-1 (OSF-1), heparin affinity regulatory peptide (HARP) and heparin-binding neurotrophic factor (HBNF), had been suggested to act as the activating ligands of mammalian ALK. However, several recent studies have identified that augmentor α (FAM150B or ALKAL2) and β (FAM150A or ALKAL1) are the bona fide ligands of ALK[76-80].

2.6 Oncogenic ALK: fusion proteins and activating mutations

As mentioned above, physiological ALK expression is restricted to the early development phase and it is only weakly expressed in the later stages of life. On the contrary, oncogenic ALK is expressed constitutively and leads to the neoplastic proliferation of cells in the region it is expressed. As described in the previous sections, constitutive activation and thereby continued expression of RTKs ensues from genetic abnormalities, mainly gene fusions (as a result of translocations) and activating mutations.

ALK fusion proteins mediate tumorigenicity via the formation of dimers by the amino-terminal part of the protein (derived from the 5’ fusion partner) which consequently leads to the activation of the

TKD of ALK. In addition, the N-terminal partner protein drives aberrant expression of the kinase.

ALK activating mutations function by synergizing with other oncogenes, by making the kinase activation ligand-independent or by increasing the expression of ALK through amplifications.

From the time when ALK was first discovered and associated with ALCL in the form of NPM-ALK oncogenic protein, several other forms of ALK have been identified predominantly because of their causal role in hematopoietic and non-hematopoietic malignancies which are discussed below in different categories based on oncogenic ALK fusion or full-length ALK driven malignancies.

3. ALK Fusions Cancers

Many ALK fusion proteins have been described in different types of cancers such as ALCL, inflammatory myofibroblastic tumours (IMTs), non-small cell lung cancer (NSCLC), diffuse large B-cell lymphomas (DLBCLs) and squamous cell carcinoma (SCC) of the esophagus.

3.1 Anaplastic Large Cell Lymphoma (ALCL)

ALCL is described under the lymphoid neoplasms category in the World Health Organization (WHO) classification[81]. It is an aggressive type of Non-Hodgkin Lymphoma (NHL) with neoplastic proliferation of lymphoid cells. ALCL was originally described and

named as Ki-1 lymphoma by Stein and colleagues as these lymphomas constantly expressed Ki-1 (CD30) antigen[82]. The original publication described ALCL as a subset of NHL characterized by anaplastic large lymphoid cells that expressed CD30, a propensity to grow cohesively and predisposition for invading lymph node sinuses[82]. The authors also concluded that Ki-1 lymphoma is a heterogeneous entity with cases representing T-cell, B-cell or null-lineages.

In the latest WHO classification, ALCL is categorised into four different categories: primary cutaneous ALCL (PC-ALCL), systemic Anaplastic Lymphoma Kinase (ALK)-positive ALCL (sALK+ ALCL), systemic ALK-negative ALCL (sALK− ALCL), and Breast Implant-Associated (BIA)-ALCL[83] (Figure 4). In primary cutaneous ALCL, the tumor is restricted only to the skin at the time of the diagnosis. More than 75% of PC-ALCL cells express CD30 antigen. Patients with PC-ALCL usually present with an asymptomatic, solitary skin tumor or nodule that may be ulcerated. Even though PC-ALCL can occur at any age, approximately 50% of PC-ALCL cases are diagnosed in patients aged above 60. It is an indolent neoplasm with good prognosis and five-year survival rates between 76% and 96%[84].

On the other hand, systemic cases of ALCL usually present with involvement of extranodal secondary sites such as soft tissues, skin, bones, lungs and liver. Most of systemic ALCLs can be distinguished based on the expression or the lack thereof of *ALK* gene, in the form of an oncogenic fusion protein with various partner genes. The most common partner gene found to be fused with *ALK* is Nucleophosmin *(NPM1)* as a result of t(2;5)(p23;q35) translocation[23,24,85,86]. While systemic ALK+ ALCL express altered forms of *ALK*, systemic ALK−

ALCL lacks *ALK* expression. Recently, two recurrent rearrangements have been identified in ALK− ALCL[87,88]. Other characteristic differences between the ALK+ and ALK− ALCL are the age of the affected individuals and overall survival (OS). ALK+ ALCL is frequent in young patients (below 30 years) in comparison to ALK− ALCL which has peak incidence in adults (40-65 years). The 5-year OS rate in ALK+ ALCL is approximately 80%, in contrast to only 48% in ALK− ALCL[89,90].

BIA-ALCL is a somewhat new entity that has only been recently recognized by the WHO[83]. BIA-ALCL cells express CD30 but lack ALK expression. BIA-ALCL typically manifests as an accumulation of seroma fluid between the implant and its surrounding fibrous capsule but could also be presented with a discrete mass originating from the fibrous capsule[91]. The clinically indolent form of BIA-ALCL has a good prognosis with 93% of the patients disease-free with a 3-year follow-up[92].

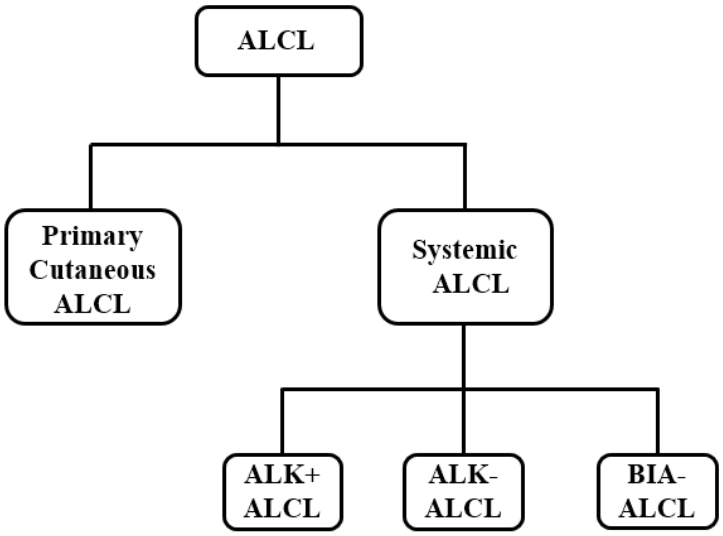

Figure 4. *Schematic representation of ALCL classification based on the revised WHO classification.*

ALK+ ALCL has a better prognosis as compared to its ALK− counterpart. Majority of ALK+ patients are treated with anthracycline-based chemotherapy, with 60% of the patients remaining relapse-free at 5 years. It has also been observed that the favourable prognosis of ALK+ lymphomas may in part be due to the young age at diagnosis[93,94]. At present, ALK+ ALCL are treated with a combination of chemotherapeutic drugs known as CHOP[93]. CHOP consists of the alkylating agent Cyclophosphamide, the intercalating agent Hydroxydaunorubicin (also known as doxorubicin or adriamycin), the tubulin inhibitor Oncovin (or vincristine), and the lympholytic corticosteroid Prednisone (or prednisolone). CHOP therapy is associated with an overall response rate of about 90%. Depending on

the stage of the disease, CHOP therapy can sometimes be followed by radiotherapy.

Patients who do not respond to or relapse very early on the initial chemotherapy regimen can be treated with allogenic stem cell transplantation (ASCT) combined with high-dose chemotherapy (HDC) conditioning regimen. The 5-year overall survival rate with such treatment is 29%. There are certain adverse prognostic factors involved with the use of HDC/ASCT that can affect the OS including the presence of chemoresistant disease at the time of transplant, severe grade 3-4 acute graft-versus-host disease, and an HLA-mismatched donor related mortality[93]. However, the PARMA study reported that the use of HDC/ASCT as a salvage treatment for relapsed patient had superior results as compared to using conventional-dose chemotherapy[95]. Albeit, resistant or refractory ALK+ ALCL patients overall have a worse prognosis[96]. In fact, the 3-year disease free survival (DFS) after the first relapse is 28% for patients with early relapse (<12 months after diagnosis) versus 68% for patients with late relapse.

3.1.1 ALK translocations in ALCL

A large number (~70-80%) of ALK+ ALCL carry NPM-ALK translocation. Immunohistochemical analyses using ALK antibody show that ALK is localized in cytoplasmic as well as nuclear regions of ALCL cells[97]. Besides NPM, several other fusion partners have been described for ALK in ALCL disease setting such as tropomyosin 3 and

4 (TPM3 and 4), TRK fused gene (TFG), 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC), clathrin heavy chain (CLTC), ALK lymphoma oligomerization partner on chromosome 17 (ALO17), moesin (MSN) and non-muscle myosin heavy chain (MYH9)[98]. A novel TRAF1-ALK translocation was also recently identified in an ALK+ ALCL case with leukemic phenotype[99].

Despite the different fusion partners, all the aforementioned fusion proteins share some common features. Across all the fusion proteins, promoter of the partner gene drives the transcription of ALK fusion protein. The localization of the fusion protein is also dictated by the partner protein. And, last but not least, oligomerization of the fusion protein is mediated through the ALK partner protein which in turn induces autophosphorylation and subsequent activation of the ALK kinase domain.

Since the most commonly found translocation in ALCL is NPM-ALK, the chimeric protein has been extensively characterized as compared to other ALK fusions in ALCL. In a typical NPM-ALK fusion protein, the NPM intron 4 is fused to the ALK intron positioned between the transmembrane and the juxtamembrane portions. The fusion protein basically contains the dimerization domain of NPM and a whole functionally active ALK kinase domain. A 680 aa long protein is encoded by the NPM-ALK transcript. Of the 680 aa, the first 117 residues come from NPM while the other 563 aa are ALK residues. Intriguingly, all ALK fusion proteins identified so far contain the same 563 aa comprising the cytoplasmic tail of ALK except a single case report of ALCL carrying a variant breakpoint located in the ALK exon,

coding for the juxtamembrane region. The 'variant' however is essentially an identical fusion protein product with a slightly smaller size differing by just 10 aa[100]. Based on these observations, it could be argued that the ALK sequences present in NPM–ALK are the minimum residues of the protein that are essential to transform a healthy cell into a malignant one.

As mentioned above, the ubiquitously expressed promoter of the partner NPM drives the transcription of NPM-ALK chimeric product, resulting in constitutive and aberrant expression of the functional ALK catalytic domain in cells in which it is normally not expressed. Transforming potential of NPM-ALK has been demonstrated both in *in vitro* and *in vivo* models. Various mouse and rat fibroblast cell lines ectopically expressing oncogenic NPM-ALK were shown to be able to form multiple foci which can grow in an anchorage-independent manner[34,101]. Furthermore, murine pro-B cell line, Ba/F3, stably transfected with human NPM-ALK oncogene gains independence from interleukin-3 (IL-3)[38]. NOD/LtSz-scid mice transfected with Fr3T3-NPM-ALK cells develop subcutaneous xenograft while mice receiving marrow infected with a retroviral construct containing the fusion protein developed tumours[38,102,103].

NPM-ALK downstream signaling includes PLC-γ, PI3K, Ras/ERK1/2 module, and the JAK/STAT pathway[32,57]. CD30 has also been shown to have a functional relationship with ALK. Activation of STAT3 via ERK1/2 pathway results in an upregulation of CD30 expression in ALK+ ALCL. CD30 expression in turn leads to the activation of NF-κB pathways, which result in apoptosis and p21-mediated cell-cycle arrest[104]. Based on the restricted expression of

CD30 in tumor cells, anti-CD30 antibodies are of great interest in ALK-positive ALCL diagnosis and treatment.

3.2 Non-small cell lung cancer (NSCLC)

Lung cancer is the most frequently diagnosed and deadliest cancer worldwide with ~12% of the total cancer cases diagnosed and accounting for approximately 1.7 million deaths per year[105]. Based on histology, lung cancer is categorized into two main groups: non-small cell lung carcinomas (NSCLCs) and small cell lung carcinoma (SCLC), accounting for 85% and 15% of lung cancers, respectively. NSCLC can be further classified into three separate subgroups: squamous cell carcinoma, adenocarcinoma and large cell carcinoma. NSCLC patients are usually diagnosed at very advanced stages leading to poor patient outcomes with a median survival time of less than one year after diagnosis[106].

Cytotoxic chemotherapy, comprised of DNA-binding drugs such as carboplatin/cisplatin combined with either a topoisomerase type II inhibitor such as etoposide or an anti-mitotic such as vinorelbine, are generally used for the treatment of NSCLC patients. Other treatments include surgery and immunotherapies. In metastatic cases, when the tumor has spread from its original site, radiotherapy in combination with chemotherapy is also used.

A large number of driver mutations have been identified in NSCLC, kirsten ras sarcoma viral homolog (KRAS) and EGFR being the two most commonly found mutated genes in lung adenocarcinoma

accounting for 30% and 15% of cases respectively[107]. Identification and targeting of EGFR mutations with small molecular inhibitors such as erlotinib and gefitinib have considerably improved survival of the subset of NSCLC patients carrying EGFR mutations.

However, involvement of an oncogenic translocation in NSCLC was identified in 2007 when Soda et al reported the presence of a fusion between echinoderm microtubule associated protein-like 4 (*EML4*) and *ALK* genes in Japanese NSCLC patients[108]. Approximately 3-7% of NSCLCs carry *ALK* rearrangements, commonly found in the adenocarcinoma subclass and mutually exclusive with *KRAS* and *EGFR* mutations[107,109]. Another characteristic feature of ALK+ NSCLC patients is that they are usually younger and light or non-smokers[110,111]. ALK+ NSCLC is only a minor fraction of the total NSCLC cases, but, given the high incidence of lung cancer cases worldwide, even this small fraction accounts for an approximately 40,000 new cases every year[112]. The comparatively higher number of ALK+ NSCLC cases as compared to ALK+ ALCL is one of the main reasons why the small molecule inhibitors targeting ALK were first only used for the treatment of NSCLC patients.

3.2.1 ALK translocations in NSCLC

Several ALK fusions have been reported in NSCLC, however as in the case of ALK+ ALCL, one fusion prevails over the others. In NSCLC, EML4-ALK is by far the most frequently found fusion. Other less commonly found fusions are KIF5B-ALK, KLC1-ALK, PTPN3-

ALK, HIP1-ALK, STRN-ALK and TPR-ALK[113-119].

EML4 is part of the echinoderm microtubule-associated protein-like proteins family. EML4 is ubiquitously expressed in a wide range of tissues, including the lung. Two different EML4 isoforms exist owing to alternative splicing, the longer isoform containing 981 aa with a molecular weight of 108 kDa, and the shorter isoform composed of 923 aa and a molecular weight corresponding to 102 kDa.

Structurally, EML4 is composed of an N-terminal basic region, a hydrophobic echinoderm microtubule-associated protein-like protein (HELP) domain, and WD repeats. EML4 is a cytoplasmic protein which is highly expressed during mitosis. EML family of proteins interact with soluble tubulin and microtubules and many of the members have been shown to associate with mitotic spindle including EML4[120]. EML4 has also been suggested to modify formation and stability of microtubules[121].

Both, the *EML4* and *ALK* genes, are located on the short arm of chromosome 2, but oriented in opposite directions. The EML4-ALK fusion occurs as a consequence of a small inversion involving 2p21 and 2p23 [inv(2)(p21;p23)][108]. The fusion protein is comprised of the N-terminal portion of EML4, encompassing the basic region, the hydrophobic HELP domain, and a portion of the WD repeat region, and the intracellular kinase domain of ALK. All fusion proteins contain the indispensable intracellular TKD of ALK, from exon 20 to 29. However, EML4 is not always truncated at the same position, which leads to EML4-ALK fusion protein variants (Figure 5). Till date, 15 different EML4-ALK chimeric variants have been reported[122] (Table 1).

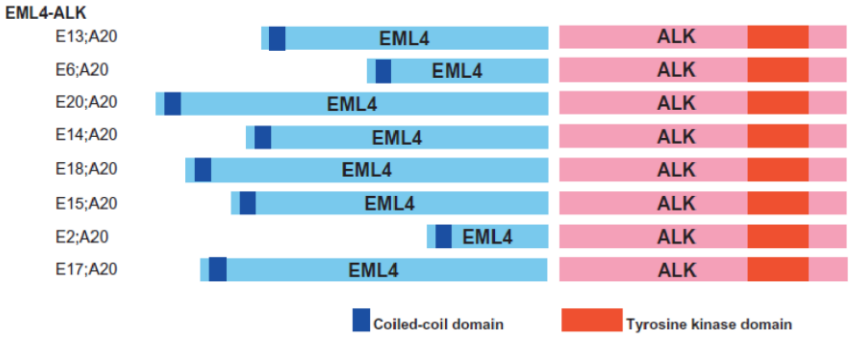

Figure 5. *EML4-ALK variants. A simplified representation of different EML4-ALK variants based on different fusion points involving different exons on EML4 gene depicted by the numbers.*

(adapted from Sasaki T, Rodig SJ, Chirieac LR, Jänne PA. The biology and treatment of EML4-ALK non-small cell lung cancer. Eur J Cancer. 2010 Jul;46(10):1773-80)

The first two variants, variant 1 and 2 (v1 and v2), of EML4-ALK were reported by Soda et al. In v1, intron 13 of EML4 is disrupted, inverted and translocated to exon 20 of ALK, whereas in v2, exon 20 of EML4 is fused to exon 20 of ALK[123]. Later, another two variants, namely, variant 3a and 3b, were identified. Variant 3a contains exons 1-6 of EML4 which are linked to exons 20-29 of ALK. The v3b differs from v3a only by an additional 33 bp sequence which are derived from an alternatively spliced exon of EML4[108,124]. The most commonly found variants are v1(E13;A20), and v3a/b (E6a/b;A20), detected in 33% and 29% of NSCLC patients, respectively[111]

Variant 1 and 2 are reported to preferentially localize in the cytoplasm with only minimal staining in the nucleus, in contrast to the variant 3a which is found equally distributed throughout the cytoplasm

and the nucleus. The differential cellular localization can be attributed to the HELP domain which is present in variants 1 and 2 but not in 3a, suggesting a role of HELP domain in retaining the fusion protein in the cytoplasm[125]. Variant 2 has been reported to have a shorter half-life compared with the other variants indicating that different portions of EML4 provide different stability to the fused protein. These findings point out that the EML4 part of the EML4-ALK fusion does not only induce aberrant kinase activation, but also affects protein localization and stability[125].

Constitutive oligomerization and consequent activation of the EML4-ALK fusion protein is mediated by EML4 via its protein-protein interactions motif. The continuous, uncontrolled kinase activity leads to tumour formation[126]. All of the EML4 domains affect the transforming potential of EML4-ALK fusion protein as deletion of basic, HELP or WD domains resulted in a marked decrease in EML4-ALK catalytic activity[108].

Table 1. *List of EML4-ALK variants outlining variant number, fusion location on EML4 gene and their respective structural features.*

EML4-ALK variant	**Gene fusion points**	**Structural features**
V1	E13;A20	EML4-TD, basic, HELP motif, incomplete TAPE, ALK-TK
V2	E20;A20	EML4-TD, basic, HELP motif, incomplete TAPE, ALK-TK
	E20;ins18A20	EML4-TD, basic, HELP motif, incomplete TAPE, ALK-TK
V3a	E6a;A20	EML4-TD, basic, No HELP or TAPE, ALK- TK
V3b	E6b;A20	EML4-TD, basic, ALK-TK
V4	E15del60;del71A20	EML4-TD, basic, HELP motif, incomplete TAPE, ALK-TK
V4′	E14;ins11del49A20	EML4-TD, basic, HELP motif, incomplete TAPE, ALK-TK
V5a	E2;A20	EML4-TD, ALK-TK
V5b	E2;ins117A20	EML4-TD, ALK-TK
V5′	E18;A20	EML4-TD, basic, HELP motif, incomplete TAPE, ALK-TK
V6	E13;ins69A20	EML4-TD, basic, HELP motif, incomplete TAPE, ALK-TK
V7	E14;del12A20	EML4-TD, basic, HELP motif, incomplete TAPE, ALK-TK
V8a	E17;ins30A20	EML4-TD, basic, HELP motif, incomplete TAPE, No functional ALK domain
V8b	E17ins61;ins34A20	EML4-TD, basic, HELP motif, incomplete TAPE, ALK-TK
	E17;ins68A20	EML4-TD, basic, HELP motif, incomplete TAPE, ALK-TK

(adapted from Bayliss R, Choi J, Fennell DA, Fry AM, Richards MW. Molecular mechanisms that underpin EML4-ALK driven cancers and their response to targeted drugs. Cell Mol Life Sci. 2016;73:1209-1224)

Transforming potential of EML4-ALK has been demonstrated both *in vitro* and *in vivo*. Subcutaneous injection of mouse 3T3 fibroblasts cells, transfected with the *EML4-ALK* fusion gene, into nude mice leads to tumour formation[108]. Ba/F3 cells transfected with human *EML4-ALK* oncogene grow independently of exogenous IL-3[125]. Additionally, the potent oncogenic activity of EML4-ALK protein was confirmed in transgenic mice which expressed EML4-ALK specifically in lung alveolar epithelial cells[123].

EML4-ALK signaling pathways have been mostly deduced based on the effects on ALK phosphorylation upon various treatments. EML4-ALK drives the phosphorylation of AKT and ERK since their phosphorylation is reduced upon ALK inhibition in the H2228 and H3122 ALK+ NSCLC cell lines[124,127,128]. However, EML4-ALK does not seem to influence AKT in Ba/F3 cell lines expressing EML4-ALK fusions. On the other hand, role of STAT3 pathway in EML4-ALK mediated oncogenesis is not so clear. While some studies have reported a significant reduction in STAT3 phosphorylation upon ALK inhibition[128,129], STAT3 phosphorylation was not affected in H2228 and H3122 cells even when ALK was inhibited[124].

3.3 Diffuse large B-cell lymphoma (DLBCL)

DLBCL is a B-cell malignancy. Few and rare cases of DLBCL have been reported to have aberrant expression of ALK, representing less than 1% of all DLBCLs. ALK+ DLBCL predominantly affects male adults with a male to female ratio of 3:1 and spans all age groups

(9-70) with a median age of 36 years[130,131]. While the primary manifestation of the disease usually involves lymph nodes, rare extranodal presentations at sites such as the tongue, nasopharynx, and stomach have also been reported. The specific subset of DLBCL with ALK expression usually have a poor prognosis and an inferior clinical outcome as compared to the typical DLBCL[132]. The ALK+ subpopulation of DLBCL has a plasmablastic and/or immunoblastic morphology, with the expression of ALK, epithelial membrane antigen (EMA), CD138 and single light-chain cytoplasmic immunoglobulin A (IgA). However, unlike the ALK+ ALCL cells, the DLBCL cells lack CD30 expression.

Most of ALK+ DLBCLs carry the t(2;17)(p23;q23) translocation leading to a fusion between *CLTC* gene on chromosome 17q23 with *ALK* gene on chromosome 2p23 and have a unique granular cytoplasmic immunohistochemical (IHC) staining pattern[133]. However, a small number of cases have also been reported to carry other ALK translocations such as NPM-ALK, SEC31A-ALK and SQSTM1-ALK[134-136].

Similar to the NPM-ALK in ALCL, the ALK fusions in DLBCL induce activation of STAT3 indicating a cross-talk between deregulated ALK and STAT3 phosphorylation[43,47].

3.4 Inflammatory myofibroblastic tumor (IMT)

IMTs are benign mesenchymal neoplasms. IMTs have a distinct cellular spindle proliferation that contain malignant myofibroblasts and

infiltrating non-malignant inflammatory cells mainly plasma cells and lymphocytes[137]. IMTs were first reported in the lung, however, they can also manifest in almost every soft tissue and viscera of the body. They are most commonly detected in lung, abdomen, pelvis and retroperitoneum but there are also reports of IMTs of soft tissue, central nervous system (CNS), and bone[138]. IMTs usually affect children and young adults but it can also develop at any age. The IMTs are mostly indolent and can be cured by surgical resection of the mass. Incomplete surgical resections can lead to a local recurrence. Moreover, a smaller percentage of IMTs can also have an aggressive phenotype and can become invasive and metastatic.

IMTs were the first non-haematological tumors where ALK fusion was reported[139]. Around 50% of IMT cases have been reported to have ALK expression. Griffin and colleagues identified two different ALK fusions in IMTs, namely TPM3-ALK and TPM4-ALK[139]. *TPM3* gene is located on chromosome 1 and encodes a non-muscle tropomyosin. In the TPM3-ALK fusion, N-terminal residues of TPM3 are fused to ALK. *TPM4* which is a homolog of *TPM3* is located on chromosome 19. In line with other ALK fusions, the fusion partner, TPM3 or TPM4 in case of IMT, are responsible for the dimerization of the fusion protein which ultimately cause constitutive autophosphorylation and activation of ALK.

Several other ALK fusion partners have also been identified in IMTs, such as ATIC-ALK[140], CLTC-ALK[141], CARS (cysteinyl-tRNA synthetase)-ALK[142], RANBP2 (Ran-binding protein 2)-ALK[141] and SEC31L1 (SEC31 homologue A)-ALK[143].

ALK expression has been correlated with a better prognosis in IMT[144,145] although the functional relevance and consequences of ALK signaling in ALK-positive IMTs are not yet fully understood.

3.5 ALK-positive systemic histiocytosis

ALK-positive systemic histiocytosis is a relatively new entity which was first described in 2008. Chan et al, reported for the first time 3 cases of a new form of histiocytosis with distinctive morphology and *ALK* expression[146]. The initial three cases of ALK-positive histiocytosis were reported in infants. The three cases showed immunoreactivity against ALK and in one, the TPM3-ALK fusion was detected. Despite the slow response to treatment, all the patients had favourable outcome even in the patient who did not undergo cytotoxic treatment.

The same group recently described analysis of 10 patients with ALK+ histiocytosis, including 3 patients from the first report[147]. Systemic disease was confined to young patients while patients with localized disease tended to be older. The group detected 2 additional ALK fusions in patient biopsies, namely, KIF5B-ALK and COL1A2-ALK. COL1A2 gene encodes the alpha 2 chain of type I collagen protein. One KIF5B-ALK-positive patient treated with the ALK inhibitor, crizotinib, had a rapid disease resolution as compared to the slower spontaneous resolution in rest of the patients.

3.6 Esophageal squamous cell carcinoma (ESCC)

ESCC is a subtype of esophageal carcinoma arising mainly from epithelium lining the gullet. ESCC are usually found in the upper two third region of the esophagus.

ALK expression in ESCC have been described by two separate groups using proteomics approach. An unreported number of ESCC cases expressed TPM4-ALK protein[148,149]. However, the association of ALK fusion in the pathogenesis of the disease is yet to be understood. As the 5-year survival rate for ESCC patients is only 5-15%, perhaps unravelling the role of ALK in the disease might pave the way for ALK based therapies and improve patient survival.

3.7 ALK rearrangements in other cancers

There are several other ALK fusions that have been reported in different types of cancer, although at low frequencies[150] (Table 2). For example, CLTC-ALK fusion has been detected in extramedullary plasmacytoma[151]. A new ALK fusion partner, vinculine protein (VCL) was found in renal cell carcinoma[152]. Moreover, EML4-ALK expression was detected in colorectal cancer as well as breast cancer specimens[153]. Adding to the list of ALK fusion partners is also a protein named C2orf44 (also known as WDCP) that was identified in a colorectal cancer sample[154]. The C2orf44-ALK fusion originates from a tandem duplication in chromosome 2. However, the biological relevance of the protein is not clear.

Table 2. *List of various ALK rearrangements in human malignancies.*

Cancer Type	ALK Fusion Partner (Chromosomal Localization)	Frequency %
ALCL	NPM1 (5q35.1), TPM3 (1q21.3), ATIC (2q35), TFG (3q12.2), TRAF1 (9q33.2), CLTC (17q23.1), RNF21 (17q25.3), TPM4 (19p13.1), MYH (22q12.3), MSN (Xq12) Additional rare rearrangements	~55% (in adults)
Breast cancer	EML4 (2p21)	N.D.
Colorectal cancer	EML4 (2p21), WDCP (2p23.3)	<1%
DLBCL	RANBP2 (2q13), EML4 (2p21), SEC31A (4q21.22), SQSTM1 (5q35), NPM1 (5q35.1)	<1%
Esophageal cancer	TPM4 (19p13.1)	N.D.
IMT	TPM3 (1q21.3), RANBP2 (2q13), ATIC (2q35), SEC31A (4q21.22), CARS (11p15.4), PPFIBP1 (12p11), CLTC (17q23.1), TPM4 (19p13.1)	Up to 50%
NSCLC	EML4 (2p21), TPR (1q31.1), CRIM1 (2p22.2), STRN (2p22.1), TFG (3q12.2), HIP1 (7q11.23), PTPN3 (9q31), KIF5B (10p11.22), KLC (14q32.3), CLTC (17q23.1)	3–7%
Ovarian cancer	FN1 (2q35)	N.D.
Renal Cell Carcinoma	VCL (10q22.2), TPM3 (1q21.2), EML4 (2p21), STRN (2p22.2)	<1%
Renal Medullary Carcinoma	VCL (10q22.2)	N.D.

(adapted from Sharma GG, Mota I, Mologni L, Patrucco E, Gambacorti-Passerini C, Chiarle R. Tumor resistance against ALK targeted therapy-where it comes from and where it goes. Cancers (Basel) 2018:10)

4. Full-length (Non-fusion) ALK-positive cancers

Full-length ALK protein can also attain oncogenic potential either by acquiring gain-of-function point mutations in the ALK TKD or by having multiple copies of the wild-type full-length ALK. While point mutations in the TKD lead to conformational changes and a ligand-independent activation of ALK, multiple copies of the wild-type ALK provide with an increased expression of the protein kinase. Role of full-length ALK, even though it is less frequently activated in tumors as compared to the translocated ALK, has been documented in several types of tumors including neuroblastomas[155], glioblastomas[156,157], rhabdomyosarcoma[158] and melanoma[159]. Additionally, breast carcinoma, malignant peripheral nerve-sheath tumors, and lipogenic tumors have been reported to have anti-ALK immunoreactivity[160,161]. However, if the source of the reactivity is full-length or fused ALK, has not been clearly identified.

4.1 Neuroblastoma (NB)

Neuroblastoma (NB) is the most commonly diagnosed solid tumor of childhood accounting for 6% of all cancers in children. It is an embryonal tumor derived from the developing neural crest cells. It often arises from the peripheral sympathetic nervous system mostly in the adrenal medulla, but it can also present as mass lesions in the neck, chest, abdomen or pelvis. NB is clinically heterogenous with some cases being quite aggressive and with rapid progression while others

undergoing spontaneous regression even with limited therapy. Most common genetic characteristics of NB include the presence of v-myc myelocytomatosis viral-related oncogene, NB-derived (MYCN), chromosomal abnormalities such as deletions of parts of chromosome arms 1p and 11q, gain of parts of chromosome 17q and triploidy[155]. MYCN amplification is usually associated with poor patient outcomes.

Evidence of full-length ALK expression in neuroblastoma was first reported by Lamant and colleagues in 2000[155]. The authors detected ALK in 22 out of 24 primary neuroblastoma patient samples. Involvement of ALK in neuroblastoma was further demonstrated by several other groups who found ALK locus amplification in NB cell lines[162] and patient samples[163]. Furthermore, a number of studies described *ALK* point mutations in familial as well as sporadic neuroblastoma[164,165]. Majority of these mutations are clustered within the ALK kinase domain and are activating in nature. *ALK* expression has been shown to be a predisposition gene. While almost 50% of the familial NB cases harbour germline gain-of-function *ALK* mutations, only 7% of sporadic NB patients were found to have activating *ALK* mutations[166]. Two mutations, F1174L and R1275Q have been characterized and shown to activate ALK via autophosphorylation[165,167]. Interestingly, the F1174L mutation mostly occur in association with *MYCN* amplification suggesting that the two genetic aberrations work in synergism to attain higher oncogenic potential[168,169]. While the presence of ALK mutations does not affect survival rate, increased ALK expression is associated with poor prognosis.

4.2 Other diseases

ALK has been implicated in many other diseases as well. For example, gain-of-function ALK mutations, L1198F and G1201E have been described in Anaplastic thyroid carcinoma[170]. Aberrant expression of ALK has also been reported in breast cancer[171,172]. Van Gaal and colleagues reported differential expression of ALK between different subtypes of rhabdomyosarcoma as a result of an elevated *ALK* gene copy number[173]. ALK transcripts have also been detected in retinoblastoma, melanoma and breast carcinoma[159].

Glioblastoma cases have also been reported to have strong ALK expression driving non-hypoxia-driven mechanism of neovascularization[174].

5. Rationale behind targeting ALK

Since the first report of ALK in the pathogenesis of ALCL[23] in 1994, ALK has been implicated in a number of other diseases. ALK transformed cells are in general strongly dependent on the kinase activity of ALK, for their growth and survival. Additionally, it has become clear that expression of normal ALK is restricted to the early stages of development and then retained at low levels in few specific tissues[32]. All these factors make ALK an ideal target to be used for pharmacological use because its blockage is catastrophic for cancer cells but irrelevant for normal tissues.

Recognition of ALK fusion proteins as a target for therapeutic intervention was also strengthened by the successful use of imatinib, a tyrosine kinase inhibitor (TKI) for the treatment of Chronic Myeloid Leukaemia (CML)[175]. A subset of CML patients, termed as Philadelphia-positive patients, were found to carry a t(9;22)(q34;q11) translocation leading to the fusion of breakpoint cluster region (*BCR*) gene present on the chromosome 22 and *ABL* gene. Five years follow-up studies of Philadelphia-positive CML patients treated with imatinib have shown that the patients achieved deep molecular responses and their overall survival was not different from the general population[176].

Another key factor that propelled the discovery and development of ALK inhibitors was implication of ALK in NSCLC. As mentioned earlier, despite the small percentage of ALK+ NSCLC cases, the overall absolute number is quite high. Availability of patient samples as well as the possibility of curing a larger population, ALK inhibitors were mainly developed and approved for use in ALK+ NSCLC patients.

Till date, around 10 ALK inhibitors have been developed (Table 3). All of them target ALK but also a few other known oncogenic target genes such as proto oncogene *ROS1*, *c-MET* and others. Most of them have been tested in clinic and are now approved for use in ALK+ NSCLC by the Food and Drug Administration (FDA) while some are still in preclinical stages. All the ALK inhibitors are described below along with their pharmacological profiles as well as clinical trial results, if any.

Table 3. *Different ALK inhibitors and their activity.*

Inhibitor	Targeted Kinase/s	Activity against Mutant Forms	Clinical Evidence	Brain Penetrance
Crizotinib (Xalkori–Pfizer)	ALK, c-MET, sROS1	L1198F (EML4-ALK)	Phase I Phase II Phase III	No
Ceritinib s(Zykadia–Novartis)	ALK, IGR-1R, INSR, STK22D	I1171T/N, L1196M, S1206C/Y, G1269A/S (EML4-ALK)	Phase I Phase II Phase III (NCT02393625)	Yes
Alectinib (Alecensa–Roche)	ALK, LTK, GAK	L1152P/R, C1156Y/T, L1196M, F1174C/Y, S1206C/Y, G1269A/S (EML4-ALK)	Phase I Phase II Phase III (NCT02075840)	Yes
Brigatinib (AP26113-Ariad)	ALK, ROS1	I1151Tins, C1156Y/T, L1196M, L1152P/R, F1174C/L/V, G1269A/S, G1202R (EML4-ALK)	Phase I Phase II Phase III (NCT02094573)	Yes
PF-06463922 (Lorlatinib-Pfizer)	ALK, ROS1	L1196M, G1269A, S1206Y, C1156Y, F1174L, L1152R, 1151Tins (EML4-ALK) L2026M, G2032R(ROS1)	Phase I Phase II (NCT01970865) Phase III (NCT03052608)	Yes (NCT02927340)
RXDX-101 (Entrectinib-Ignyta)	ALK, ROS1, TrkA, TrkB, TrkC	C1156Y, L1196M (EML4-ALK)	Phase I (ALKA-372-001, STARTRK-1; NCT02097810)	Yes
ASP3026 (Astellas Pharma)	ALK, ACK, ROS1	L1196M (EML4-ALK) I231N(NPM-ALK) L256Q(NPM-ALK)	Phase I (NCT01284192)	Not Described.
X-376 and Ensartinib (X-396) (Xcovery)	ALK, MET	L1196M, C1156Y (EML4-ALK)	Phase I/II (X-396) (NCT01625234) Phase III; NCT02767804 (ongoing)	Yes
CEP-37440 (Teva)	ALK, FAK	Not Described.	Phase I (NCT01922752)	Not Described.
Belizatinib (TSR-011) (Tesaro)	ALK, TrkA/B/C	Not Described.	Phase I/IIa (NCT02048488)	Not Described.

(adapted from Sharma GG, Mota I, Mologni L, Patrucco E, Gambacorti-Passerini C, Chiarle R. Tumor resistance against ALK targeted therapy-where it comes from and where it goes. Cancers (Basel) 2018:10)

6. First Generation ALK inhibitor

6.1 Crizotinib: The first generation ALK inhibitor

Crizotinib also known as PF-2341066 or Xalkori^{TM} (IUPAC name [(R)-3-[1-(2,6dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1Hpyrazol-4-yl)-pyridin-2-ylamine) is an orally available ATP-competitive and selective small molecule inhibitor developed by Pfizer (Figure 6). It was originally synthesized as a c-Met inhibitor. Ironically, cross-sectional studies against different RTKs revealed that crizotinib had off-target effects on ALK, at pharmacologically relevant concentrations[177].

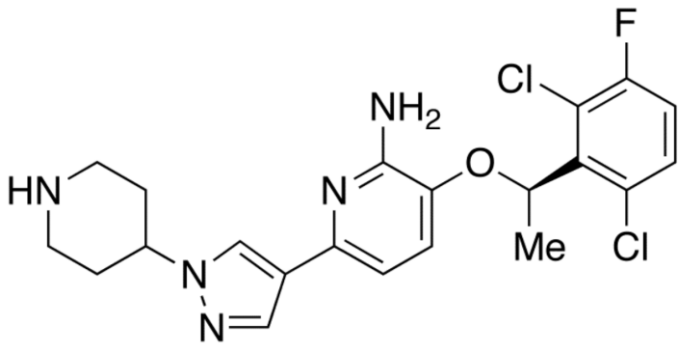

Figure 6. *Structure of crizotinib.*

(adapted from Roskoski R Jr. Anaplastic lymphoma kinase (ALK): structure, oncogenic activation, and pharmacological inhibition. Pharmacol Res. 2013 Feb;68(1):68-94)

Crizotinib binds in the ATP pocket of MET kinase in a DFG-in conformation, forming classical hydrogen bonds (Hb) with hinge region residues[178]; in addition, its phenyl ring forms a π-π interaction with the A-loop. Crystal structure of crizotinib bound to ALK revealed a similar binding mode, with conserved Hb to the hinge region, but lacking the π stacking to A-loop, which might explain lower activity of the compound against ALK compared to MET (Figure 7). However, since ALK had already been implicated in ALCL, NSCLC and IMT around that time, crizotinib was then pursued as an ALK inhibitor intended for use in ALK malignancies.

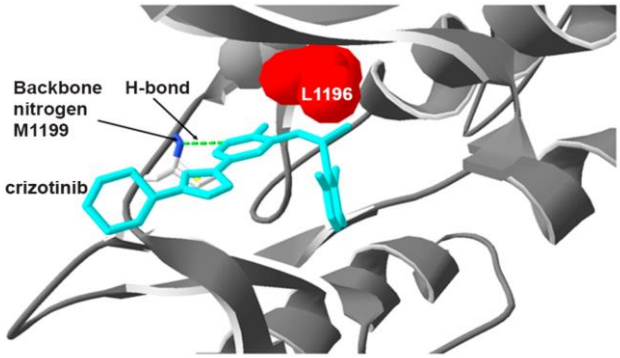

Figure 7. *Close view of Crystal structure of ALK in complex with crizotinib (PDB: 2XP2). crizotinib binds in the active site of wild-type ALK. The gatekeeper residue L1196 is shown as red surface while crizotinib as cyan sticks. The green dashed line indicates the hydrogen bond between the backbone nitrogen of M1199 (indicated in sticks).*

(adapted from Sharma GG, Mota I, Mologni L, Patrucco E, Gambacorti-Passerini C, Chiarle R. Tumor resistance against ALK targeted therapy-where it comes from and where it goes. Cancers (Basel) 2018:10)

Crizotinib inhibited phosphorylation of wild-type c-MET with a mean IC_{50} (drug concentration able to inhibit 50% of cell growth) of 11 nmol/L across a panel of human tumor and endothelial cell lines. It also showed similar activity against a number of c-MET mutants with IC_{50} ranging from 2-19 nmol/L, however c-MET activation loop mutants had a significant increase in the IC_{50} values reaching up to 127 nmol/L[177]. Kinase selectivity assays showed that crizotinib was selective for three RTKs; c-MET, ALK and RON, all three of which had an IC_{50} lower than 100nM in the cells.

In vitro studies revealed that crizotinib was able to inhibit cell proliferation of ALCL, NSCLC as well as NB cell lines by inducing G1-S phase cell cycle arrest, while apoptosis was limited only to the lymphoma cell lines. Immunodeficient mice bearing Karpas 299 (an ALCL patient derived cell line) tumor xenografts regressed upon crizotinib treatment[179]. Crizotinib treatment also led to significant reduction in the phosphorylation and thereby inhibition of ALK, PLC-γ, and STAT3 activity while AKT and ERK phosphorylations could only be reduced with higher doses of crizotinib and only partially inhibited. Based on the promising *in vitro* and *in vivo* results crizotinib entered into clinical trial.

6.1.1 Crizotinib for the treatment of ALK+ NSCLC

Phase I dose escalation study[180] of crizotinib (ClinicalTrials.gov Identifier: NCT00585195) enrolled 37 patients carrying ALK, c-MET and ROS abnormalities in any type of solid tumor refractory to standard

therapies. In terms of safety and toxicity, crizotinib was found to be well tolerated in patients, usually with grade 1 or grade 2 treatment related adverse events (TRAEs). Most commonly observed TRAEs included nausea, emesis, fatigue and diarrhoea. Severe side effects of grade 3 and grade 4 were also reported with elevated transaminases and neutropenia. Most of the TRAEs could be reversed upon crizotinib discontinuation. Based on the results of this study, 250 mg of crizotinib b.i.d was established as the maximum tolerated dose (MTD)[180].

The single arm phase I study of crizotinib (PROFILE 1001) showed impressive results in ALK+ NSCLC patients refractory to previous treatments with an objective response rate (ORR) of 61% and a median progression-free survival (PFS) of 9.7 months[181]. The authors reported that the results were superior in the patients who received crizotinib as first-line therapy with a median PFS of 18.3 months which prompted the multicentre, open-label, single-arm phase II trial (PROFILE 1005) of the drug. PROFILE 1005 recruited ALK+ NSCLC patients who had relapsed after ≥1 line of chemotherapy. Final results of the study were published recently which reaffirmed efficacy of crizotinib with an ORR of 54 and 41% in the central- and local-testing ALK-detection subgroups, respectively[181,182].

Subsequent phase III trials, PROFILE 1007[183] and PROFILE 1014[184] further confirmed the superiority of crizotinib over standard chemotherapy regimens in advanced stage ALK+ NSCLC. In PROFILE 1007 study, 347 ALK+ NSCLC patients who had relapsed on platinum-based chemotherapy were randomised to receive crizotinib or second-line chemotherapy. Patients who received crizotinib were reported to have an ORR of 65% in comparison to an ORR of 20% in

patients who received second-line chemotherapy. The median PFS was also higher (7.7 months versus 3 months) in patients treated with crizotinib[183]. Similar trend was observed in PROFILE 1014 study, in which treatment naïve ALK+ NSCLC patients were recruited to receive either crizotinib or pemetrexed plus platinum chemotherapy. Concordant with the previous studies, PFS was longer (10.9 versus 7 months) and ORR (74% versus 45%) were higher in patients receiving crizotinib as compared to patients receiving chemotherapy, respectively[184]. However, both the studies did not show any significant difference in terms of overall survival in the patients who received crizotinib which could be because of the treatment crossover to crizotinib, which was permitted upon progression to chemotherapy in both studies.

Over the years, few other uncommon TRAEs in patients receiving crizotinib have also been observed which include interstitial lung disease (ILD), bradycardia, QTc prolongation, renal cysts and decreased total testosterone in males which were all reversible upon discontinuation of the drug or a drug holiday period. The above-mentioned studies characterized and established the efficacy and safety profile of crizotinib and eventually led to the approval of crizotinib by FDA for the treatment of locally advanced or metastatic ALK-positive NSCLC in 2011.

6.1.2 Crizotinib for the treatment of ALK+ cancers other than NSCLC

Given the evidence of the involvement of ALK in other diseases, organically, crizotinib has also been evaluated to treat diseases other than NSCLC. In 2010, Butrynski and colleague reported the use of crizotinib in a RANBP2-ALK-positive IMT patient[185]. Crizotinib led to a rapid and substantial partial response in the patient that lasted for 6 months, however additional lesions were detected in the patient indicating that the tumor burden was still increasing while the patient was still on crizotinib. Few other reports have also described the use of crizotinib, with varying degree of response from partial to complete, in ALK+ IMT patients[186-188].

Activity of crizotinib was also described in 2 ALK+ ALCL patients with impressive short-term therapeutic outcomes[189]. Based on the encouraging results, a phase 1b open-label study (PROFILE 1013) was initiated to evaluate the safety and antitumor activity of crizotinib in ALK+ malignancies including advanced ALCL, IMT and other ALK+ tumors excluding NSCLC[187]. The investigators of the study reported an ORR of 52.9% (95% CI, 27.8–77.0) and PFS at 2 years of 63.0% (95% CI, 35.3–81.4) in the lymphoma group that consisted of 17 evaluable patients. In contrast, the ORR in 'other tumors' group was quite low with 11.8% (95% CI, 1.5–36.4) which could be attributed to various factors such as different ALK signaling events in different tumor types and genetics of the tumor cell. An interesting finding of the study was that the progression/relapse occurred only in the initial 90

days of crizotinib treatment in the lymphoma group indicating that the initial 3 months of crizotinib treatment are crucial.

Moreover, crizotinib was also approved in the United states and European Union for the treatment of ROS1+ NSCLC patients with advanced disease. Additionally, a number of clinical trials are currently ongoing to evaluate the efficacy of crizotinib in various ALK+ cancers such as in melanoma, breast cancer, gastric cancer (ClinicalTrials.gov identifier: NCT01121588)[190].

7. Second Generation ALK Inhibitors

Despite the remarkable activity of crizotinib in ALK+ cancers, resistance invariably emerges and most of the patients relapse usually within the first two years of treatment. However, in many cases, the resistant tumor is still driven by oncogenic ALK. It has been reported that around 30% of ALK+ NSCLC patients acquire secondary mutations in the TKD of ALK leading to resistance against crizotinib[112], as described below in detail.

Another limitation in improving the survival of NSCLC patients is the presence of brain metastasis (BM) at baseline which is observed in approximately 20% of ALK+ NSCLC patients at diagnosis[191]. Presence of drug-efflux transporters in the central nervous system (CNS) limits the blood-brain barrier penetration of pharmacological compounds which makes CNS as one of the leading progression sites in NSCLC patients. Since crizotinib is a substrate for the ATP-binding cassette (ABC) drug efflux transporters, P-glycoprotein and ABC

subfamily G member 2, its accumulation in the brain is limited. Henceforth, CNS progression was the most common progression site in patients treated with crizotinib[192]. In a retrospective analysis of patients with untreated BM pooled together from PROFILE 1005 and 1007 studies revealed that the intracranial efficacy of crizotinib was inferior as compared to its systemic efficacy (ORR of 18% versus 53%)[193]. Moreover, no significant improvement in intracranial time to progression was detected in PROFILE 1014 study with crizotinib treatment as compared to chemotherapy[194].

These factors led to the development of next-generation ALK inhibitors that are designed to be more potent, selective and structurally different in order to overcome crizotinib resistance. At present, nine new ALK inhibitors have been reported. Most of these new ALK inhibitors have demonstrated potent and durable activity in ALK+ NSCLC.

7.1 Ceritinib (LDK378; Zykadia; Novartis)

Ceritinib is a selective ALK inhibitor which is 20-fold more potent than crizotinib[195]. Ceritinib was synthesized from another original compound, NVP-TAE684, by making some significant structural changes in order to improve the kinase selectivity and reduce the formation of reactive metabolites which led to toxicity[195,196] (Figure 8).

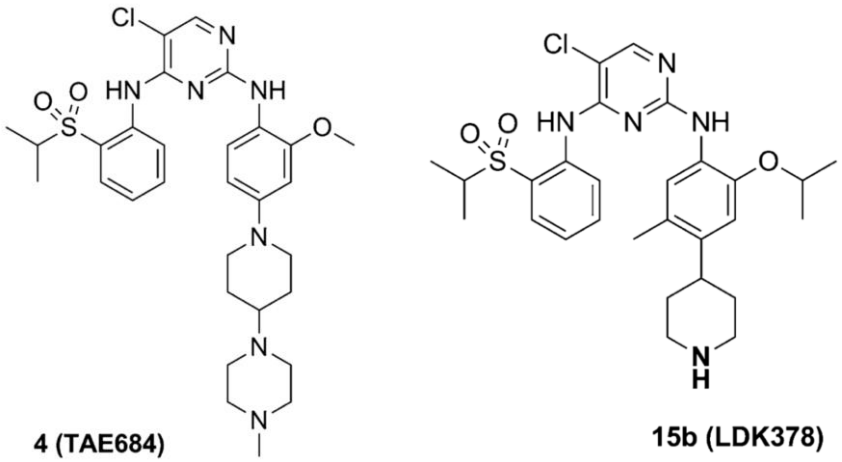

Figure 8. *Structure of the original compound NVP-TAE684 and the new compound, ceritinib (LDK378).*

(adapted from Marsilje,TH, Pei,W, Chen,B, Lu,W, Uno,T, Jin,Y, Jiang,T, Kim,S, Li,N, Warmuth,M, et al. Synthesis, structure-activity relationships, and *in vivo* efficacy of the novel potent and selective anaplastic lymphoma kinase (ALK) inhibitor 5-chloro-n2-(2-isopropoxy-5-methyl-4-(piperidin-4-yl)phenyl)-n4-(2-(isopropylsulf onyl)phenyl) pyrimidine-2,4-diamine (LDK378) currently in phase 1 and phase 2 clinical trials. J. Med. Chem. 2013, 56, 5675–5690)

Kinase selectivity and pharmacokinetic profile assays of ceritinib showed potent activity against ALK with an IC_{50} value of 20nM in ALK+ ALCL and NSCLC cell lines[195]. Ceritinib was also found to be active against insulin-like growth factor 1 receptor (IGF-1R), IR and ROS1 but at comparatively higher doses (5-11 fold higher). *In vitro* studies demonstrated that ceritinib inhibited mutated NPM-ALK harbouring crizotinib resistant mutations L1196M, G1269A, I1171T and S1206Y in ALCL cell line models, although it was not effective against the G1202R and F1174C mutants[195]. This preferential

activity of ceritinib can be explained by the structural design of the compound. For example, the glycine to alanine substitution at position 1269 (G1269A) in ALK creates steric hindrance for the halogenated phenyl ring of crizotinib but not in ceritinib binding. Similarly, ceritinib interacts equally well with Leucine1196 as with Methionine1196. Both, crizotinib-sensitive and crizotinib-resistant tumors of ALK+ NSCLC mouse xenograft models showed marked tumor regression upon ceritinib treatment[195,197].

Consequently, ceritinib was evaluated in a phase I study (ASCEND-1) for its safety and antitumor activity in ALK+ NSCLC and other cancers harbouring ALK alterations[198]. The established maximum tolerated dose for ceritinib was 750 mg daily. Updated results of the trial were published recently, where the authors reported an ORR of 56% and median PFS of 6.9 months with ceritinib treatment in patients who had relapsed on crizotinib[199]. On the other hand, a higher ORR and longer PFS, 72% and 18.4 months, were observed in ALK-inhibitor naïve patients indicating that ceritinib was more potent in comparison to crizotinib. Ceritinib was also reported to induce intracranial responses.Most commonly observed adverse events of ceritinib treatment included diarrhoea, vomiting, dehydration, elevated aminotransferase levels, and hypophosphatemia[199].

Based on the activity and safety results of ASCEND-1, two phase II trials (ASCEND-2 and ASCEND-3) were initiated in ALK+ NSCLC patients previously treated or untreated with an ALK TKI. Results from both the trials confirmed durable antitumor activity and safety of ceritinib in ALK+ NSCLC patients including those who had brain metastases[200,201]. Ceritinib was subsequently assessed in a

randomised, open-label phase III (ASCEND-4) trial as a first line treatment in ALK+ NSCLC versus platinum-based chemotherapy[202]. Results showed that the median PFS in ceritinib arm was double in comparison to the chemotherapy arm (16.6 months versus 8.1 months). Another phase III trial of ceritinib, ASCEND-5, compared the activity of ceritinib to standard chemotherapy in ALK+ NSCLC patients who had progressed following previous chemotherapy and crizotinib[203]. In line with previous studies, ASCEND-5 study showed that ceritinib was superior to standard chemotherapy in terms of PFS. However, the OS was not different between the two treatment groups. Following the results of these studies, ceritinib was approved at a daily dose of 750 mg in a fasted state in first-line for ALK+ metastatic NSCLC.

All the clinical trials found that most of the TRAEs with ceritinib treatment were gastrointestinal (GI) toxicity related. To investigate this, a multicenter, randomized open-label study ASCEND-8 was started. The study compared the safety profile of ceritinib at lower doses (450 mg or 600 mg) taken daily with a low-fat meal and ceritinib 750 mg taken daily in fasted state in patients with ALK+ NSCLC[204]. The investigators found that a lower dose of ceritinib (450 mg) taken with food reduced the number of GI toxicity related adverse events (AEs). Most of the GI toxicities in the 450 mg dose arm were of low grade and a lower number of patients needed dose adjustment or drug interruption in comparison to the 600 mg ceritinib arm. The results indicated that a lower dosage of 450 mg ceritinib taken with food maintains the same exposure as the currently approved dose of 750 mg fasted but with a more favourable GI toxicity profile[204].

7.2 Alectinib (CH5424802; Chugai-Roche)

Alectinib is a benzo[b]carbazole derivative, highly selective, potent and orally available inhibitor of ALK[205] (Figure 9). In cell-free assays, IC_{50} of alectinib against ALK was 1.9nM[206]. Apart from ALK, alectinib was also found to have activity against LTK and GAK kinases. Preclinical studies indicated that alectinib was active against various crizotinib-resistant mutations such as C1156Y, F1174L and G1269A as well as the gatekeeper L1996M mutation but was less effective against the G1202R mutation[205,207].

X-ray crystal structure studies of alectinib bound-ALK showed that alectinib binds to the ATP binding site of ALK by forming a canonical Hb with Methionine1199 (M1199) and in the DFG-in mode. Alectinib seems to be embedded in a stabilizing global Hb network which can probably compensate for any single mutation at the binding site. In silico studies showed that alectinib establishes a π interaction with Leu1196, which is maintained even when leucine is mutated to methionine[205].

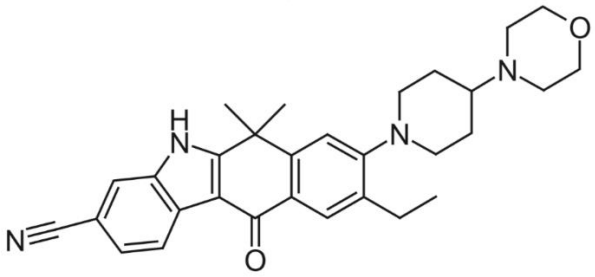

Figure 9. *Structure of alectinib.*

(adapted from Kinoshita K, Asoh K, Furuichi N, et al. Design and synthesis of a highly selective, orally active and potent anaplastic lymphoma kinase inhibitor (CH5424802). Bioorg Med Chem. 2012;20(3):1271–80)

Alectinib was evaluated in clinical settings in a multicenter, single-arm, open-label, phase I/II trial (AF-001JP) conducted in a crizotinib-naïve Japanese population[208]. The phase I part of the trial investigated dosage of alectinib. Escalating doses starting from 20 mg and going up to 300 mg twice daily were evaluated in 24 patients. Since alectinib was well tolerated and no dose limiting toxicities (DLT) or grade 4 AEs were detected at the highest dose, 300 mg twice daily was selected as the acceptable dosage for further evaluations in phase II study. In the phase II part of the study, an ORR of 93.5% was reported in TKI-naïve ALK+ NSCLC patients with alectinib treatment. TRAEs included dysgeusia, increased aspartate aminotransferase (AST), increased blood bilirubin, increased blood creatinine, rash, constipation, and increased alanine aminotransferase. Most of the events were of grade 1 or grade 2, with grade 3 AEs reported only in 12 (26%) patients, with 5 experiencing serious AEs. However, no grade 4

AEs were observed[208]. In addition, alectinib also showed activity in patients with CNS involvement.

After the initial dose finding study of alectinib in crizotinib-resistant ALK rearranged NSCLC in the US[209] and Canadian[210] population, 47 patients were treated with a 600 mg twice daily dose of alectinib. The updated results of the study showed an ORR of 52% and a median duration of response of 13.5 months. Median PFS was reported as 8.1 months and although 1-year OS was not reached at the time of data cut-off, it was estimated to be 71%. Alectinib showed a remarkable effect on the brain metastases with an intracranial response rate of 75% including a CNS disease control rate (DCR) of 89% in the patients who were not previously treated with radiotherapy for their CNS lesions. Consistently with the Japanese study, the phase II trial reported that alectinib was well tolerated with mostly grade 1 or 2 toxicities[210].

Another phase II global study (ClinicalTrials.gov Identifier: NCT01801111), evaluated the safety and efficacy of alectinib in 138 crizotinib-refractory ALK+ NSCLC patients[211]. With a 600 mg twice daily dosage, an ORR of 48% and 50% were reported from the North American and global study respectively. Similar, previously observed, AEs were reported from both the studies. A higher intracranial response rate was noticed in the North American study in comparison to the global study (75% versus 57%, respectively).

Subsequently, two phase III clinical trials were designed to compare alectinib versus crizotinib for the treatment of advanced ALK+ NSCLC. In the ALEX trial, 303 ALK rearranged NSCLC patients were randomised to receive either alectinib (600 mg b.i.d) or

crizotinib (250 mg b.i.d). While the median PFS was not reached in the alectinib arm at the time of data cut-off (95% CI, 17.7 months to not estimable), PFS was 11.1 months in the crizotinib arm (95% CI, 9.1–13.1). In line with the phase II studies, alectinib exhibited a superior intracranial response in comparison to crizotinib[212].

On the other hand, the Japanese phase III trial of alectinib (J-ALEX) evaluated the efficacy of alectinib versus crizotinib in chemotherapy naïve or treated advanced ALK+ NSCLC patients[213]. 207 patients were randomly assigned to receive oral alectinib (300 mg b.i.d) or crizotinib (250 mg b.i.d). A longer PFS was found to be associated with alectinib treatment with a median PFS not reached at data cut-off (95% CI, 20.3–not estimated) as compared to crizotinib treatment where PFS was 10.2 months (95% CI, 8.2–12.0).

Alectinib was granted a breakthrough therapy designation by the FDA for ALK+ NSCLC patients upon progression on crizotinib, while it was approved in Japan in 2014 for the treatment of ALK-rearranged NSCLC patients.

7.3 Brigatinib (AP26113; Ariad)

Brigatinib is a next-generation, potent ALK/ROS1/EGFR inhibitor. It has been shown to have a pan-ALK inhibitory activity, since it was able to block all crizotinib-resistant mutants in pre-clinical models[214-216]. However, the ALK G1202R mutant, one of the most resistant ALK mutations, was only inhibited at significantly higher doses[216,217]. *In vitro*, it was reported to be 10-fold more potent and

selective than crizotinib when tested in both lung and ALCL cell lines, as well as in Ba/F3 EML4-ALK cell model[218]. Structurally, brigatinib is comparable to NVP-TAE684, with modifications in the original sulfonyl group, which is replaced by a phosphine-oxide moiety. Based on the structure activity relationship (SAR) data, the replaced group confers higher selectivity to the compound[219] (Figure 10).

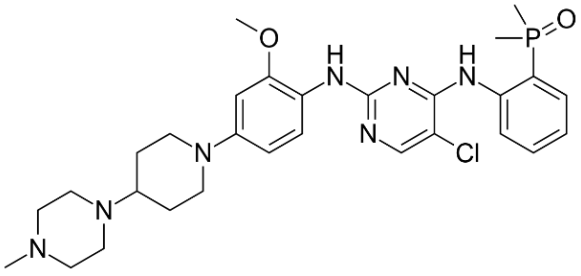

Figure 10. *Structure of brigatinib.*

(adapted from Huang, WS, Liu, S, Zou, D, Thomas, M, Wang, Y, Zhou, T, Romero, J, Kohlmann, A, Li, F, Qi, J, et al. Discovery of brigatinib (AP26113), a phosphine oxide-containing, potent, orally active inhibitor of anaplastic lymphoma kinase. J. Med. Chem. 2016, 59, 4948–4964)

Results from a phase I/II study (ClinicalTrials.gov identifier: NCT01449461) of brigatinib were recently reported[220]. The study was designed to evaluate the efficacy and safety of brigatinib in patients with advanced malignancies other than leukaemia. Three dose regimes were identified from the phase I study: 180 mg daily, 90 mg daily and 180 mg daily with a 7-day lead-in at 90 mg daily, which were then subsequently evaluated in the phase II study. The phase II expansion

study was divided into five histologically and molecularly defined cohorts based on prior treatments, cancer types and CNS involvement. An ORR of 62% with a median PFS of 13.2 months was reported in crizotinib pre-treated ALK+ NSCLC patients' cohort with brigatinib treatment. The ongoing phase II, randomized, open-label, multicenter international study (ALK in Lung Cancer Trial of brigatinib; ALTA, ClinicalTrials.gov identifier: NCT02094573), aimed to investigate the efficacy and safety of two different brigatinib dosage regimens (90 mg daily and 180 mg daily) in crizotinib-treated ALK+ locally advanced or metastatic NSCLC patients; reported an investigator-assessed ORR of 45% and 54% in the 90 mg daily and 180 mg daily dosage groups, respectively, after a median follow-up of 8 months[221]. An interesting and encouraging finding of the study was the observed confirmed partial response in one patient with the G1202R mutation from the 180 mg daily group. Most commonly observed treatment-emergent adverse events (TEAEs) with brigatinib treatment included GI symptoms, headache and cough of low grade. Serious AEs of grade >3 included hypertension, increased blood creatine phosphokinase, pneumonia and increased lipase. Pulmonary AEs (dyspnea, hypoxia, cough, pneumonia, and pneumonitis) were observed early usually within 24-48 h of treatment initiation[220,221]. Although, all the pulmonary AEs in the ALTA study occurred only at 90 mg brigatinib dose while no such events occurred after escalation to 180 mg dose. The FDA granted an accelerated approval to brigatinib in April 2017 for the treatment of ALK+ metastatic NSCLC patients.

A phase III trial, ALTA-1L (ClinicalTrials.gov identifier: NCT02737501) is ongoing to compare the efficacy and safety of

brigatinib with crizotinib as a first-line treatment in patients with ALK+ metastatic NSCLC. Additionally, brigatinib is also planned to be evaluated in sequential strategy after ceritinib or alectinib treatment in a phase II study (ClinicalTrials.gov identifier: NCT02706626).

8. Third Generation ALK inhibitor

8.1 Lorlatinib (PF-06463922; Pfizer)

Lorlatinib is the latest addition to the list of ALK TKIs. It is a highly selective, potent and brain penetrant inhibitor of ALK and ROS1. Structurally, it is a macrocyclic compound designed primarily to overcome the ALK-TKI resistant mutations along with enhanced CNS activity[222] (Figure 11).

In biochemical assays, lorlatinib inhibited wild-type ALK with a mean inhibitory constant (Ki) of less than 0.07nM. Additionally, it potently inhibited crizotinib-resistant ALK mutants at low nanomolar concentrations (Ki < 0.1-0.9nM), including the extremely resistant G1202R[223]. Lorlatinib showed greater inhibition in comparison to crizotinib and alectinib in *in vitro* cell line models as well as in xenograft mouse models expressing ALK fusion kinase. It was also shown to have a superior activity against the intracranial EML4-ALK indicating the ability of lorlatinib to cross the blood brain barrier (BBB)[223].

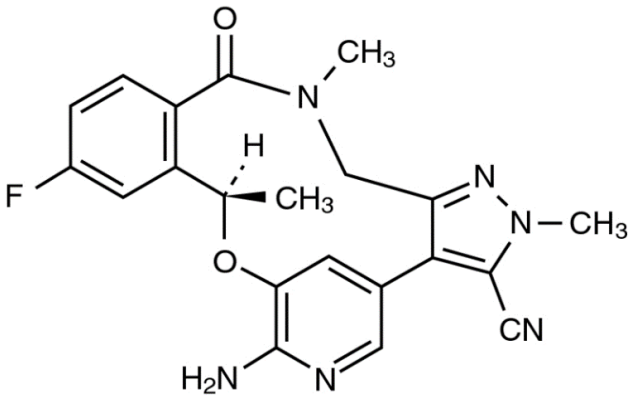

Figure 11. *Structure of lorlatinib.*

(adapted from Johnson TW, Richardson PF, Bailey S, et al. Discovery of (10R)- 7-amino-12-fluoro-2,10,16-trimethyl-15-oxo-10,15,16,17-tetrahydro-2H-8,4(metheno)pyrazolo[4,3-h][2,5,11]-benzoxadiazacyclotetradecine-3-carbonitrile (PF-06463922), a macrocyclic inhibitor of anaplastic lymphoma kinase (ALK) and c-ros oncogene 1 (ROS1) with preclinical brain exposure and broad-spectrum potency against ALK-resistant mutations. J Med Chem. 2014;57(11):4720–44)

Activity of lorlatinib against ROS1 was also evaluated in biochemical assays and was found to be sub nanomolar (Ki < 0.025nM), and more potent than crizotinib, ceritinib and alectinib[224]. Cellular proliferation was inhibited with lorlatinib treatment in HCC78 and Ba/F3 cells engineered to express ROS1 at IC_{50} values of 1.3 and 0.6nM. Lorlatinib also showed potent activity against cells expressing crizotinib-refractory ROS1 mutants, such as ROS1 G2032R (similar to ALK G1202R) and the ROS1 G2026M (similar to ALK L1196M) gatekeeper mutant.

Significant reduction in tumor volumes was observed in mouse models bearing wild-type and mutant ROS1-fusion xenograft tumors.

Moreover, lorlatinib was also tested in a glioblastoma mouse model expressing fused in glioblastoma (*FIG*)-*ROS1* fusion gene and led to considerable reduction in the tumor size following 7-day treatment, indicating that lorlatinib indeed has BBB penetrant activity[224]. Lorlatinib has also been tested in *in vitro* and *in vivo* models of neuroblastoma with different ALK mutations and was reported to have superior anti-tumor activity than crizotinib[225,226].

Organically, lorlatinib was tested in the clinic. A multicenter, single-arm, first in human phase I trial evaluated the safety, MTD and anti-tumor activity of lorlatinib in patients with ROS1 or ALK-rearranged NSCLC. To find the MTD, different doses of lorlatinib were evaluated in the dose escalation phase (10-200 mg once daily or 35-100 mg twice daily). Most of the patients enrolled in the study had previously received first and second-generation ALK TKIs. A response rate of 46% (95% CI, 31%–63%) and a median PFS of 9.6 months were reported with lorlatinib treatment. Interestingly, the median PFS was higher in patients who had received only one previous TKI treatment (13.5 months) as compared to the patients who had received two or more ALK TKIs (9.2 months). Comparison of blood and cerebral spinal fluid (CSF) samples from some patients revealed high concentrations of lorlatinib in the CSF suggesting that lorlatinib can effectively cross the BBB to induce substantial and durable CNS responses[227]. Most commonly observed AEs with lorlatinib treatment were hypercholesterolemia, hypertriglyceridemia, peripheral neuropathy and peripheral edema. 100 mg once daily was selected as the recommended dose of lorlatinib for phase II study. Based on the phase I and preliminary phase 2 results, lorlatinib received accelerated approval

from the FDA in November 2018, for the treatment of patients with ALK+ metastatic NSCLC whose disease has progressed on crizotinib and at least one other ALK inhibitor for metastatic disease, or alectinib as the first ALK inhibitor therapy for metastatic disease, or ceritinib as the first ALK inhibitor therapy for metastatic disease.

Preliminary results from the ongoing phase II (ClinicalTrials.gov identifier: NCT01970865) studies of lorlatinib were published recently[228]. The study recruited 276 NSCLC patients which were further divided into 6 different expansion (EXP) cohorts, ALK+ patients who had received or not received prior therapies (EXP 1-5) and ROS1-positive patients (EXP6). In line with the previous results, lorlatinib achieved impressive overall and intracranial activity in treatment-naïve (ORR of 90%, intracranial response rate 66.7%) as well as in heavily pretreated (up to three previous ALK TKIs) ALK+ NSCLC patients (ORR of 47%, intracranial response rate 63%)[228]. Similar results were obtained in the ROS1+ patients (EXP 6) with an overall ORR of 36.2% and intracranial ORR of 56% with crizotinib-naïve patients showing higher responses[229]. The AE profile was similar to as previously reported in phase I/II trial.

9. Other novel ALK TKIs under development

Other than the above mentioned ALK TKIs, there are some additional novel TKIs which are under development (pre-clinical or clinical) (Table 3).

Entrectinib is an orally available tyrosine kinase inhibitor that has activity against neurotrophic receptor tyrosine kinase (NTRK) fusions. However, it was originally developed as an ALK inhibitor and showed significant activity against ALK (including the L1196M and C1156Y crizotinib-resistant mutations), ROS1, and TRK, in pre-clinical models. It was also shown to retain activity in the brain and efficiently inhibited the intracranial ALK-rearranged tumor cells[230,231]. Two phase I studies evaluated the efficacy and safety of entrectinib in patients with advanced solid tumors carrying *NTRK1/2/3*, *ROS1* or *ALK* gene aberrations (point mutations, amplifications or copy number variants). Responses with entrectinib treatment were only seen in the treatment-naïve patients carrying *NTRK1/2/3*, *ROS1* or *ALK* gene fusions[232]. Therefore, entrectinib is being further evaluated only in that particular "eligible population" in the phase II study.

ASP3026 is an oral, selective, potent ALK/ROS1 kinase inhibitor. Preclinical studies showed that the compound effectively inhibited ALK activity and growth of EML4-ALK positive cells. It also reduced tumor growth in the xenograft models carrying the L1196M ALK mutation[233]. Results of the Phase I study of ASP3026 described an ORR of 44% in 15 ALK+ NSCLC patients with a median PFS of 5.9 months[234]. However, in 2015, further development of ASP3026 were stopped due to strategic reasons.

Ensartinib (X-396) is an aminopyridazine-based ALK inhibitor. *In vitro* studies showed that ensartinib has a 10-fold increased potency as compared to crizotinib in inhibiting ALK+ NSCLC cells. Additionally, ensartinib was able to inhibit the commonly found crizotinib-resistant L1196M and C1156Y mutations. Phase I/II study of

ensartinib reported 60% response rate (RR) overall in ALK+ NSCLC patients. Improved RR (80%) was associated in TKI treatment-naïve patients with ensartinib treatment[235].

CEP-28122 is a dual ALK/FAK inhibitor. Preclinical studies demonstrated that it is highly selective against ALK (IC_{50} = 1.9nM) and ALK mutations. A structurally similar, but improved, in terms of pharmacological activity and kinase selectivity, compound CEP-37440 was recently reported to have activity against ALK and FAK[236]. Phase I trial is ongoing to determine the MTD of CEP-37440 in patients with advanced or metastatic solid tumors (ClinicalTrials.gov identifier: NCT01922752).

Belizatinib (TSR-011), another inhibitor of ALK and TRK A/B/C kinases is currently being evaluated in a phase I/II trial (ClinicalTrials.gov identifier: NCT02048488). Preliminary results from the trial showed that at a total daily dose of ≥120 mg, responses were observed in 60% of ALK inhibitor-naïve and in 50% of patients who had progressed on crizotinib. The most commonly detected grade 1–2 AEs included fatigue, constipation, QTc prolongation, diarrhoea and headache[237].

10. Resistance to ALK inhibitors

Despite the impressive results with ALK inhibitors' treatment, most of the patients develop resistance after a certain period of time. However, resistance is not a new phenomenon, instead, it has been observed against almost all targeted therapies especially in the case of

TKI treatment. Resistance can be either intrinsic/primary, where despite the presence of the oncogenic target gene, the TKI fails to achieve a response, or it can be acquired/secondary where after an initial response to the treatment, the tumor relapses. While intrinsic resistance is still not fully understood, acquired resistance mechanisms have been elucidated and characterized in the past few years as the use of crizotinib and other ALK inhibitors have expanded. Acquired resistance mechanisms against ALK can be broadly classified into two categories; ALK-dependent or ALK-independent resistance mechanisms.

10.1 Intrinsic/primary resistance

There is not much evidence regarding the lack of response against ALK TKIs despite the presence of the oncogenic ALK. In an *in vitro* study, Heuckmann and colleagues compared the effect of crizotinib and TAE684 on Ba/F3 cells ectopically expressing different variants of EML4-ALK (v1, v2, v3a and v3b). The group reported that the different parts of the *EML4* fused to *ALK* affect the stability of the fusion protein and protein degradation mediated by the inhibitor, thereby controlling drug sensitivity[125]. Additionally, the fluorescence in situ hybridization (FISH)-based diagnosis of ALK+ NSCLC lacks the ability to detect the specific variant of *EML4* involved in the fusion and could therefore, in part, explain the heterogeneous response to ALK inhibitors. Moreover, gene alterations in *KRAS* or *EGFR* have also been implicated in providing tumor cells with primary resistance. Doebele et

al described ‘emergence of a separate oncogenic driver’ that can occur in a subpopulation of ALK+ NSCLC cases where the minority of cells acquire activating mutations in a gene other than *ALK* such as *KRAS* which can drive their growth and proliferation. In such cases, ALK TKI does not lead to tumor regression since the cells are not completely dependent on ALK[238].

10.2 Acquired/secondary resistance

Majority of the resistance cases against ALK TKIs fall into the acquired resistance category as most of the ALK+ patients initially respond to the inhibitor but invariably relapse after a few months of response. Although secondary resistance culminates from a complex process, it always eventually leads to the constitutive activation of the growth-promoting or survival pathways escaping the effect of the inhibitor. This escape can be attained in two different ways; either by modifying the target rendering the drug binding ineffective or by utilizing alternate signaling pathways. Both escape mechanisms are described in detail below.

10.2.1 ALK-Dependent Resistance Mechanisms

Resistance mechanisms in which the target, in this case ALK, is used to circumvent the inhibitor’s effect are classified as ALK-dependent (“On-target”) resistance mechanisms. In this type of resistance

mechanisms, the tumor cells are still driven by the (modified) target oncogene.

a) Secondary mutations of ALK

Secondary mutations acquired in the ALK TKD are the most common mechanism of resistance identified in almost one third of the patients, who relapse on ALK TKIs. From a structural point of view, mutations in the TKD re-activate the kinase despite the inhibitor's presence, and signals through the downstream signaling pathways leading to an unaffected growth and proliferation of tumor cells. Usually the mutations occur in the drug-binding region of the kinase rendering the drug binding either unachievable or ineffective; however, some ALK mutations have also been described that lie away from the active site of the kinase.

Point mutations against crizotinib

The first clinical case of resistance against crizotinib was reported in 2010, only 2 years after the start of crizotinib use in clinic. Choi and colleagues identified two point mutations, L1196M and C1156Y, in the pleural effusion of an ALK+ NSCLC patient who relapsed after 5 months of crizotinib treatment[239]. Mutations in the conserved gatekeeper residue is a common resistance mechanism against TKIs. For example, the T790M and T315I mutations in the EGFR and ABL kinases confer resistance against gefitinib, erlotinib (EGFR TKIs) and imatinib, dasatinib, nilotinib and bosutinib (BCR-

ABL TKIs), respectively. The gatekeeper residue is located at the bottom of ATP-binding pocket of the ALK kinase. Substitution of the smaller gatekeeper residue leucine at position 1196 with a larger methionine residue interferes with the drug binding as it blocks the access of the inhibitor to the adjacent hydrophobic pocket[240] (Figure 12). Moreover, methionine substitution leads to an increase in the enzyme activity by strengthening the hydrophobic R-spine which in turn promotes formation of the active protein conformation[241]. *In vitro* studies showed that the EML4-ALK L1196M mutant cells had comparatively higher phosphorylation levels supporting that the gatekeeper mutation confers drug resistance by enhancing the protein activity[242]. Interestingly, a different gatekeeper mutation, L1196Q where leucine was replaced by a glutamine residue, was detected *in vitro* in ALCL cells at higher crizotinib doses[243].

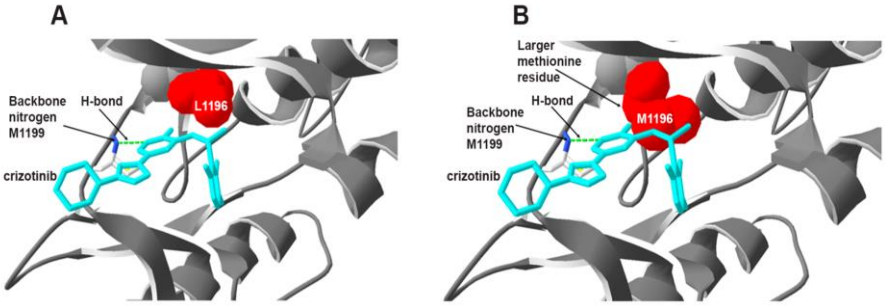

Figure 12. *Comparison of wild-type and L1196M mutant EML4-ALK. (A) Crizotinib bound to wild-type EML4-ALK is shown along with the important interactions. (B) Crizotinib bound to the mutant EML4-ALK is shown with the evident steric clash between the drug and larger methionine residue.*
(adapted from Sharma GG, Mota I, Mologni L, Patrucco E, Gambacorti-Passerini C, Chiarle R. Tumor resistance against ALK targeted therapy-where it comes from and where it goes. Cancers (Basel) 2018:10)

On the other hand, the C1156Y mutation, unlike the gatekeeper mutation, has never been reported for other tyrosine kinases. The 1156 residue lies at the N-terminal domain away from the ATP binding site and hence the role of the mutant was obscure in the beginning. However, after its first report, some authors used molecular docking simulations to suggest that the mutation hampers interaction of crizotinib with EML4-ALK by lowering the drug-target binding affinity[244]. Moreover, conformational changes in the 1122-1130 loop fragment were also predicted in the mutant form of EML4-ALK (Figure 13).

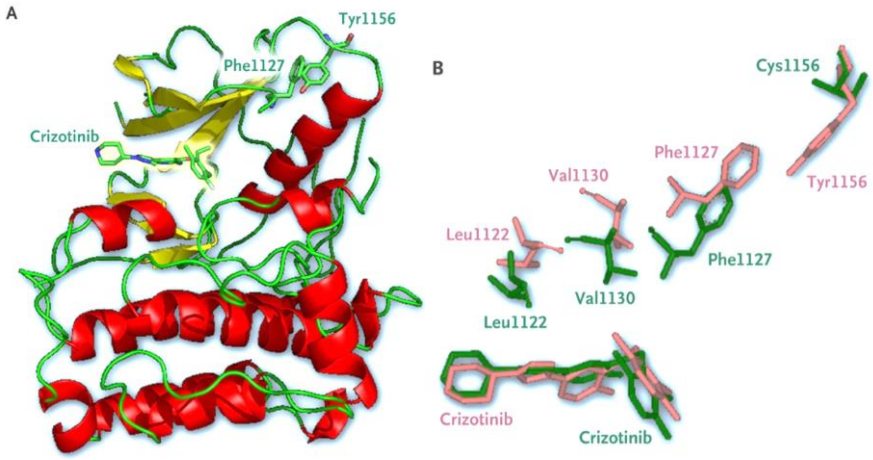

Figure 13. *Molecularly docked structure of the C1156 (wild-type) or Y1156 mutant in EML4-ALK with Crizotinib. (A) Position of the 1156 residue and interaction of crizotinib with EML4-ALK and other depicted residues. (B) Selected residues of wild-type (in green) and C1156Y mutant (in pink) EML4-ALK superimposed are shown.*
(adapted from Shen, L, Ji, HF. More on crizotinib. N. Engl. J. Med., 364 (2011)

Further studies using molecular dynamics showed that the C1156Y mutation also displaces crizotinib in addition to affecting the binding affinity between the drug and the target. Altogether, the changes reduce the interactions between crizotinib and active-site residues ultimately leading to resistance[245].

Another mutation, G1269A, was identified in two ALK+ NSCLC patients upon relapse on crizotinib[246]. Glycine1269 is located at the end of the ATP-binding pocket of ALK, exactly before the DFG activation loop and is in direct contact with crizotinib. Substitution of glycine with a comparatively larger alanine residue presumably affects crizotinib binding as a result of steric hindrance. Ba/F3 cells transduced with the G1269A EML4-ALK mutant were resistant to crizotinib even at higher doses compared to the wild-type cells. Expectedly, phosphorylation of downstream signaling molecules such as ERK, STAT3 and AKT was also not affected even at high doses of crizotinib in the G1269A mutant cells[246].

Furthermore, a L1152R mutation was identified in a NSCLC patient whose disease progressed after a brief radiographic response of 3 months with crizotinib treatment[247]. The mutation results in a change at position 1152 replacing leucine with arginine residue. Functional studies showed that the mutant cells were significantly resistant to crizotinib in comparison to the wild-type control cells. Based on the available ALK crystal structures, it is unclear how the L1152R mediates resistance against ALK inhibitor as the 1152 residue does not seem to be in direct contact with the ATP-binding pocket.

In addition, crizotinib resistant secondary mutation F1174L was reported in an IMT patient harbouring the RANBP2-ALK

translocation[248]. The mutation leads to an increase in ALK phosphorylation as well as downstream signaling. Moreover, it was also found to block apoptosis in RANBP2-ALK transduced Ba/F3 cells. Although the structural basis of F1174L mediated resistance is not clear, it is suggested that the mutation might promote the active conformation of the kinase and hence affects the binding of crizotinib. Another variant at the same residue position, F1174V was also detected in an ALK+ NSCLC patient[249]. It should be noted that the activating F1174L mutation had already been identified in neuroblastoma[164,167].

In another study involving 18 ALK+ NSCLC patients who relapsed on crizotinib, 22% were found to have acquired resistant mutations[250]. Apart from the already known gatekeeper mutation, the new mutations identified were G1202R, S1206Y and an insertion of threonine at 1151 position (1151Tins). Both, G1202R and S1206Y residues are located in the solvent front of ALK kinase domain near crizotinib-binding site, hence, presumably, the mutations affect the crizotinib affinity for mutant ALK forms. On the other hand, the 1151 residue is situated at the α-helix C-loop of N-terminal lobe, far away from the crizotinib-binding site. However, it has been predicted based on molecular modelling studies that the 1151Tins may change the affinity of ALK for ATP and conversely affect crizotinib binding to ALK[251]. The 1151Tins and G1202R mutations are extremely resistant to crizotinib.

A number of other point mutations have been described in the literature that confer resistance to crizotinib in diseases other than NSCLC (Figure 14). For example, an I1171N mutation was first described in ALCL crizotinib-resistant cell line and later identified also

in an ALK+ ALCL patient who progressed on crizotinib[243,244]. The hydrophobic I1171 residue lies distant from the ATP-binding site and forms the hydrophobic R-spine along with other residues. It connects the two lobes, N-lobe and C-lobe, of the ALK kinase. Mutation at the 1171 residue destabilizes the inhibitor-bound DFG-in conformation of the kinase and favours the stability of active conformation of ALK indicating that they confer resistance to crizotinib independent of its structure and binding mode[243]. Other mutations of ALK identified, mostly in neuroblastoma, include, R1275Q, F1245L, Y1278S, F1174C/V[167,168].

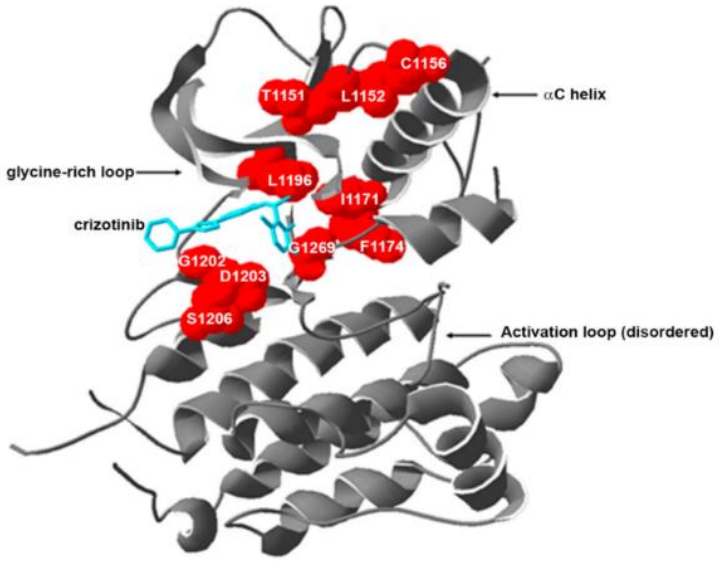

Figure 14. *Crystal structure of ALK (in grey) bound with crizotinib (in light blue) with key residues that confer resistance against crizotinib highlighted in red. Also, a few important regulatory regions are shown and indicated with arrows.*

(adapted from Sharma GG, Mota I, Mologni L, Patrucco E, Gambacorti-Passerini C, Chiarle R. Tumor resistance against ALK targeted therapy-where it comes from and where it goes. Cancers (Basel) 2018:10)

Point mutations against next generation ALK inhibitors

Despite the improved potency and efficacy of the next generation ALK inhibitors, even the new inhibitors succumb to resistant point mutations.

During pre-clinical studies, ceritinib inhibited the most common crizotinib resistant mutations, including L1196M, G1269A, S1206Y, and I1171T, however it was not efficient against some other mutations such as C1156Y, G1202R, 1151Tins, L1152R, and F1174C/V[252]. While the G1202R mutation mediates resistance by the same mechanism as in crizotinib, the other mutations are located at N-terminal lobe of the ALK catalytic domain flanking the opposite ends of the αC-helix and do not directly interfere with the inhibitor binding. Presumably the mutations affect the αC-helix mobility and bring about conformational changes in the catalytic domain. Two of these mutations, G1202R and F1174C were also identified in EML4-ALK+ NSCLC patients after they progressed on ceritinib[252]. An $ALK^{G1202del}$ mutation was also identified 2 patients' samples resistant to ceritinib[253]. Functional studies revealed that the G1202del mutation confers moderate level of resistance to ceritinib. Additionally, a novel secondary mutation, G1123S was identified in the post-ceritinib biopsy sample of a NSCLC patient who relapsed after a partial response to ceritinib[254]. Mutation at the same residue, G1123S/D had also been identified in neuroblastoma cell line and was found to be resistant against TAE684[255]. The G1123 residue is positioned in the glycine-rich loop and could perturb interactions between ALK and the inhibitor. Interestingly, the mutation was sensitive to alectinib treatment[254].

Another mutation, L1152R, was detected in an ALK rearranged adenocarcinoma relapsed patient sample following a 4-month duration of response to ceritinib[256]. Again, the patient responded to alectinib after ceritinib failure indicating that L1152R is sensitive to alectinib.

Although alectinib could overcome a number of resistant mutations found against crizotinib and ceritinib, secondary acquired mutations were also identified against alectinib. Katayama et al, described two secondary mutations, V1180L and I1171T, in cell lines and a primary tumor sample resistant to alectinib, respectively[257]. The V1180L mutation was later identified also in an ALK+ NSCLC patient refractory to alectinib[253]. From *in vitro* studies, it was found that V1180L is more resistant to alectinib as compared to I1171T. Structural modelling studies suggest the position of V1180 to be near the gatekeeper L1196 residue while I1171 resides in the αC-helix of ALK kinase domain. Substitution of valine with a leucine at the 1180 position creates a steric hindrance. The methyl group of leucine residue is not compatible with the multicyclic alectinib rings. On the other hand, the replacement of isoleucine with a threonine at codon 1171 distorts the αC-helix and displaces the glutamic acid at 1167 downwards. This shift impedes the hydrogen bond between the cyano group of alectinib with E1167, eventually decreasing the binding affinity of alectinib to the mutant form[257]. *In vitro*, structurally distinct inhibitors were found to overcome alectinib resistant mutations.

Pre-clinical studies showed that brigatinib was effective against all the crizotinib-resistant mutations, except L1188F where it was moderately active, and was more potent in inhibiting several mutations as compared to other second generation ALK inhibitors such as

ceritinib or alectinib[258]. In another study, a cohort of ALK+ NSCLC patients who progressed on brigatinib were found to have G1202R, D1203N, S1206Y/C, E1210K secondary mutations with G1202R being the most commonly identified mutation[253]. Additionally, mutations against brigatinib were found in ALK+ ALCL cell lines including L1198F, F1174V, F1174V+L1198F, L1122V+L1196M, S1206C[259]. Interestingly, most of the brigatinib mutations could be overcome by reverting back to the use of crizotinib, other second generation ALK TKIs or different inhibitors such as heat shock protein 90 (HSP90) inhibitors[259].

With the expansion in the use of ALK TKIs, another phenomenon that has come to light is the emergence of compound mutations upon sequential ALK inhibitor treatment[253]. It is not surprising that certain mutational profiles are unique to specific inhibitors considering that there are structural differences among the available ALK TKIs (Table 4).

The first clinical evidence of the compound mutation phenomenon came from the finding of a double mutation, C1156Y and L1198F in an advanced ALK+ NSCLC patient who was sequentially treated with crizotinib, ceritinib and lorlatinib[260]. Lorlatinib showed enthusiastic results in terms of overcoming resistant mutations acquired against previous ALK TKIs including the most commonly found and extremely resistant mutation G1202R[227]. However, as is the case with other ALK inhibitors, resistance eventually develops even against lorlatinib. The compound mutation identified in the patient after failing lorlatinib ($ALK^{C1156Y/L1198F}$) was paradoxically sensitive to crizotinib[260].

Table 4. *ALK inhibitors and their respective sensitive and resistant mutations.*

TKI	Sensitive Mutants	Resistant Mutants	Disease	Evidence (*In vitro*/ *In vivo*/Clinical)
Crizotinib	L1198F	I1151Tins	NSCLC	Clinical
		L1152R	NSCLC	Clinical
		C1156Y	NSCLC	Clinical
		I1171T/N	NSCLC	Clinical
		F1174L	IMT	Clinical
		L1196M	NSCLC	Clinical
		L1196Q	NSCLC	Clinical
		L1198P	EML4-ALK Ba/F3 cells	*In vitro*
		G1202R	NSCLC	Clinical
		D1203N	NSCLC	Clinical
		S1206Y	NSCLC	Clinical
		G1269A	NSCLC, IMT	Clinical
Ceritinib	G1269A, I1171T, S1206Y, L1196M	R1275Q	Neuroblastoma	*In vitro*
		L1152P/R	NSCLC	*In vitro*/Clinical
		D1203	NSCLC	Clinical
		G1202R	NSCLC	Clinical
		F1174C/V	NSCLC	Clinical
		L1198F	NSCLC	*In vitro*
		C1156Y/T	NSCLC	*In vitro*
		G1123S/D	NSCLC	Clinical/*In vitro*
Alectinib	G1269A, S1206Y, L1152R, F1174L, 1151Tins	I1171T	NSCLC	Clinical
		V1180L	NSCLC	Clinical
		G1202R	NSCLC	Clinical
Brigatinib	G1269A, S1206Y, L1152R, F1174C, 1151Tins, I1171T, D1203N, E1210K, F1245C	F1174V+L1198F	ALCL	*In vitro*
		G1202R	NSCLC	Clinical
		S1206C/F	NSCLC	Clinical
Lorlatinib	L1196M, G1202R, G1269A	L1198F+C1156Y	NSCLC	Clinical
		G1202R+L1196M	NSCLC	Clinical
		E1210K+D1203N+L1196M	NSCLC	Clinical
		I1171N+L1198F	NSCLC	Clinical

(adapted from Sharma GG, Mota I, Mologni L, Patrucco E, Gambacorti-Passerini C, Chiarle R. Tumor resistance against ALK targeted therapy-where it comes from and where it goes. Cancers (Basel) 2018:10)

Since lorlatinib has not been used in patients for a long time, there are not many clinical cases of resistance against lorlatinib reported in the literature. In an accelerated mutagenesis screen[261] aimed to characterize mutations that could develop upon lorlatinib treatment, the authors reported to have identified only compound mutations and no single ALK point mutations including $ALK^{G1202R/L1196M}$, $ALK^{G1202R/L1198F}$ and $ALK^{L1196M/L1198F}$ (Figure 15).

The authors later also detected compound mutations such as $ALK^{G1201R/L1196M}$, $ALK^{E12010K/D1203N/G1269A}$, $ALK^{I1171N/L1198F}$ in ALK+ NSCLC patients who progressed after undergoing treatments with first, second and third generation ALK TKIs.

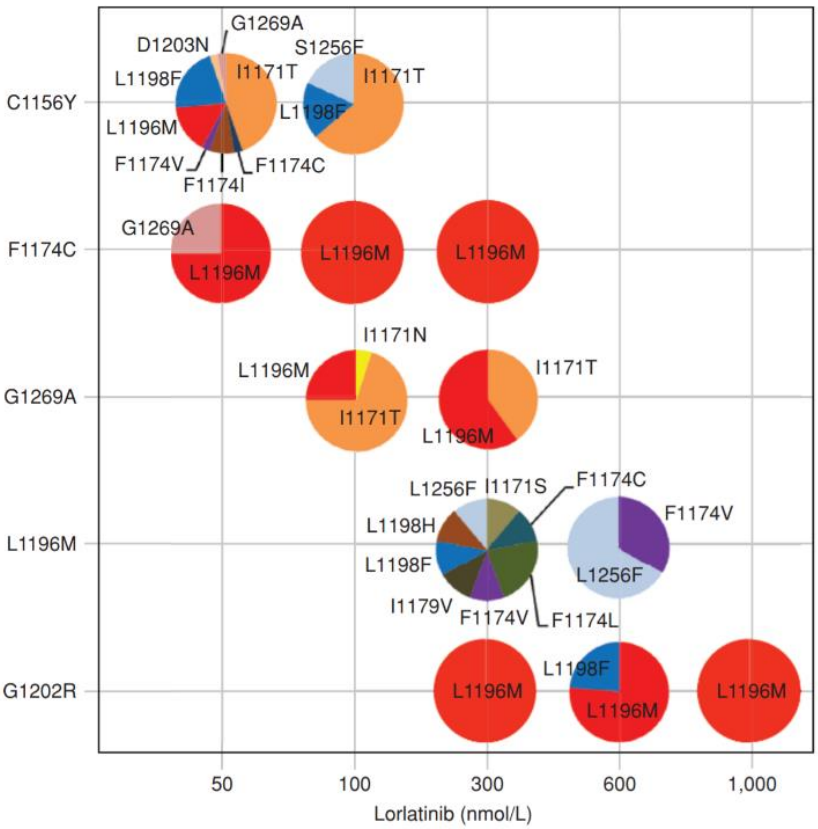

Figure 15. *Emergence of compound ALK mutations against lorlatinib. Different compound ALK mutations identified are depicted. The respective lorlatinib concentration (on x-axis) at which the mutants were identified and the single mutant EML4-ALK models (on y-axis) that were used for the mutagenesis screen.*

(adapted from Yoda, S. et al. Sequential ALK inhibitors can select for lorlatinib-resistant compound ALK mutations in ALK-positive lung cancer. Cancer Discov. https://doi.org/10.1158/2159-8290.CD-17-1256 (2018)

b) Amplification of *ALK* gene

Another, less frequent, ALK-dependent mechanism of resistance is the amplification of *ALK* gene. *ALK* amplification is frequent (~10%) in primary neuroblastoma and cell lines. Overexpression of full-length *ALK* leads to anti-apoptotic signaling and tumor growth in primary glioblastoma[262]. On the other hand, amplification of oncogenic fusion *ALK* is not a common event from a pathogenesis point of view but is rather observed as a resistance mechanism. Copy number gain (CNG) in *EGFR* has been regarded more to be a predictor of EGFR-TKI sensitivity[263] while on the contrary there is evidence of an increased *BCR-ABL* fusion gene copy number to serve as a resistance mechanism[264]. Amplification of *ALK* is similar to BCR-ABL increased expression as it seems to confer resistance against ALK TKIs. *In vitro* studies found that partially crizotinib resistant ALK+ NSCLC cell lines carried *ALK* amplification without any secondary resistant mutations[218]. Later, amplification of *EML4-ALK* fused gene was detected in a patient who had progressed on crizotinib[250]. Same year, another study reported 2 patients who had a copy number increment of rearranged *ALK* gene per cells following crizotinib failure[246]. One of these patients also had an ALK secondary point mutation (G1269A) along with *ALK* CNG. Several other studies have also implicated *ALK* CNG in mediating TKI resistance[218,253,265]. Recently, it was reported that CNG is a more common resistance mechanism in ALK+ NSCLC patients as compared to patients carrying *ROS1* gene rearrangements. ALK+ patients were found to have both an increase in the number of rearranged copies per cell as well as an

increase in the number of cells with detectable *ALK* rearrangement[266]. However, it is still not clear under which circumstances/factors, amplification of *ALK* gene alone is enough to render the tumor cells resistant.

Genomic amplification of *ALK* locus as a resistance mechanism has also been observed in ALCL cell lines[259,267]. Ceccon and colleagues described that NPM-ALK was overexpressed in brigatinib-resistant ALCL cells because of *ALK* amplification[259]. Intriguingly, overexpression of the oncogenic NPM-ALK creates a dependency on TKI (drug-addiction) in the tumor cells for their growth and proliferation[267]. Surprisingly, removal of drug from these drug-addicted cells leads to apoptotic death. The authors described that the apoptosis was mediated by the activation of the DNA damage response pathway due to an unbalanced NPM-ALK signaling[267].

10.2.2 ALK-Independent resistance by activation of bypass signaling pathways

One third of crizotinib-resistant ALK+ NSCLC cases harbour 'on-target' modifications in terms of secondary mutations and are still driven by the 'driver' gene (*ALK*). However, approximately, 40-50% of ALK+ NSCLC cases resistant against second generation ALK TKIs do not carry any 'on-target' modifications and falls under the 'ALK-independent/off-target' resistance mechanisms category[268]. As indicated by the name, ALK-independent resistance mechanisms

employ strategies to sustain the tumor growth, proliferation and survival using genes/pathways other than *ALK*.

One of the characteristic features of oncogenic kinases is that most of them share many common downstream signaling pathways and effectors. The communal access of oncogenic kinases to these critical signaling pathways enable them to circumvent/bypass the inhibition and sustain the continued growth and proliferation of tumor cells even in the presence of a 'driver gene' inhibitor. Several reports have described the activation of bypass signaling pathways as mediating resistance against ALK inhibitors in ALK+ NSCLC (Figure 16).

One of the first signaling pathways implicated in ALK resistance was EGFR activation[247,250,269,270]. In *in vitro* studies, characterization of crizotinib and TAE684 resistant EML4-ALK+ NSCLC cell lines identified an increase in the phosphorylation of EGFR in comparison to the parental inhibitor-sensitive cell lines[247]. Interestingly, the resistant cells did not carry any secondary resistant mutations suggesting that EGFR activation was sufficient to mediate resistance against ALK inhibition. In addition to the increased phosphorylation of EGFR protein, elevated *EGFR* mRNA expression and sustained activation of downstream ERK and AKT signaling was also detected in the resistant cells. Evaluation of paired pre- and post-crizotinib patient samples confirmed the same results, with evidence of increased EGFR activation in 4 out of 9 patients[247]. Since the resistant cells were not found to have any *EGFR* mutations or amplifications, it was proposed that the activation of EGFR might be a consequence of either the EGFR receptor or its ligand upregulation [250,269,270]. Paracrine EGFR ligands such as EGF, TGF-α and heparin-binding EGF-like

growth factor (HB-EGF) were also found to cause crizotinib resistance[270]. Concomitant presence of activating mutations of *EGFR* and *ALK*-translocations have been detected in TKI refractory patients[250,271]. Additionally, it was also observed that ALK+ NSCLC resistant cells harbouring EGFR activation were cross-resistant to almost all the ALK-TKIs, i.e. crizotinib, alectinib, ceritinib and brigatinib[269]. Although, the EGFR-mediated resistance could be overcome by combination treatment, with ALK and EGFR inhibitors[250,269].

Furthermore, human epidermal growth factor receptor 2 (HER2) has also been identified in causing ALK TKI resistance[250,271,272]. In a systemic large-scale functional study aimed to identify ALK resistance mediators using open reading frame (ORF) library, neuregulin-1 (NRG-1), an activating ligand of HER3, was found as the strongest inducer of ALK resistance. Activation of HER3 leads to the formation of a heterodimer with HER2 which in turn activates further downstream signaling[271]. Combined use of ALK and HER2 inhibitors (TAE684 and lapatinib, respectively) was able to overcome the resistance in NSCLC cell lines. The *in vitro* findings were also corroborated in patients, when researchers found EGFR and HER2 as the most enriched gene expression signatures in crizotinib-resistant tumors as compared to the crizotinib-naïve tumors[271]. However, use of EGFR, HER2/HER3 and ALK inhibitors have not yet been demonstrated in clinic.

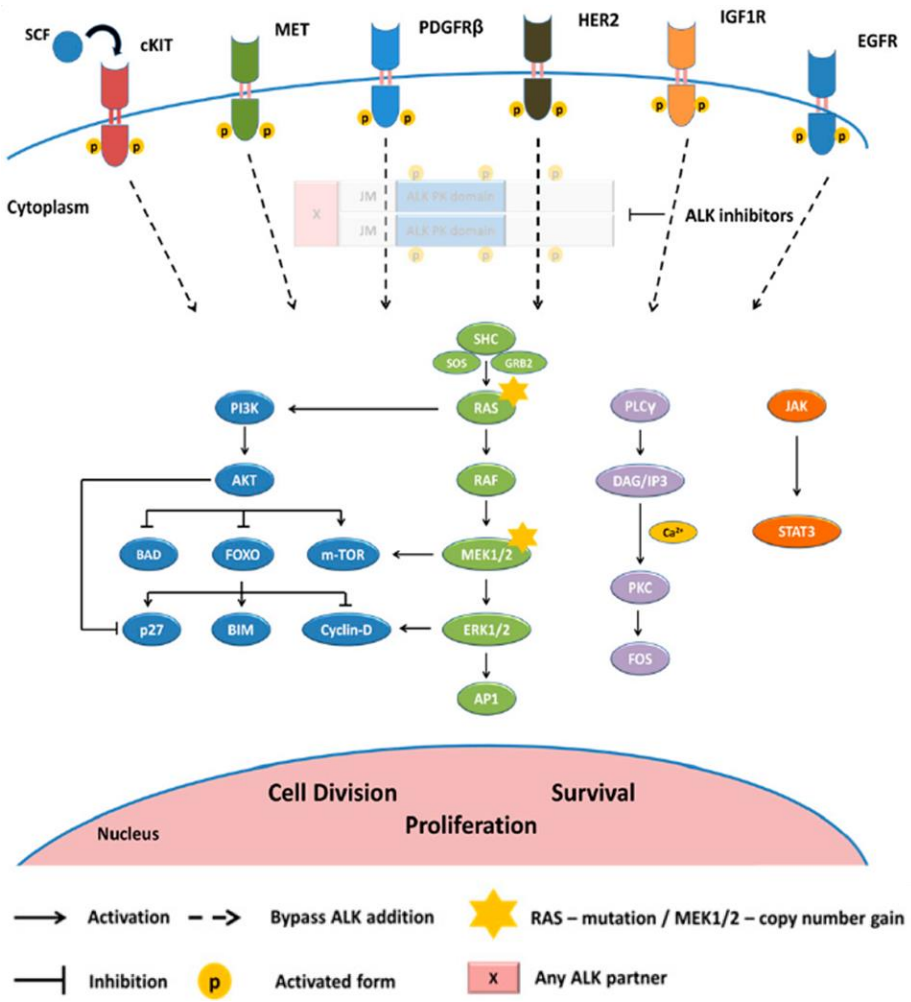

Figure 16. *Activation of bypass signaling pathways as an ALK resistance mechanism. A number of other kinases and downstream effector molecules can be activated and sustain tumor cell growth, proliferation and survival when oncogenic ALK is inhibited with ALK inhibitors.*

(adapted from Sharma GG, Mota I, Mologni L, Patrucco E, Gambacorti-Passerini C, Chiarle R. Tumor resistance against alk targeted therapy-where it comes from and where it goes. Cancers (Basel) 2018:10)

Amplification of the protooncogene *c-KIT* has also been associated with crizotinib resistance. In a study of 18 patients who acquired resistance to crizotinib, 2 patients had a clear overexpression of the *KIT* gene histologically as well as at protein levels in the resistant tumor samples[250]. Out of the 2 patients with *KIT* amplification, one patient carried *KIT* amplification with increased expression of the *KIT* ligand, stem cell factor (SCF), in the stromal cells, while the other patient had focal *KIT* amplification along with a secondary *ALK* mutation. Functional studies showed that the ALK+ NSCLC H3122 cells engineered to overexpress wild-type *KIT*, but in the absence of exogenous SCF, were sensitive to crizotinib treatment indicating that *KIT* amplification mediated resistance requires both, activation of the receptor and presence of the ligand. The authors also demonstrated that the use of imatinib (inhibitor of *ABL*, *KIT* and *PDGFR*) with crizotinib sensitized the resistant H3122 cells overexpressing *KIT* to crizotinib[250].

Based on the 'exceptional responders' profile of a patient, researchers uncovered the role of IGF-1R activation in ALK resistance[273]. The patient had an exceptional response to IGF-1R monoclonal antibody (mAB) after failing erlotinib. Molecular analyses revealed that the patient carried *ALK* rearrangement and was therefore enrolled in the crizotinib trial and had a partial response to crizotinib. *In vitro* analyses revealed IGF-1R pathway upregulation in crizotinib-resistant cells as a consequence of increased level of IRS-1, an adaptor protein that binds to IGF-1R or ALK, and downregulation of IGF binding protein 3 (IGFBP3), known to block IGF-1–induced activation of IGF-1R. Consequently, analyses of paired pre- and post-crizotinib treatment tumor samples showed increased levels of phospho-IGF-1R

(pIGF-1R) in 4 out of 5 resistant samples. Increased expression of IRS-1 was also detected in 2 of these resistant samples. Interestingly, one patient harboured the G1202R ALK resistant mutation along with increased pIGF-1R levels. The authors suggested that combined use of ALK and IGF-1R inhibitors can overcome the resistance caused by upregulation of IGF-1R[273].

Reactivation of downstream effector molecules can also serve as an ALK resistance mechanism. In a compound drug screening experiments using patient-derived NSCLC cell lines, a MAPK (MEK) inhibitor; selumetinib (AZD6244), was identified as a potent hit when used in combination with ceritinib to overcome ceritinib resistance. Further analyses revealed an activating mutation MAP2K1 K57N that led to MAPK activation[274]. Another study aimed to establish optimal upfront co-targeting strategy, identified RAS-MEK as the critical downstream effector of EML4-ALK in ALK-rearranged NSCLC cell lines and mouse xenograft models. The authors demonstrated that using a polytherapy approach in the form of dual ALK and MEK inhibitors was not only able to enhance the effect of ALK inhibitor, but it also forestalled the emergence of resistance[275].

Several other bypass mechanisms implicated in ALK TKI resistance also include, *PIK3CA* mutations[253,274], SRC activation[274], *KRAS* mutations and increase in copy number[246,275,276], loss of *DUSP6*[275]. An NGS based study analyzed 27 post-2nd generation ALK TKI- resistant specimens and detected mutations in *TP53, DDR2, BRAF, FGFR2, MET,* and *NRAS* genes[258].

Given the expanding use of technology, more novel resistance mechanisms will probably be identified. Additionally, a rational and

systematic comparison of paired pre- and post-TKI tumor samples will also foster our knowledge of clinically relevant resistance mechanisms against ALK TKIs.

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CHAPTER II

Lorlatinib Treatment Elicits Multiple On- and Off-Target Mechanisms of Resistance in ALK-Driven Cancer

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Abstract

Targeted therapy changed the standard of care in ALK-dependent tumors. However, resistance remains a major challenge. Lorlatinib is a third-generation ALK inhibitor that inhibits most ALK mutants resistant to current ALK inhibitors. In this study, we utilize lorlatinib-resistant anaplastic large cell lymphoma (ALCL), non–small cell lung cancer (NSCLC), and neuroblastoma cell lines *in vitro* and *in vivo* to investigate the acquisition of resistance and its underlying mechanisms. ALCL cells acquired compound ALK mutations G1202R/G1269A and C1156F/L1198F *in vitro* at high drug concentrations. ALCL xenografts selected *in vivo* showed recurrent N1178H (5/10 mice) and G1269A (4/10 mice) mutations. Interestingly, intracellular localization of NPM/ALK^{N1178H} skewed towards the cytoplasm in human cells, possibly mimicking overexpression. RNA sequencing of resistant cells showed significant alteration of PI3K/AKT and RAS/MAPK pathways.

Functional validation by small-molecule inhibitors confirmed the involvement of these pathways in resistance to lorlatinib. NSCLC cells exposed *in vitro* to lorlatinib acquired hyperactivation of EGFR, which was blocked by erlotinib to restore sensitivity to lorlatinib. In neuroblastoma, whole-exome sequencing and proteomic profiling of lorlatinib-resistant cells revealed a truncating NF1 mutation and hyperactivation of EGFR and ErbB4. These data provide an extensive characterization of resistance mechanisms that may arise in different ALK-positive cancers following lorlatinib treatment.

Significance: High-throughput genomic, transcriptomic, and proteomic profiling reveals various mechanisms by which multiple tumor types acquire resistance to the third-generation ALK inhibitor lorlatinib. *CancerRes;78(24);6866–80.©2018AACR*

Introduction

Activation of the anaplastic lymphoma kinase (ALK) is involved in the pathogenesis of different cancers, including anaplastic large cell lymphoma (ALCL), non–small cell lung cancer (NSCLC) and neuroblastoma[1]. ALK inhibitors (ALKi) were developed for specific treatment of ALK-positive patients[1,2]. Crizotinib demonstrated superior activity compared with chemotherapy in NSCLC and showed exceptional response rates in refractory ALCL and inflammatory myofibroblastic tumor (IMT) patients[3-5]. Unfortunately, the selection of drug-resistant clones has limited the long-term efficacy of crizotinib, especially in NSCLC[1,6,7]. The knowledge of resistance mechanisms guided the quest for new drugs to overcome crizotinib failure.

Several novel compounds were developed, by improving potency, selectivity, and brain penetration. Among these, lorlatinib (PF-06463922, a third-generation ALKi) showed activity against most drug-resistant mutants, including the highly refractory G1202R mutant[8-11]. Indeed, ceritinib-resistant patient derived cells carrying EML4-ALK mutations were shown to be sensitive to lorlatinib, while cells without ALK mutations were resistant[12], suggesting that resistance to this drug might arise from ALK-independent processes bypassing ALK

dependency, as observed in a fraction of patients with NSCLC treated with other ALKi[6,7,13,14]. In such cases, drug combinations could provide effective therapeutic options[15,16]. On the other hand, compound mutations may also represent a big challenge, still poorly characterized. Indeed, a C1156Y/L1198F mutation was found in a patient relapsed on lorlatinib[17]. Therefore, understanding the mechanisms leading to tumor escape is a key to the development of better therapeutic choices.

In this work, we investigated the spectrum of possible resistance mechanisms arising during lorlatinib treatment in ALK-dependent tumors. To this end, we kept ALCL, NSCLC, and neuroblastoma cells under selective pressure until drug-resistant clones evolved, *in vitro* and *in vivo*, from the original cell population.

Materials and Methods

Chemicals and cell lines

Lorlatinib and crizotinib were provided by Pfizer. Ceritinib, erlotinib, afatinib, alectinib, and trametinib were purchased from Selleck Chemicals. Karpas-299, SUP-M2, and HEK-293T cells were purchased from DSMZ, where they were routinely verified using genotypic and phenotypic testing to confirm their identity. H3122 and H2228 cell lines were provided by Dr. Claudia Voena (University of Turin, Turin, Italy). CLB-GA cells were provided by Dr. Valérie Combaret (Léon Bérard Cancer Centre, Lyon, France). Mycoplasma testing is routinely conducted on all cell lines in the laboratory.

Antibodies

The following antibodies were purchased from Cell Signaling Technology: ALK (31F12), EGFR, S6 ribosomal protein (RPS6), p44/42 MAPK (Erk1/2), STAT3, HER4/ErbB4 (111B2), AKT, phospho-ALK (Tyr1604), phospho-ALK (Tyr1278), phosphoEGFR (Tyr1068), phospho-RPS6 (Ser240/244), phospho-STAT3 (Tyr705), phospho-p44/42 MAPK (Erk1/2; Thr202/Tyr204), phospho-HER4/ErbB4 (Tyr1284; 21A9), phospho-AKT (Ser473), phospho-AKT (Thr308; D25E6). Anti-phosphotyrosine (PY20) and anti-actin antibodies were purchased from Sigma-Aldrich, and anti-tubulin was from Abcam. All antibodies were used at 1:1,000 dilution, except anti-actin (1:2,000).

In vitro selection of lorlatinib-resistant cells

Lorlatinib-resistant cell lines were established *in vitro* by exposing cells to gradually increasing drug concentrations, as described previously[18]. To monitor cell culture growth, ALCL cell number and viability were tracked by Trypan Blue count every other day, while confluency was estimated under the microscope for adherent cells. Every time the cells resumed proliferation rates comparable with parental cells, drug concentration was increased.

In vivo studies

For selection of resistant tumors *in vivo*, 6-week-old female *scid* mice (C.B.17/IcrHanHsd-Prkdc) were purchased from Envigo Laboratories (San Pietro al Natisone, Udine, Italy) and kept under standard conditions following the guidelines of the University of

Milano-Bicocca ethical committee for animal welfare. The protocol was approved by the Italian Ministry of Health and by the Institutional Committee for Animal Welfare. Lorlatinib was suspended in 0.5% carboxymethylcellulose/0.1% Tween80. Ten million Karpas-299 cells were injected subcutaneously into the left flank of the mice. Once tumors reached an average size of 200mm3, mice were randomized to receive vehicle alone (4 mice) or lorlatinib (10 mice; starting dose 0.1mg/kg), orally, twice a day. Tumor size was evaluated three times a week with a caliper, using the formula: tumor volume (mm3) = (d2 × D/2), where D is the longest and d is the shortest diameter. After 21 days, mice were shifted to receive 0.25 mg/kg twice a day. On day 37, lorlatinib was increased to 0.5mg/kg twice a day. After that, each mouse was followed individually, and dosage was increased every time the tumor relapsed or stabilized after partial regression (Supplementary Figure S1). Treatment was stopped at three different doses: 0.5 mg/kg (4 mice), 1 mg/kg (3 mice), or 2 mg/kg (3 mice). To confirm drug resistance of NSCLC and neuroblastoma cells *in vivo*, subcutaneous tumors (parental or resistant, 106 cells) were established in nude mice and treated with 1mg/kg twice a day (NSCLC) or 1.5 mg/kg twice a day (neuroblastoma) for two weeks.

PCR, detection of mutations, and next-generation sequencing

Total RNA was extracted using TRIzol (Invitrogen). Real-time quantitative PCR for NPM-ALK and ABCB1 was performed with Brilliant-III Ultra-Fast SYBR Green QPCR Master Mix (Agilent). Primers are reported in Supplementary Table 1. TaqMan qPCR for EGFR (Hs01076090_m1), ERBB4 (Hs00955522_m1), and ABCG2

(Hs01053790_m1) was performed using Brilliant-II QPCR Master Mix (Agilent) and probe mixes from Thermo Fisher Scientific. The beta-glucuronidase (GUS) gene was used as a reference (probe 5′-CCAGCACTCTCGTCGGTGACTGTTCA-3′). For mutation analysis, ALK kinase domain was amplified by High Fidelity Taq Polymerase (Roche) using primers shown in Supplementary Table 1. Purified PCR products were sent for Sanger sequencing to GATC Biotech or cloned by TOPO TA Cloning Kit (Invitrogen) and sequenced. Compound mutations were always confirmed by clonal Sanger sequencing. For ultra-deep sequencing, NPM-ALK kinase domain was amplified from parental and resistant cells using High Fidelity Taq Polymerase (Roche) and NPM-ALK KD primers (Supplementary Table 1). Amplicons were purified from agarose gel and sent to GalSeq srl for sequencing at 10,000x mean coverage. Fastq files were aligned onto the reference ALK transcript. Integrative Genomics Viewer (IGV; Broad Institute, Cambridge, MA) was used to visualize the data and annotate variants. Resistant cell–specific mutations were identified by filtering against parental cells' data. Whole-exome sequencing (WES) and RNA-sequencing were performed as described previously[19]. Briefly, genomic DNA was extracted from control and resistant cells using PureLink Genomic DNA Mini Kit (Invitrogen) and sent to GalSeq srl for sequencing at mean coverage 80x. Fastq files were aligned on reference human genome (hg38) and analyzed by CEQer2, an evolution of CEQer[20]. Variants were called if present in >25% of resistant cells reads and <5% of control reads. Synonymous and noncoding substitutions were discarded. Variants with <20x coverage in either control or case samples were filtered out. For RNA-sequencing, total RNA was

extracted from three independent vehicle-treated control K299 xenografts, and three lorlatinib-resistant tumors (AS4, BS1, and BD1) using TRIzol reagent, following the standard protocol. Samples were sent to Galseq srl for polyA selection, library preparation, and paired-end sequencing at approximately 50 million clusters per sample. Fastq sequences were aligned to the human genome (GRCh38/hg38) and raw counts were generated using STAR[21]. Differential gene expression analysis was performed with DESeq2 tool[22]. Functional enrichment for GO biological processes was performed with the Gene Set Enrichment Analysis software[23]. Heatmaps were produced with GENE-E (Broad Institute, Cambridge, MA). Mutations were validated by Sanger sequencing, using the primers described in Supplementary Table 2. The NGS data discussed in this publication have been deposited in NCBI's Sequence Read Archive and are accessible through accession number PRJNA491639.

Western blotting and phospho-RTK array

Cell lysates were prepared in Laemmli buffer and run on SDS-PAGE with specific antibodies (Supplementary Methods). The Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems) was used to evaluate phosphorylation of 49 human receptor tyrosine kinases. For this assay, cell lysates from parental and resistant cells were prepared in RIPA buffer (NaCl (150 mM), Nonidet P-40 (1%), Sodium deoxycholate 0.5%, SDS (0.1%), Tris (25 mM, pH 7.4)) with protease and phosphatase inhibitors and 300 µg of total proteins were processed according to manufacturer's instructions. Specific antibodies

are spotted in duplicate, with positive and negative control spots on membrane corners.

Proliferation, apoptosis, and colony assays

Cells (10,000/well) were incubated in the presence of the indicated compounds for 72 hours. Cell growth was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay System (Promega). Dose–response curves were generated using GraphPad Prism software. The IC_{50} value was calculated as the concentration inhibiting 50% of vehicle-treated control response (absolute IC_{50}). For cells showing a bell-shaped dose–response curve, the IC_{50} was calculated relative to the maximal response or peak (relative IC_{50}). The relative resistance (RR) index is defined as the fold shift of IC_{50} value as compared with control[24]. Apoptosis was determined after 72 hours, using the eBioscience AnnexinV Apoptosis Detection Kit FITC (Thermo Fisher Scientific) and analyzed on a FACSCalibur flow cytometer (BD Biosciences). For soft-agar colony assay, cells were suspended with drugs in RPMI:low melting agarose as described previously[25]. Colonies were counted after three weeks.

Immunofluorescence microscopy

Cells were washed with PBS and fixed with 4% p-formaldehyde in 0.12 mol/L sodium phosphate buffer, pH 7.4, and incubated for 1 hour with primary antibody (ALK, 31F12) diluted 1:100 in GDB buffer (0.02 mol/L sodium phosphate buffer, pH 7.4, 0.45 mol/L NaCl, 0.2% bovine gelatin) followed by 1-hour incubation with an Alexa 488-conjugated secondary anti IgG antibody. After washing with PBS,

coverslips were stained with DAPI and mounted on glass slides with a 90% glycerol/PBS solution. HEK-293T cells, seeded on glass coverslips coated with poly-D-lysine (0.1 mg/mL), were transfected with pcDNA6.2_GFP-NPM/ALK, WT, or N1178H. After 72 hours, cells were washed with PBS, fixed, stained with DAPI, and directly mounted on glass slides with a 90% glycerol/PBS solution. For quantification, >300 cells in at least 10 acquired fields were blindly analyzed per sample. Images were acquired using an LSM 710 inverted confocal microscope (Carl Zeiss) and analyzed using a specific macro with ImageJ software to measure the fraction of nuclear ALK mean intensity over the total signal. Statistical analysis was performed using a one-way ANOVA test for K299 and *ex vivo*–derived cell lines or an unpaired two-tailed *t* test for HEK293T–transfected cells.

Results

ALK-dependent mechanisms driving resistance in ALCL cells *in vitro* and *in vivo*

Two human ALCL cell lines, Karpas-299 (K299) and SUP-M2 (SUPM2), were selected *in vitro* until (approximately 4 months) they could propagate at 100 nmol/L lorlatinib. Both resistant lines (K299-LR100 and SUPM2-LR100) showed about 100-fold increased IC_{50} compared with parental cells (Figure 1A and B). Analysis of ALK phosphorylation suggested that reactivation of ALK may account for resistance (Figure 1C and D). To investigate how the resistant

populations evolve upon further increase of drug concentration, K299-LR100 and SUPM2-LR100 cells were then challenged with higher lorlatinib doses, up to 1 μmol/L. The new K299-LR1000 and SUPM2-LR1000 cell lines displayed a drug-addicted phenotype (Figure 1E and F), that is, their viability progressively decreased at lower lorlatinib doses[26]. They showed persistent ALK phosphorylation at high drug concentrations (Figure 1G and H) and increased NPM/ALK transcript level (Figure 1I and J). Mutational analysis revealed a G1202R substitution in approximately 25% of the cells in both LR100 populations (Figure 1K and L, top left charts). In addition, 15% of K299-LR100 developed a G1269A mutation (mutually exclusive with G1202R), while SUPM2-LR100 also carried a compound C1156F/L1198F mutation (25%). Interestingly, increasing lorlatinib concentration led to a progressive disappearance of the G1202R mutation in SUPM2-resistant cells, while the percentage of cells carrying the C1156F/L1198F mutation markedly increased, until they represented almost the entire SUPM2-LR1000 cell population (Figure 1L). Similarly, within K299 cell line, the G1269A mutant gradually emerged as the predominant clone, upon lorlatinib increase (Figure 1K). Noticeably, while the G1202R mutant tended to be outgrown by the G1269A clone up to 0.5 μmol/L lorlatinib, its frequency rose again at the highest dose (1 μmol/L) and the sum of G1269A- and G1202R-mutated reads exceeded 100%, suggesting that a compound mutation may have evolved. Clonal sequencing confirmed that the two mutations are on the same allele. Thus, the G1269A clone had independently acquired a second G1202R hit. No single G1202R mutant was found in K299-LR1000 cells. Overall, the identified NPM/ALK mutations may

well explain resistance in these cells. These data suggest that, despite the anticipated pan-ALK– inhibitory potency of lorlatinib, *in vitro*–selected ALCL cell lines quickly develop ALK mutants. At high concentrations, a highly resistant double mutant clone becomes predominant. Overexpression of mutated NPM/ALK is also likely to contribute to both drug resistance and drug dependency.

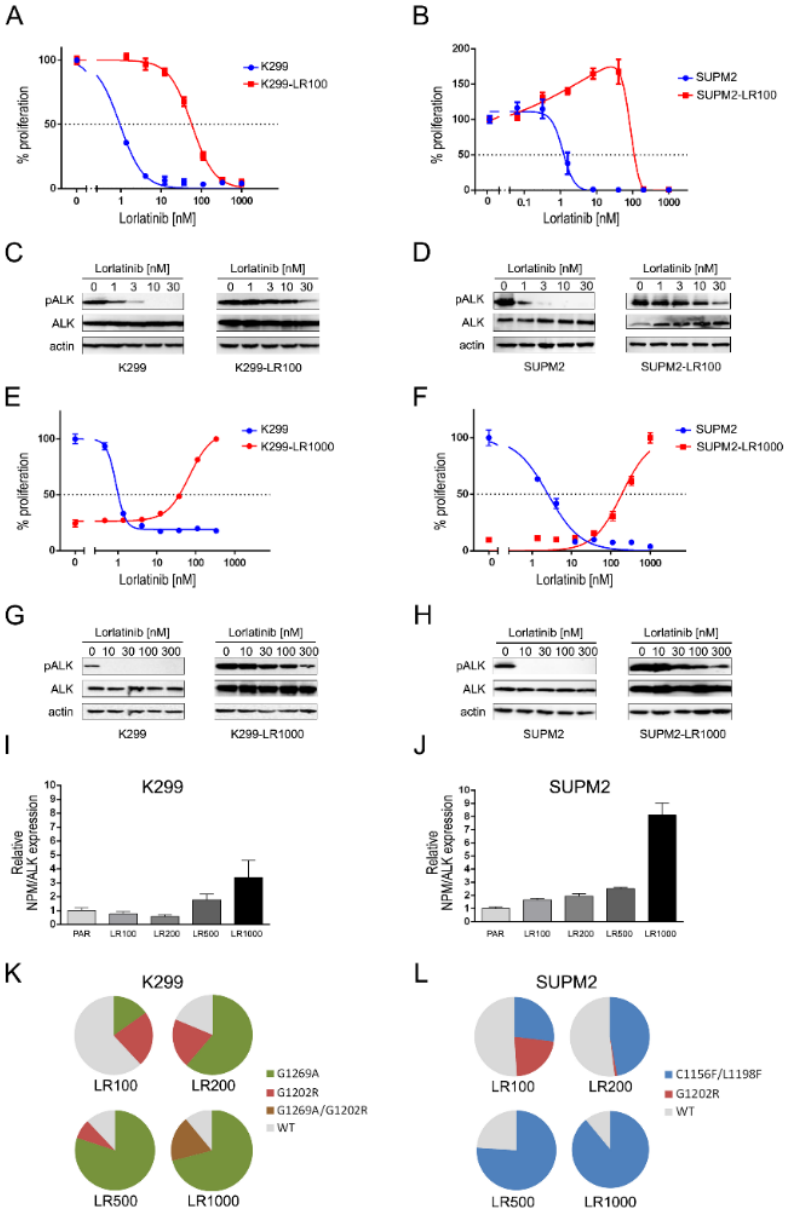

Figure-1. *Lorlatinib-resistant ALCL in vitro.* ***A–D****, Characterization of LR100-resistant cells: proliferation (****A*** *and* ***B****) and Western blot analysis (****C*** *and* ***D****) of K299 (****A*** *and* ***C****) and SUPM2 (****B*** *and* ***D****) parental*

versus resistant cells. ***E–H****, Characterization of LR1000-resistant cells: proliferation (****E*** *and* ***F****) and Western blot analysis (****G*** *and* ***H****) of K299 (****E*** *and* ***G****) and SUPM2 (****F*** *and* ***H****) parental versus resistant cells.* ***I*** *and* ***J****, NPM/ALK expression in parental and resistant K299 (****I****) and SUPM2 (****J****) cells during selection. LR100, LR200, LR500, and LR1000 indicate cells growing at 100, 200, 500, 1,000 nmol/L lorlatinib, respectively.* ***K*** *and* ***L****, Deep sequencing of NPM/ALK kinase domain in resistant K299 (****K****) and SUPM2 (****L****) cells during selection.*

Next, we assessed the emergence of lorlatinib-refractory tumors *in vivo*. Preliminary experiments showed that 0.5 mg/kg twice a day induces full regression of parental K299 xenografts (Figure 2A). Subsequently, mice bearing established K299 tumors were treated with lorlatinib at a suboptimal dose of 0.1 mg/kg (Figure 2A; Supplementary Figure S1). After 21 days, mice were shifted to 0.25 mg/kg and tumors regressed, but then relapsed. Hence, the mice underwent successive rounds of dosage increase. Each time, the tumors progressed after initial response. Three independent groups were sacrificed at different steps (0.5, 1, or 2 mg/kg lorlatinib). Finally, lorlatinib-resistant xenografts were excised and characterized. Proliferation assays confirmed resistance to lorlatinib, although at variable degrees. Sensitivity correlated with the dose at which the animals were sacrificed (Figure 2B). The IC_{50} values of the established *ex vivo* cell lines ranged from 6 to 175-fold the IC_{50} of parental cells (Figure 2C–E; Table 1). Interestingly, in few cases, the cells showed a bell-shaped proliferation curve and a slightly drug-addicted behaviour (e.g., AS4 and AS6).

Therefore, these cells were kept in culture in the presence of 3–10 nmol/L lorlatinib. However, in contrast to previously described drug-addicted cells[26], NPM/ALK transcript levels were not substantially upregulated (Table 1; Supplementary Figure S2). Analysis of NPM/ALK phosphorylation suggested that resistance was partially dependent on ALK activity, as in most resistant cell lines phospho-ALK was higher, in presence of the drug, compared with parental cells (Figure 2F). However, in several cases, relative resistance (RR) to ALK inactivation (as determined from Western blot analysis) did not correlate with cell proliferation data, suggesting that other mechanisms may be in place (Table 1; Figure 2). Sequencing of ALK kinase domain by ultradeep and Sanger sequencing revealed the copresence of multiple NPM/ALK–mutant clones in most xenografts.

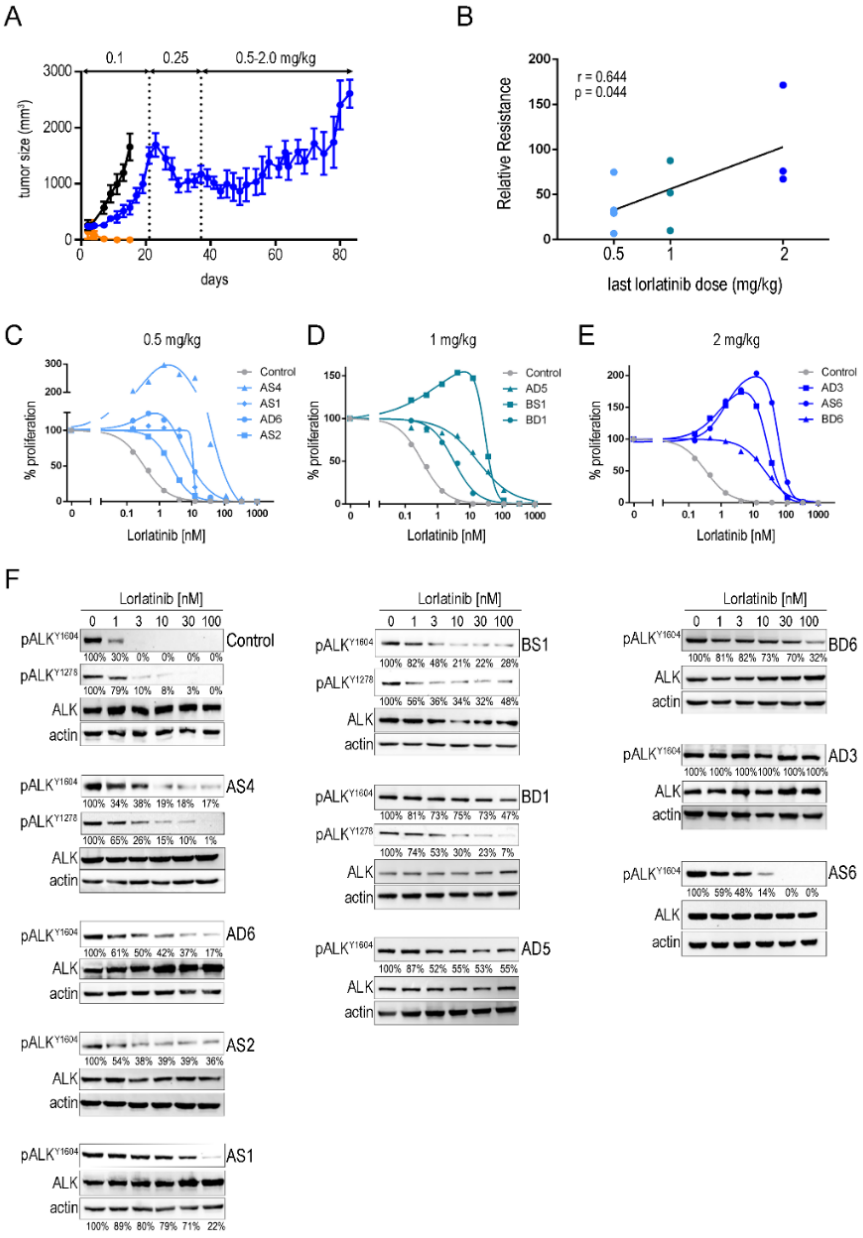

Figure-2. *Lorlatinib-resistant ALCL in vivo.* ***A****, Tumor growth of vehicle-treated mice (black) and mice treated with increasing lorlatinib doses (blue; dosing is indicated at the top) or with upfront 0.5 mg/kg*

lorlatinib since day 0 (orange). Dotted lines indicate the days of drug concentration switches. Between 0.5 and 2mg/kg, each mouse followed an own timing according to individual response to the drug (Supplementary Figure. S1). ***B****, Correlation between ex vivo* IC_{50} *values and in vivo dose reached by each xenograft.* ***C–F****, Ex vivo characterization of resistant xenografts: dose–response lorlatinib curves (****C–E****) grouped by dose reached in vivo (****C****, 0.5 mg/kg;* ***D****, 1 mg/kg;* ***E****, 2 mg/kg) and Western blot analysis of NPM/ALK dephosphorylation (****F****). Densitometry analysis of p-ALK/ALK signals is shown as a percentage of untreated cells*

Several recurrent mutations were identified, including N1178H (5/10 mice), G1269A (4 mice), I1171T (2 mice, both from the 2mg/kg group), G1202R (3 mice, across different dose groups), and L1196M (2 mice). In particular, G1269A was a minor subclone in the 0.5mg/kg group and became more frequent in animals treated at higher doses, up to 100% cells in mouse AS6. Thus, G1202R and G1269A mutations recurred both *in vitro* and *in vivo* and G1269A similarly expanded under increased lorlatinib concentrations, suggesting a possible critical role in the context of resistance to lorlatinib therapy in ALCL. In some cases, the observed mutations could not explain the resistant phenotype, based on their sensitivity to lorlatinib. For example, AS4 and BS1 carried a L1196M mutation, which is considered sensitive to lorlatinib[8,10]. Indeed, NPM/ALK phosphorylation was only mildly resistant to lorlatinib in these two cell lines, but the cells showed >100 RR in cell growth assays. The BD1 cell line showed contrasting results, that is,

persistence of the carboxy-terminal phospho-Tyr1604 but inhibition of phospho-Tyr1278 (the first activation loop tyrosine to be phosphorylated upon kinase activation[27], suggesting that NPM/ALK was indeed inhibited by lorlatinib, but either tyrosine 1604 is phosphorylated by other kinases in these cells, or its dephosphorylation by phosphatases is impaired or slower. This discrepancy was not observed in two other lines (AS4 and BS1) or in the control. Given the heterogeneity of NPM/ALK mutations in the relapsing tumors, ectopic cell models can help elucidate the relative contribution of single mutants within a population. Analysis of transduced Ba/F3 cells expressing various NPM/ALK mutants confirmed that C1156F, I1171T, G1202R, and G1269A cause a significant loss of lorlatinib sensitivity (Table 1; Supplementary Figure S3A and S3B). However, the Ba/F3 cell model could not explain high resistance of cells carrying a L1196M substitution, nor the observed high prevalence of N1178H mutation, which was found in 5 of 10 mice (50%), across all three dosage groups, at relevant frequency. Minor subpopulations carried N1178H in combination with G1269A (in AD5 and AD6 xenografts at 18% and 3%, respectively) but the majority of cells only harboured a single N1178H variant. Ba/F3 cells expressing an NPM/ALK^{N1178H} mutant showed a low RR index, indicating that the mutation does not confer resistance *per se*, in this cellular model (Supplementary Figure S3A and S3B). Because it is unlikely that half of the animals developed this mutation by chance, we sought to determine the possible mechanism of NPM/ALK^{N1178H} action in human cells. We noticed that this mutant has an inverted cytosol to nuclear distribution ratio, compared with normal NPM/ALK: BD1 cells, carrying a homogeneous

N1178H mutation (100% frequency), showed <20% nuclear localization (Figure 3A; Supplementary Figure S4).

Table-1. Ex-vivo characterization of resistant xenografts.

ID	Dose	PROLIFERATION		pALK-Y1604		pALK-Y1278		qN/A	MUTATIONS		BaF3-N/A	
	(mg/kg)	IC_{50} (nM)	RR	IC_{50} (nM)	RR	IC_{50} (nM)	RR	(fold)	Variant	Freq (%)	IC_{50} (nM)	RR
CTRL	vehicle	0.33	**1**	0.96	**1**	1.6	**1**	1	WT	100	1.4	**1**
AS4	0.5	26[a](52)	**79 (158)**	1.3	**1.3**	1.6	**1**	0.8	L1196M	50	5.6	**4**
									N1178S	2	nd	-
AD6	0.5	10	**30**	5.5	**5.7**	nd	-	1	N1178H	54	2.9	**2.1**
									P1153S	3	nd	-
									P1153H	3	nd	-
									N1178H/G1269A	3	nd	-
									C1156F	2	183	**130**
									T1151A	2	nd	-
AS2	0.5	2.3	**7**	4.3	**4.5**	nd	-	1.2	N1178H	74	2.9	**2.1**
									G1202R	24	79	**56**
AS1	0.5	11	**33**	43	**45**	nd	-	1.6	N1178H	90	2.9	**2.1**
									G1202R	10	79	**56**
BS1	1	30[a](38)	**91 (115)**	5.2	**5.4**	3.9	**2.4**	0.6	L1196M	50	5.6	**4**
AD5	1	17	**52**	54	**56**	nd	-	1.7	N1178H	64	2.9	**2.1**
									N1178H/G1269A	18	nd	-
									E1241G	18	nd	-
									N1178H/C1156Y	9	nd	-
BD1	1	3.2	**10**	164	**171**	4.5	**2.8**	1	N1178H	100	2.9	**2.1**
BD6	2	23	**70**	53	**55**	nd	-	0.8	G1202R	37	79	**56**
									G1269A	22	140	**100**
									C1156Y	5	2.8	**2**
									N1178H	5	2.9	**2.1**
									I1171T	4	173	**123**
AD3	2	26[a](39)	**79 (118)**	>100	**>100**	nd	-	0.5	I1171T	82	173	**123**
									I1171T/E1210K	9	67	**48**
AS6	2	58[a](105)	**176 (318)**	2.5	**2.6**	nd	-	3	G1269A	100	140	**100**

NOTE: For each of the established cell lines, lorlatinib dose at tumor excision, activity of lorlatinib on cell proliferation and ALK phosphorylation (IC_{50} and corresponding RR values) are reported; RR = 1 for control), ALK mutations identified and their frequency, IC_{50} and RR of mutants when expressed in Ba/F3 cells, and relative NPM/ALK expression (qN/A; control = 1) are reported. IC_{50} values are expressed in nmol/L. RR values are shown in bold text. Abbreviation: N/A, NPM/ALK.

[a]For cells showing a bell-shaped dose–response curve, the relative IC_{50} (and corresponding RR) is shown (see Materials and Methods). Absolute IC_{50}/RR values are reported in parentheses.

The same was observed in two additional K299-derived cell lines previously selected *in vitro* under different inhibitors and harbouring 100% N1178H mutant [K1, resistant to ASP3026[9]; K300, resistant to brigatinib[28]]. In contrast, control cells and AS4 (carrying a L1196M substitution) showed the expected ratio. AS2 cells, harbouring a mixture of different mutants including an N1178H subclone, showed an intermediate localization ratio. To further validate these observations, wild-type or N1178H mutant GFP-NPM/ALK were expressed in HEK-293T cells and their localization was analyzed. Cells expressing the mutant showed stronger cytoplasmic signal, while fluorescence was barely detected in the nucleus. In contrast, wild-type NPM/ALK was equally distributed in both compartments, as expected (Figure 3B and C).

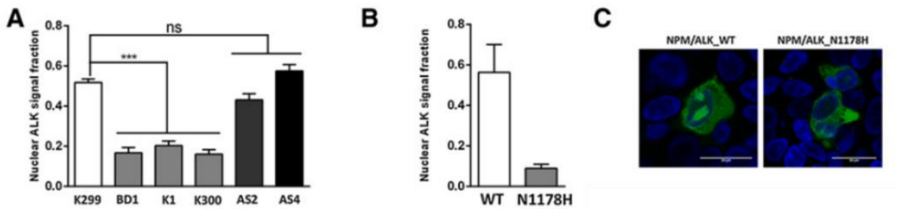

Figure-3. *Analysis of resistant ALCL xenografts.* ***A–C,*** *Relative ALK nuclear localization signal in parental and mutant K299-derived cells (**A**) and in transfected HEK-293T cells (**B**); representative micrographs of HEK-293T$^{GFP\text{-}NPM/ALK}$ cells are shown (**C**).* ****, P<0.001. Scale bar, 20 µm.*

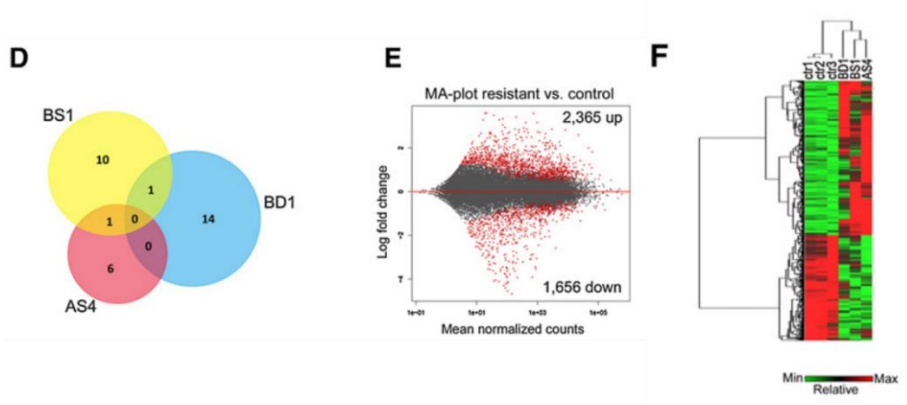

Figure-3. *Analysis of resistant ALCL xenografts.* ***D,*** *Number of mutations identified in three tumors by WES, filtered against control cells data.* ***E,*** *MA plot of differentially regulated genes in resistant versus control samples. Red dots, significantly modulated genes.* ***F,*** *Heatmap of hierarchical clustering of three resistant and three control tumor mRNA profiles.*

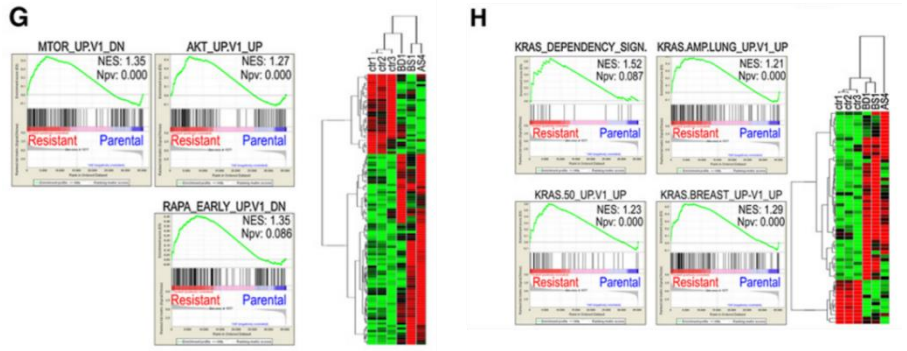

Figure-3. *Analysis of resistant ALCL xenografts.* ***G*** *and* ***H,*** *Gene-set enrichment analysis of resistant versus control xenografts shows enriched AKT/mTOR and RAS/MAPK signatures in lorlatinib-resistant tumors. Heatmaps of corresponding genes are shown on the right.*

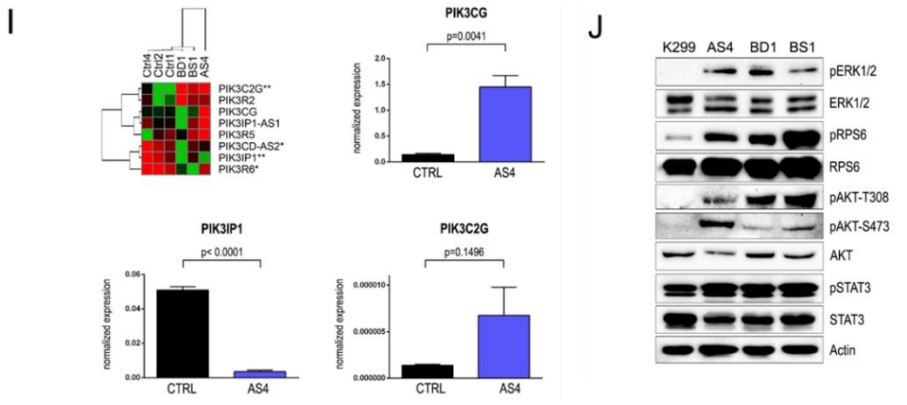

Figure-3. *Analysis of resistant ALCL xenografts.* ***I,*** *Heatmap and qPCR of selected PI3K family member genes.* **, P<0.05;* ***, P<0.01.* ***J,*** *Western blot analysis of PI3K/AKT/mTOR, RAS/MAPK, and STAT3 pathways activation in resistant versus control xenografts.*

ALK-independent mechanisms of resistance

Activation of PI3K/AKT and RAS/MAPK pathways in ALCL xenografts.

To investigate alternative, ALK-independent mechanisms driving resistance in the xenografts that carried ALKi-sensitive mutants and/or showed a significant fraction of wild-type ALK sequence, a global next-generation sequencing (NGS) approach was taken. Two cell lines harbouring the L1196M mutation (BS1 and AS4) and one carrying an N1178H mutation (BD1) were analyzed by whole-exome and mRNA sequencing, using three control xenografts as a reference. Comparative exome data showed an average of 11 (range 7–15) mutations in resistant tumors (Figure 3D; Supplementary Table 3).

Only two mutated genes (other than ALK) were shared by at least two samples: $ESYT3^{L296M}$ in BS1 and AS4 cells, carrying the same NPM/ALK mutation; thus, it might represent a passenger substitution in a preexisting L1196M clone. Conversely, two different RHOBTB2 mutations arose independently in BS1 (I158V) and BD1 (V126F) cells (Supplementary Figure S5). RHOBTB2 is a tumor suppressor that is upregulated during drug-induced apoptosis and inhibits AKT[29,30]. Thus, its inactivation may contribute to drug resistance. Valine 126 is a highly conserved residue within the GTPase domain of RHOBTB2 and its change to phenylalanine is predicted to be deleterious. Further studies are ongoing to validate the role of these substitutions in resistance to ALK inhibition.

Differential gene expression analysis from RNA-seq data revealed approximately 4,000 significantly dysregulated genes in BS1, BD1, and AS4 cells compared with controls (Figure 3E). Unsupervised hierarchical clustering showed that the three resistant samples clustered together, separated from controls (Figure 3F). In particular, BS1 and AS4 cells were more closely related while BD1 was a little more distant, in line with their ALK mutational status. Gene-set enrichment analysis suggested two significantly enriched types of signature in the resistant samples, pointing to PI3K/AKT/mTOR (Figure 3G) and KRAS/MAPK pathways (Figure 3H). Analysis of gene expression data by connectivity map tool[31] indicated two AKT inhibitors among the three top negatively connected perturbagens, suggesting that AKT inhibition may lead to reversal of the biological state encoded in the query signature (i.e., resistance to lorlatinib). When closely looking at the PI3K/AKT pathway, several members of the PI3K gene family appeared

deregulated, especially in AS4 cells, including PIK3CG (encoding for the PI3K-p110γ catalytic subunit), PIK3C2G (class II PI3K-C2γ), and PIK3IP1, a negative regulator of PI3K/AKT signaling[32] (Figure 3I). At protein level, all three resistant tumors showed hyperactivated MAPK and PI3K/AKT pathways compared with control, as indicated by phosphorylated (p-)ERK1/2 and by p-S6 and p-AKT, respectively (Figure 3J).

Curiously, an inverted ratio between AKT p-Ser473 and p-Thr308 signals was noted in AS4 compared with the other two xenografts, which might imply different substrate specificity[33]. In contrast, STAT3 was highly activated both in control and in resistant cells. These results suggested that MAPK and PI3K pathways might provide ALK-independent survival cues bypassing ALK inhibition.

To validate these findings, AS4 cells were treated with the pan-PI3K inhibitor pictilisib (GDC-0941), alone or in combination with lorlatinib, to see whether inhibition of PI3K pathway could overturn resistance. AS4 cells were slightly more sensitive than parental K299 to the PI3K inhibitor alone (Figure 4A). Moreover, pictilisib was able to partially reverse resistance to lorlatinib, as shown by a significant shift of the dose–response curve (approximately 5-fold IC_{50} reduction, $P = 0.0365$; Figure 4B). When the cells were treated with a combination of lorlatinib and pictilisib during a 7-day course, we observed a significant reduction of viability, compared with single-agent treatments (Figure 4C).

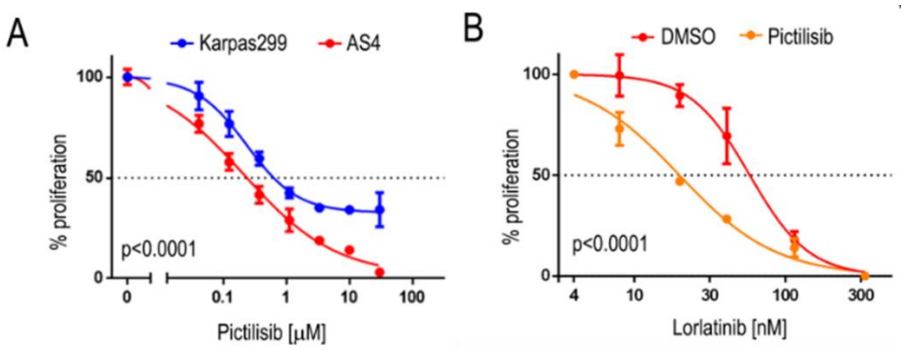

Figure-4. *Validation of PI3K/AKT and RAS/MAPK pathways as mediators of resistance.* ***A,*** *Proliferation of control and AS4 cells treated with pictilisib.* ***B,*** *Proliferation of AS4 cells treated with lorlatinib alone or combined with 1 μmol/L pictilisib.*

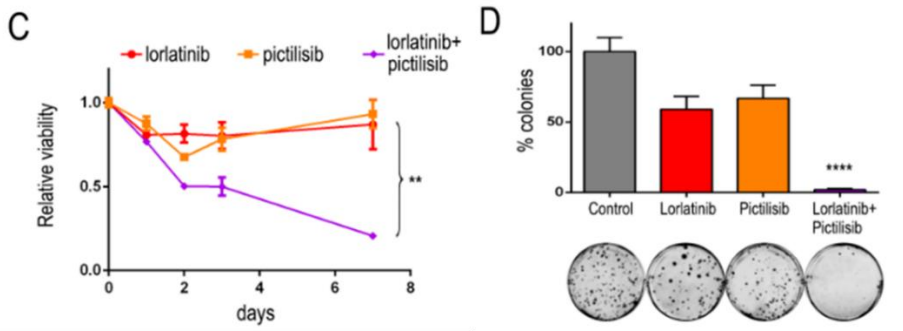

Figure-4. *Validation of PI3K/AKT and RAS/MAPK pathways as mediators of resistance.* ***C,*** *Time-course of single versus combined treatment in AS4 cells (50 nmol/L lorlatinib; 1 μmol/L pictilisib).* ***D,*** *Colony-forming assay with AS4 cells treated with vehicle, 100 nmol/L lorlatinib, 1μmol/L pictilisib, or combination. Colonies were counted after 21 days. Representative images are shown below.* ***, P<0.01;* *****, P<0.0001*

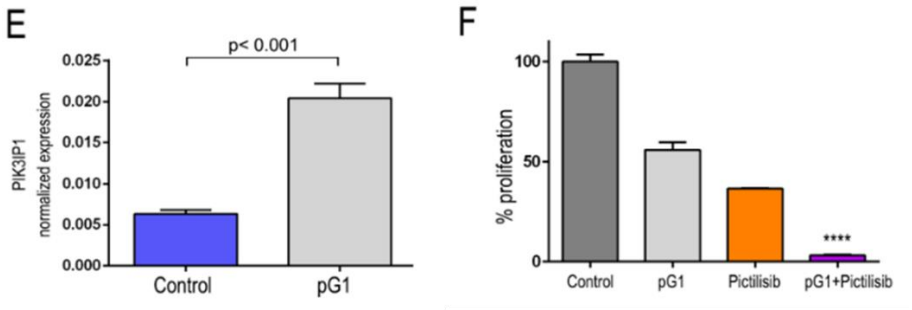

Figure-4. *Validation of PI3K/AKT and RAS/MAPK pathways as mediators of resistance.* ***E,*** *qPCR of PIK3IP1 in AS4 cells at baseline (Control) and after 24-hour serum starvation (pG1).* ***F,*** *Cell growth of AS4 cells treated with vehicle (Control), vehicle after pG1, 1 µmol/L pictilisib, or 1 µmol/L pictilisib after pG1.* ***, P<0.01;* *****, P<0.0001.*

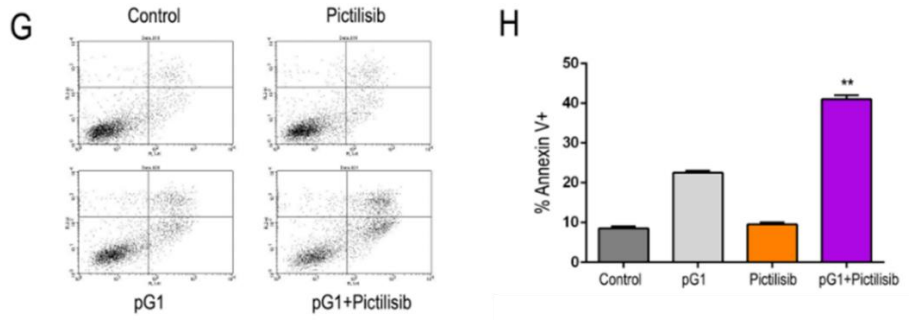

Figure-4. *Validation of PI3K/AKT and RAS/MAPK pathways as mediators of resistance.* ***G*** *and* ***H****, Apoptosis by AnnexinV/propidium iodide staining in AS4 cells treated as in* ***F****; representative plots (****G****) and quantification (****H****) from two experiments are shown.* ***, P<0.01.*

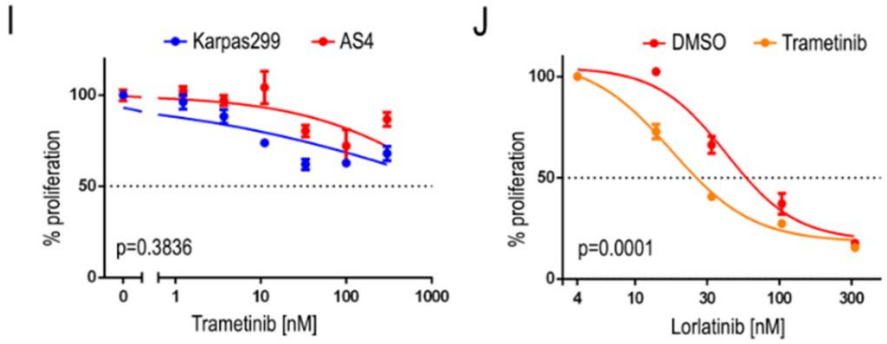

Figure-4. *Validation of PI3K/AKT and RAS/MAPK pathways as mediators of resistance.* ***I,*** *Proliferation of control and AS4 cells treated with trametinib.* ***J,*** *Proliferation of AS4 cells treated with lorlatinib alone or combined with 10 nmol/L trametinib.*

Moreover, the combination was highly effective in suppressing anchorage independent colony growth (Figure 4D). It has been shown that prolonged G1 arrest (pG1) sensitizes lymphoma cells to PI3K inhibition through induction of PIK3IP1 expression[34]. Given the striking reduction of PIK3IP1 in AS4 cells compared with parental cells, we induced AS4 cell-cycle arrest via serum deprivation and then treated cells with vehicle or pictilisib for 72 hours after readdition of serum. After 24 hours pG1, PIK3IP1 levels increased 4-fold in AS4 cells (Figure 4E). Treatment with the PI3K inhibitor under these conditions led to near complete suppression of cell growth (Figure 4F) and concomitant induction of cell death (Figure 4G and H), while pictilisib treatment in nonarrested cells had only modest effects. The second most enriched pathway in our dataset was RAS/MAPK;

therefore, we investigated the activity of the MEK inhibitor trametinib in lorlatinib-resistant cells. Single agent efficacy was comparable in parental and resistant cells (Figure 4I). However, trametinib induced a small but significant shift in sensitivity to lorlatinib when used in combination (Figure 4J), suggesting that MAPK pathway may partially contribute to resistance to ALK inhibition in these cells. A triple combination of lorlatinib, pictilisib, and trametinib further reduced cell growth, in an additive manner (Supplementary Figure S6). These data suggest that resistance to lorlatinib in ALCL xenografts follows complex trajectories involving both ALK-dependent and -independent pathways, coexisting in the same cell and contributing to drug resistance.

EGFR activation in ALK-positive NSCLC cells.

To broaden the landscape of molecular mechanisms that may hamper lorlatinib therapy, we selected *in vitro* two drug-resistant ALK-positive NSCLC cell lines, employing the usual protocol of selection[18]. Two NSCLC cell lines carrying different EML4/ALK variants were used: H3122 (v1) and H2228 (v3a/b). It has been suggested that variant 3 is less sensitive to ALK kinase inhibition[35]. We did not observe significant differences between the two cell lines in a 72-hour proliferation assay (Figure 5A and B). However, when put under lorlatinib selection, H2228 developed drug resistance much more slowly (Figure 5C). For several weeks, these cells appeared as stalled, neither dying, nor growing, until they finally started to grow at a normal pace in the presence of the drug. Ultimately, it took seven months to

select a population growing at 100 nmol/L lorlatinib. In contrast, H3122 underwent a rapid selection of the most resistant subclone and reached 100 nmol/L drug in half the time. In both cases, the selected cells (LR100) were highly resistant to lorlatinib (Figure 5A and B). To confirm resistance *in vivo*, H3122 and H3122-LR100 xenografts were established in mice. While parental cells rapidly regressed under lorlatinib treatment, resistant cells failed to do so (Figure 5D). However, phosphorylation of EML4/ALK was fully inhibited by lorlatinib at low nanomolar doses in both drug-resistant cell lines, while a downstream target, such as the ribosomal protein S6 (RPS6), was phosphorylated, suggesting the activation of alternative survival pathways that bypass ALK kinase inhibition (Figure 5E and F). Indeed, both H2228- and H3122-derived resistant cells showed activation of EGFR, which was not inhibited by lorlatinib, but could be effectively blocked by erlotinib (Figure 5G and H). Interestingly, H3122 parental cells showed rapid activation of EGFR upon lorlatinib treatment (10 nmol/L, 4 hours), suggesting an adaptive mechanism that may explain the rapid evolution of the resistant clone in this cell line. In line with these observations, treatment of H3122-LR100 with erlotinib resensitized the cells to lorlatinib (Figure 5I). Moreover, an upfront combined ALK/EGFR block could prevent the emergence of a resistant clone from H3122 cells, while single treatments eventually allowed cells expansion (Figure 5J and K). In contrast, H2228-LR100 were insensitive to EGFR inhibition (Figure 5L), which indicates that they have accumulated additional genetic lesions that may account for drug resistance. The long latency for development of resistance maybe responsible for this phenotype. We asked whether the MAPK pathway might be involved.

To test this hypothesis, H2228-LR100 cells were treated with trametinib in combinations with lorlatinib and erlotinib. Indeed, the triple combination caused remarkable growth inhibition (Figure 5M). Altogether, these results show that NSCLC cells can survive lorlatinib therapy by switching to ALK independency.

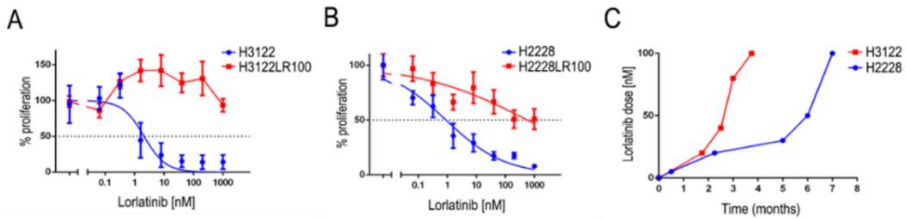

Figure-5. *Lorlatinib-resistant NSCLC.* ***A*** *and* ***B,*** *Proliferation of parental and resistant H3122 (****A****) and H2228 (****B****) cells treated with lorlatinib.* ***C,*** *Development timeline of H3122 and H2228 lorlatinib-resistant cells. Symbols correspond to drug dose increases.*

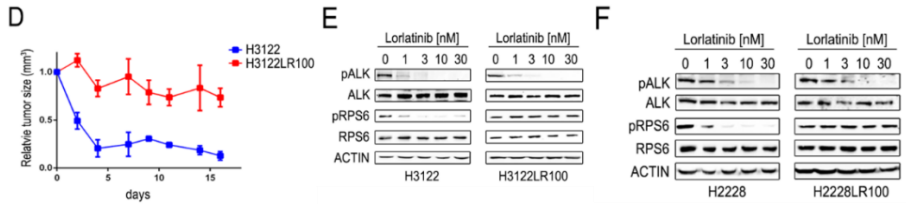

Figure-5. *Lorlatinib-resistant NSCLC.* ***D,*** *Tumor growth of parental and resistant H3122 xenografts treated with lorlatinib.* ***E*** *and* ***F,*** *Western blot analysis of parental versus resistant H3122 (****E****) and H2228 (****F****) cells treated with lorlatinib.*

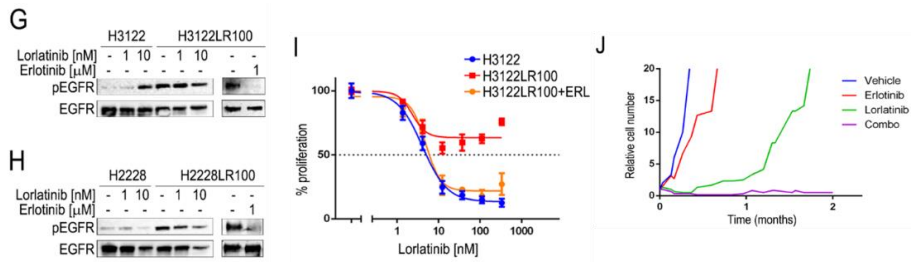

Figure-5. *Lorlatinib-resistant NSCLC.* ***G*** *and* ***H****, Phospho-EGFR activation in resistant versus parental H3122 (****G****) and H2228 (****H****) cells treated with vehicle, lorlatinib, or erlotinib.* ***I,*** *Proliferation of H3122 and H3122-LR100 cells treated with lorlatinib, with or without 1 μmol/L erlotinib.* ***J,*** *Selection of drug-resistant H3122 cells under lorlatinib (10 nmol/L), erlotinib (1 μmol/L), or combination.*

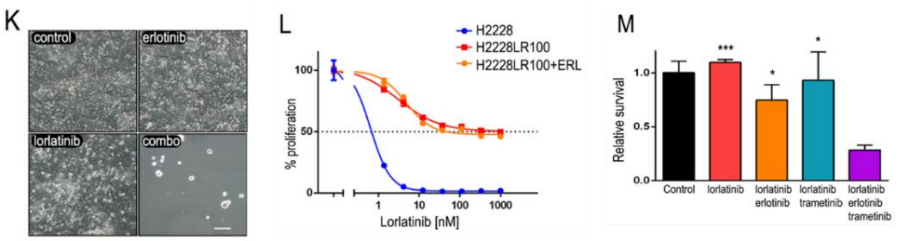

Figure-5. *Lorlatinib-resistant NSCLC.* ***K,*** *Photographs of cells taken during the selection shown in* ***J****. Scale bar, 50 μm.* ***L,*** *Proliferation of H2228-LR100 cells treated with lorlatinib, with or without 1 μmol/L erlotinib.* ***M,*** *Survival of H2228-LR100 cells treated with the indicated inhibitors (lorlatinib, 100 nmol/L; erlotinib, 1 μmol/L; trametinib, 100 nmol/L). Asterisks refer to comparisons with the triple combination (* **, P<0.05;* ****, P<0.001).*

ErbB4 activation and NF1 loss in neuroblastoma cells.

Two non MYCN–amplified neuroblastoma cell lines expressing full-length ALK with activating mutations, SH-SY5Y (ALK^{F1174L}) and CLB-GA (ALK^{R1275Q}) were tested for sensitivity to lorlatinib. SH-SY5Y proved relatively resistant to treatment ($IC_{50} \approx 300$ nmol/L) and did not appear to undergo any relevant population selection up to 2 µmol/L; therefore, they were not further analyzed. In contrast, CLB-GA cells were highly sensitive to lorlatinib (Figure 6A). After several passages in the presence of the drug, a resistant cell line was selected, showing no perturbation of cell growth up to 1 µmol/L lorlatinib *in vitro* (CLB-GA-LR1000; Figure 6A). These cells grew fast under lorlatinib treatment *in vivo* as well, while parental tumors fully regressed (Figure 6B). However, ALK kinase was effectively inhibited by the drug, even though basal phosphorylation was a little higher in the resistant cells (ALK dephosphorylation IC_{50}, 41 vs. 57 nmol/L; Figure 6C). No additional mutations were found in the ALK kinase domain and neither ALK nor MYCN expression levels were upregulated (Figure 6C; Supplementary S7A and S7B). Interestingly, global anti-phosphotyrosine blotting showed a marked increase of tyrosine kinase cascade signal in CLB-GA-LR1000 cells, further confirmed by downstream p-S6 levels (Figure 6C). This result suggested that an alternative tyrosine kinase may have taken over as a survival pathway. Indeed, phospho-RTK array analysis showed hyperactivation of EGFR and ErbB4 kinases in lorlatinib-resistant neuroblastoma cells (Figure 6D), probably as a result of kinase overexpression, as EGFR and ERBB4 transcript levels were approximately 3- and 300-fold higher in

CLB-GA-LR1000 than in parental cells, respectively (Supplementary Figure S7C and S7D). These results suggest that activation of ErbB family pathway may be involved in lorlatinib resistance in neuroblastoma cells, as a bypass track. Further genetic analysis by WES revealed that CLB-GA-LR1000 cells had acquired additional mutations in important genes related to cell growth and survival (Supplementary Table 4; Supplementary Figure S5). In particular, a heterozygous truncating NF1 mutation (Figure 6E) is predicted to cause aberrant activation of the RAS/MAPK pathway[36]. Accordingly, ERK1/2 appeared to be more active in drug-resistant compared with parental cells (Figure 6F). Treatment of CLB-GA-LR1000 cells with the pan-ErbB inhibitor afatinib efficiently shut down ErbB4 kinase activation (Figure 6G) but, surprisingly, had only minor effects on cell growth and survival and showed poor synergism with lorlatinib. In contrast, the MEK1/2 inhibitor trametinib blunted ERK1/2 activation (Figure 6H) and restored full sensitivity to lorlatinib (Figure 6I), suggesting that MAPK pathway activity contributes to drug resistance in these cells. Indeed, a marked difference was evident between the observed and the predicted effects of the combination according to Bliss independence model ($\Delta fa = -0.34$). When the cells were challenged with a three-wise drug combination, cell viability was further reduced; however, the effect of adding afatinib to a lorlatinib/trametinib treatment was only additive ($\Delta fa = -0.01$), again indicating limited contribution of EGFR/ErbB4 to drug resistance.

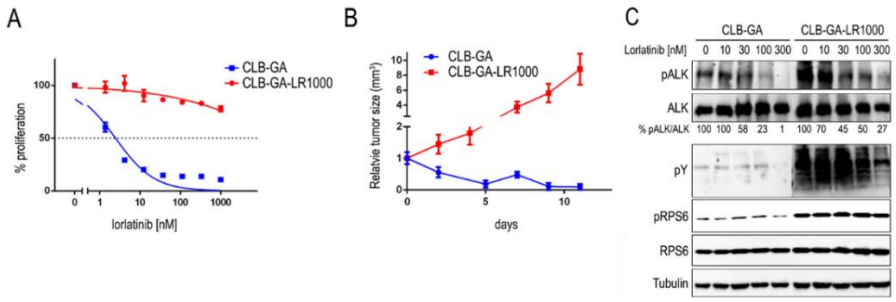

Figure-6. *Lorlatinib-resistant neuroblastoma.* ***A,*** *Proliferation of parental and resistant CLB-GA cells treated with lorlatinib.* ***B,*** *Tumor growth of parental and resistant CLB-GA xenografts treated with lorlatinib.* ***C,*** *Western blot analysis of parental versus resistant CLB-GA cells treated with lorlatinib. Normalized pALK is reported.*

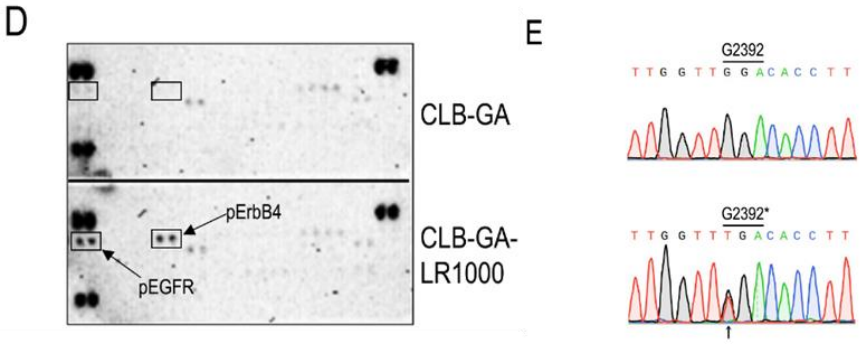

Figure-6. *Lorlatinib-resistant neuroblastoma.* ***D,*** *Hyperactivation of EGFR and ErbB4 in CLB-GA-LR1000 cells shown by phospho-RTK arrays. Phospho-EGFR and phospho-ErbB4 spots are framed and indicated by arrows.* ***E,*** *Sanger sequencing chromatogram showing the NF1 truncating mutation (indicated by an arrow) found in lorlatinib-resistant cells. The corresponding sequence from parental cells is shown for a comparison.*

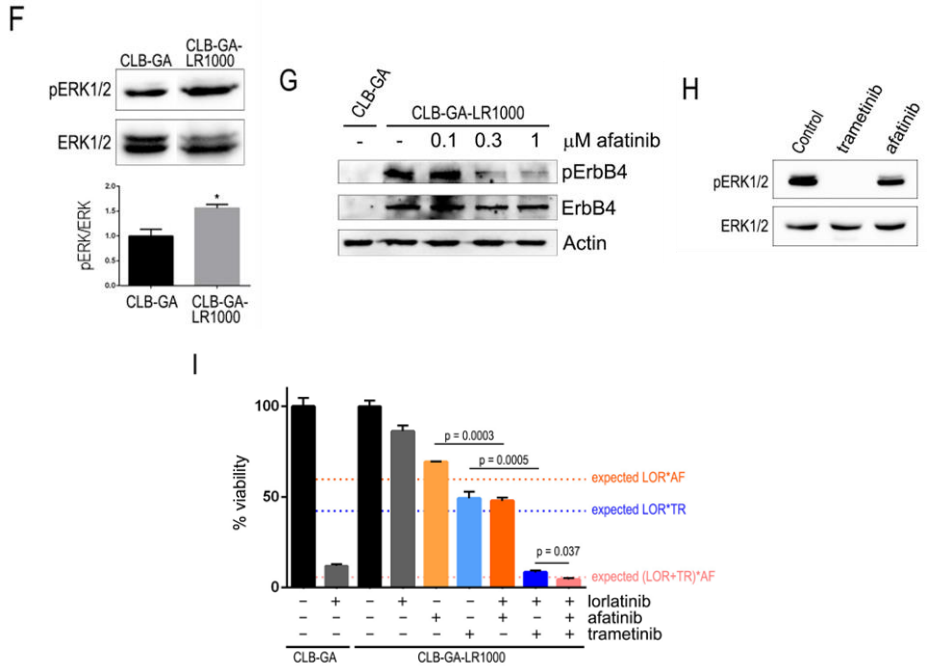

Figure-6. *Lorlatinib-resistant neuroblastoma.* ***F,*** *Phosphorylation of ERK1/2 in parental and resistant CLB-GA cells; normalized quantification is shown below.* ***G,*** *Western blot analysis confirmation of ErbB4 aberrant activation in CLB-GA-LR1000 cells and its modulation by afatinib.* ***H,*** *ERK1/2 inhibition by trametinib or afatinib.* ***I,*** *Drug combinations overcome resistance to lorlatinib in CLB-GA-LR1000 cells. Dotted lines indicate the predicted effect of combinations according to the Bliss Independence model, typed using the same colors as the corresponding combination histogram. When the observed residual viability of the combination is lower than that expected by Bliss model, the combination is supra-additive (i.e., synergistic). Addition of trametinib to lorlatinib (dark blue bar) restores full sensitivity in CLB-GA-LR1000 cells, while afatinib shows a limited effect. LOR*AF, lorlatinib + afatinib combo; LOR*TR, lorlatinib + trametinib combo; (LOR+TR)*AF, triple combo.*

Discussion

Drug resistance is a current limitation of tyrosine kinase inhibition therapy. In highly heterogeneous disease, drug pressure selects resistant clones that cause tumor relapse. Despite these drawbacks, targeted treatments have the potential to control the disease for long time. To better exploit this possibility, we need to fully understand the routes of drug resistance and devise strategies to prevent or overcome it. This study presents an array of potential mechanisms by which ALK-dependent tumors may elude targeted inhibition by the third-generation ALKi lorlatinib (Supplementary Figure S8). In ALCL models, ALK kinase domain mutations were observed in the majority of cases, both *in vitro* and *in vivo*. In contrast to previous drugs, mostly selecting single-point mutants, lorlatinib-resistant cells progressively accumulated compound mutations. Interestingly, we identified a C1156F/L1198F mutant, nearly identical to the C1156Y/L1198F mutation found in a NSCLC patient relapsed on lorlatinib[17]. In our experiments, C1156F and C1156Y responded differently as single mutants in Ba/F3 cells (Supplementary Figure S3A and S3B), but the double mutation is likely to increase resistance in both cases. A G1269A variant repeatedly emerged from K299 cells both *in vitro* and in mice, indicating that it may represent a major challenge to lorlatinib therapy. In Ba/F3 cells, this mutation conferred a significant shift in sensitivity to lorlatinib. Similarly, Zou and colleagues reported an important loss of activity on EML4/ALK^{G1269A} phosphorylation in Ba/F3 cells[36]. In contrast, Gainor and colleagues described G1269A as sensitive to lorlatinib[7]. Despite different results in different settings, this

mutant was greatly enriched in a highly resistant population growing at a drug concentration not achievable clinically[37], which speaks in favour of a considerably less sensitive variant. Its association with a second hit (G1202R) is likely to further enhance drug resistance. Thus, compound mutants are predicted to become a big complication under this potent inhibitor. While this manuscript was in preparation, a similar scenario was described in EML4/ALK–positive Ba/F3 cells and in patients[38]. Interestingly, the authors found one patient who progressed on lorlatinib carrying a G1269A mutation, and one patient with a compound G1269A/G1202R mutation, providing clinical relevance to our findings.

An N1178H mutation was frequently found in our relapsed mice. We observed that N1178H-mutant ALCL cells accumulate more NPM/ALK kinase in the cytoplasm, as compared with wild-type cells, suggesting an altered cyto-nuclear shuttling. Whether this is due to inefficient dimerization with normal NPM1, or preferential binding to cytoplasmic proteins, is unclear at present and is under investigation. We speculate that the aberrant cytoplasmic localization of N1178H mutants functionally mimics an overexpressed fusion kinase, since the cytoplasmic fraction of NPM/ALK is thought to be the oncogenic driver[26], thus leading to resistance. This behaviour may also explain the curious difference in Tyr1604 versus Tyr1278 uniquely observed in N1178H-mutated BD1 cells.

Several xenografts that relapsed *in vivo* under lorlatinib treatment carried ALK mutations that, alone, would not fully explain tumor resistance. Fusion transcript levels were not sufficiently high to support drug resistance. As a comparison, we earlier described

brigatinib-resistant cells expressing 15- to 25-fold more transcript[28]. Therefore, we sought to identify other determinants of resistance. Through an unbiased NGS approach, the PI3K/AKT/mTOR signaling was found to be commonly altered in three lorlatinib-resistant xenografts. Several catalytic and regulatory subunits of the PI3K family were deregulated, including p110γ and PI3K-C2γ, ultimately pointing to aberrant activation of proliferative and survival signals. PI3K-p110γ has been shown to control T-cell survival via activation of AKT and ERK 1/2[39]. It was also found upregulated in crizotinib-resistant cells and its role in driving resistance is under investigation (manuscript in preparation). PI3K-C2γ is a class II PI3K isoform that activates AKT2[40]. Abnormal activation of PI3K/AKT signaling has been linked to drug resistance in different diseases[41-43]. Indeed, a pan-PI3K inhibitor restored sensitivity to lorlatinib. Further analysis indicated also a RAS/MAPK signature upregulated in resistant tumors, leading to partial efficacy of a MEK inhibitor. The MAPK and PI3K pathways have been shown to be highly interconnected by cross-talks and feedbacks, highlighting an intricate network supporting survival signals[44]. These data suggest that several mechanisms may coexist in a resistant cell, each contributing part of the phenotype, including on-target mutations that do not seem, alone, to support growth (Supplementary Figure S8). For example, the gatekeeper L1196M is considered sensitive to lorlatinib; however, mechanistically, it shows a nonnegligible loss of sensitivity (approximately 4-fold) compared with wildtype, which may have an impact when combined with other survival means.

While ALK mutations appeared to be a driving force of drug resistance in ALCL (albeit associated with additional off-target mechanisms), NSCLC and neuroblastoma cells did not develop any ALK substitution in this setting. Rather, they seemed to prefer the activation of bypass tracks, such as EGFR, ErbB4, and RAS signals (Supplementary Figure S8). The reason why ALCL cells acquired multiple ALK mutations while NSCLC and neuroblastoma cells did not is unclear. Either ALCL cell lines harbour a larger pool of preexisting ALK-mutated cells, or solid tumors have easier access to alternative survival pathways that relieve addiction to the primary oncogene. In patients with lung cancer progressing on crizotinib, only one-third of cases are confirmed to harbour an ALK mutation or amplification, while the remaining patients show either bypass or unknown mechanisms[6]. The more potent inhibitors are employed, the more frequently ALK-independent mechanisms are likely to develop. Curiously, lorlatinib-resistant H2228 cells emerged much more slowly compared with H3122. This slow kinetics is reminiscent of the evolution of drug-tolerant cells[45]. Indeed, this difference reflected on the sensitivity of the two resistant cell lines to inhibition of EGFR.

The concept of complex resistance mechanisms was again recapitulated in neuroblastoma cells, in which both *EGFR* and *ERBB4* genes were markedly upregulated and hyperactivated. In addition, the RAS/MAPK pathway was deregulated by an NF1 truncating mutation. Consequently, a combined ALK/ MEK/pan-HER inhibition approach was required to completely suppress cell growth. ERBB family signaling has been associated with resistance to ALKi in ALK-positive NSCLC[46,47], but not in neuroblastoma. On the other hand, loss of NF1

and MAPK reactivation has been linked to chemotherapy resistance and disease outcome in patients with neuroblastoma[36,48,49]. The combined activation of these two pathways conferred high resistance to lorlatinib. Further characterization of CLB-GALR1000 cells revealed an increased expression of multidrug resistance transporters, ABCB1 and ABCG2, which impaired uptake of fluorescent substrates (Supplementary S9A−S9C). However, treatment with verapamil did not impact on lorlatinib sensitivity (Supplementary Figure S9D), in line with the proposed lack of interaction of lorlatinib with efflux pumps[8]. Recently, ABCB1 was shown to limit brain accumulation of lorlatinib[50]. Therefore, more studies are needed to clarify its possible involvement in resistance to lorlatinib.

In conclusion, we show here that multiple resistance mechanisms may coexist in tumors, making it difficult to devise second-line therapies. This points to the importance of strategies to prevent rather than treat resistance, to allow enough time to tumor cells to develop additional ways to resist treatment. Combinatorial approaches may be effective in such cases. However, given the complexity of potential survival pathways leading to resistance, it is hard to design preventive combinations at the personalized level. In NSCLC, the high frequency of EGFR bypass mechanisms suggests that first-line ALK/EGFR combined inhibition may be beneficial, although toxicity might be an issue. Also, the MAPK pathway appears to be a common axis contributing to ALK independence across the three different tumor types. We envisage that precise definition of drug resistance mechanisms will lead to a better control of disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Redaelli, C. Gambacorti-Passerini, L. Mologni

Development of methodology: C. Mastini, L. Mologni

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Redaelli, M. Ceccon, M. Zappa, G.G. Sharma, C. Mastini, M. Mauri, M. Nigoghossian, F. Farina, L. Mologni

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Redaelli, M. Zappa, G.G. Sharma, M. Mauri, M. Nigoghossian, L. Massimino, N. Cordani, R. Piazza, L. Mologni

Writing, review, and/or revision of the manuscript: S. Redaelli, M. Ceccon, L. Massimino, F. Farina, L. Mologni

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Mologni

Study supervision: C. Gambacorti-Passerini, L. Mologni

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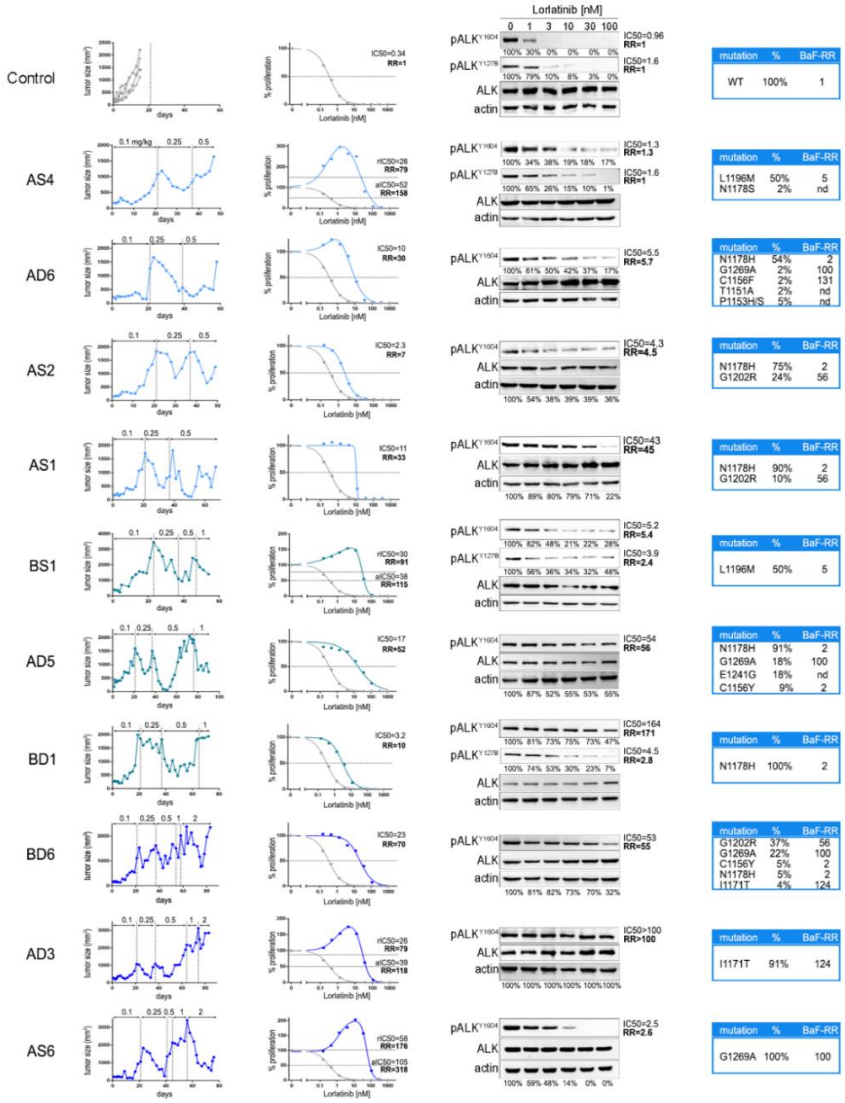
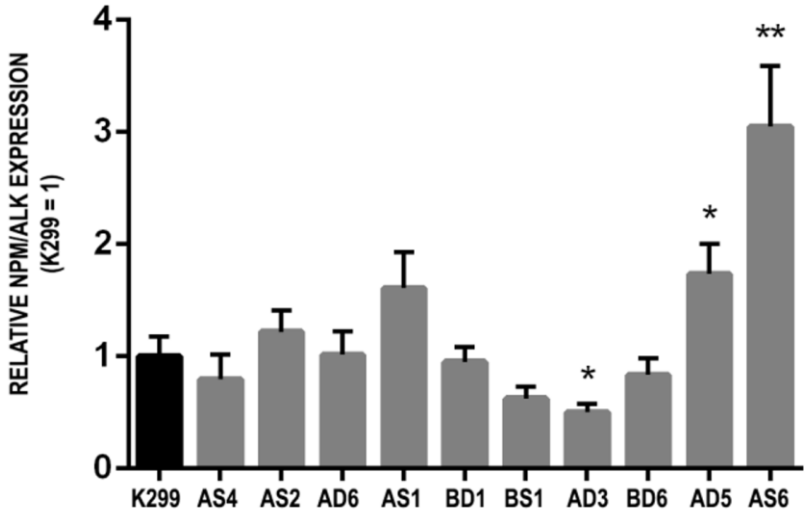
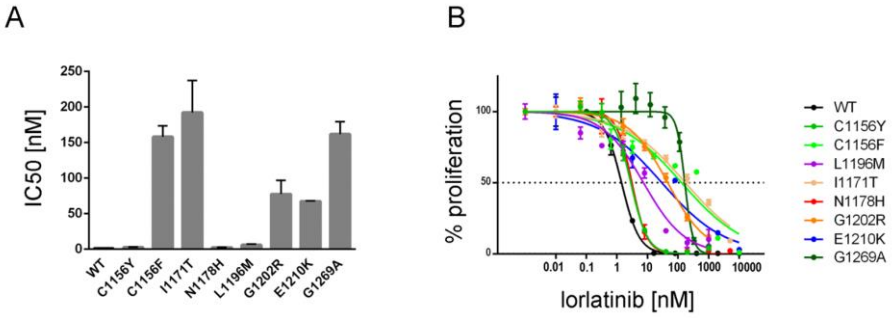
SUPPLEMENTARY DATA



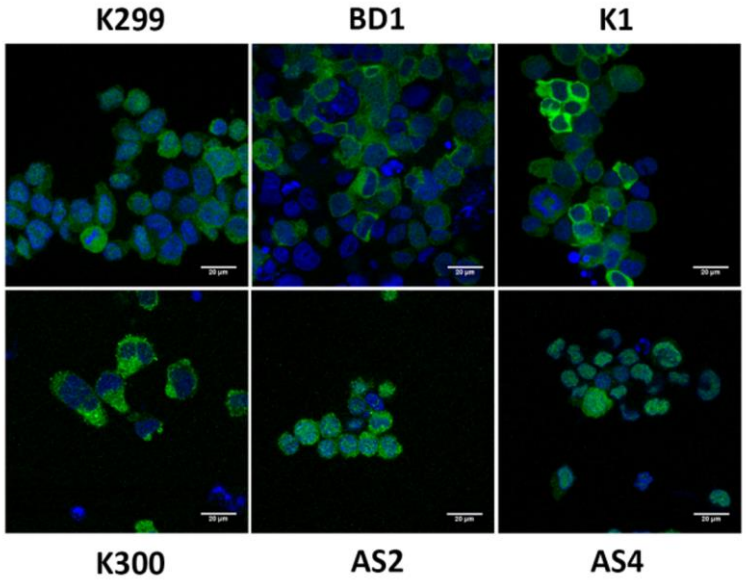
Supplementary Figure S1. *Resistance to lorlatinib in vivo. For each xenograft, the following data are reported, from left to right: mouse ID; tumor growth curve with drug doses; ex vivo cell proliferation curve with IC50 and corresponding RR values (a dotted line across the graph indicates 50% inhibition [absolute IC50]; for bell-shaped curves, a second dashed line shows 50% decrease from the peak [relative IC50]; Western analysis of ALK phosphorylation with relative IC50 and RR values calculated by densitometry; list of NPM/ALK mutations identified by deep-sequencing, with their frequency within the sample and their RR value observed when expressed in Ba/F3 cells.*



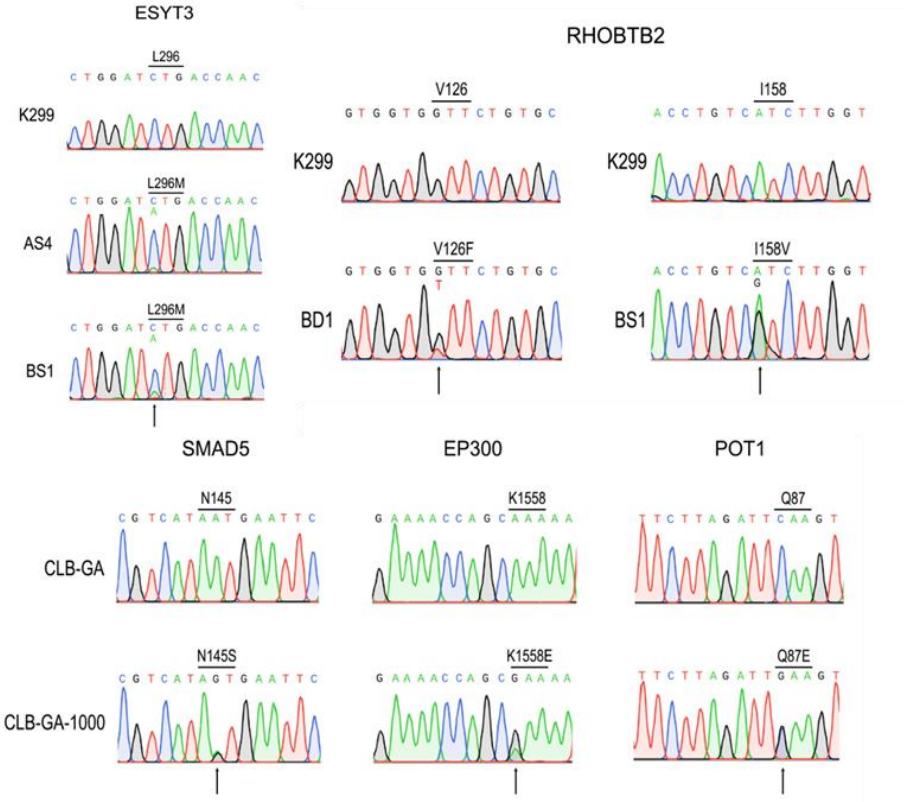
Supplementary Figure S2. *Quantitative real-time PCR analysis of NPM/ALK expression in resistant xenografts, relative to control K299. Mean±SEM is reported.* **, P<0.05;* ***, P<0.01.*



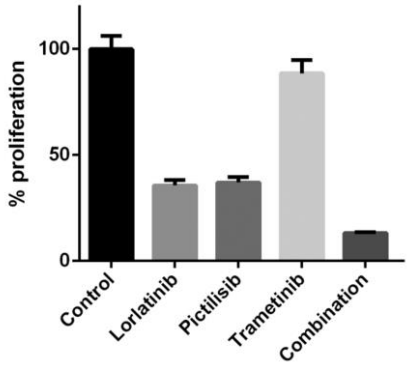
Supplementary Figure S3. *Activity of lorlatinib on Ba/F3 cells expressing NPM/ALK mutants. (**A**) Histogram showing* IC_{50} *values obtained by dose-response curve fitting. Experiments were performed at least three times. (**B**) Representative dose-response curves.*



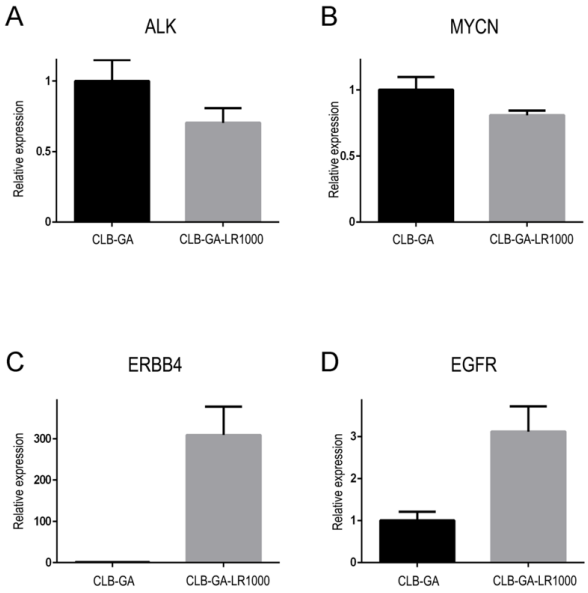
Supplementary Figure S4. *NPM//ALK- N1178H mutant shows altered cyto-nuclear localization. Micrographs showing ALCL cells stained with anti-ALK antibody (green). Nuclei are stained with DAPI (blue).*



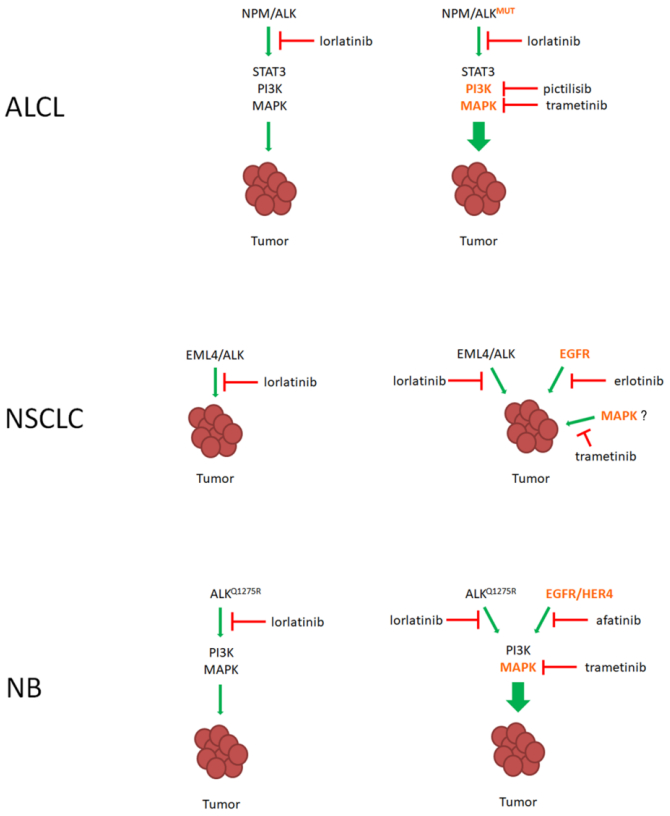
Supplementary Figure S5. *Sanger sequencing chromatograms showing the mutations (arrows) found in lorlatinib-resistant cells. The corresponding sequence from parental cells is shown, for comparison.*



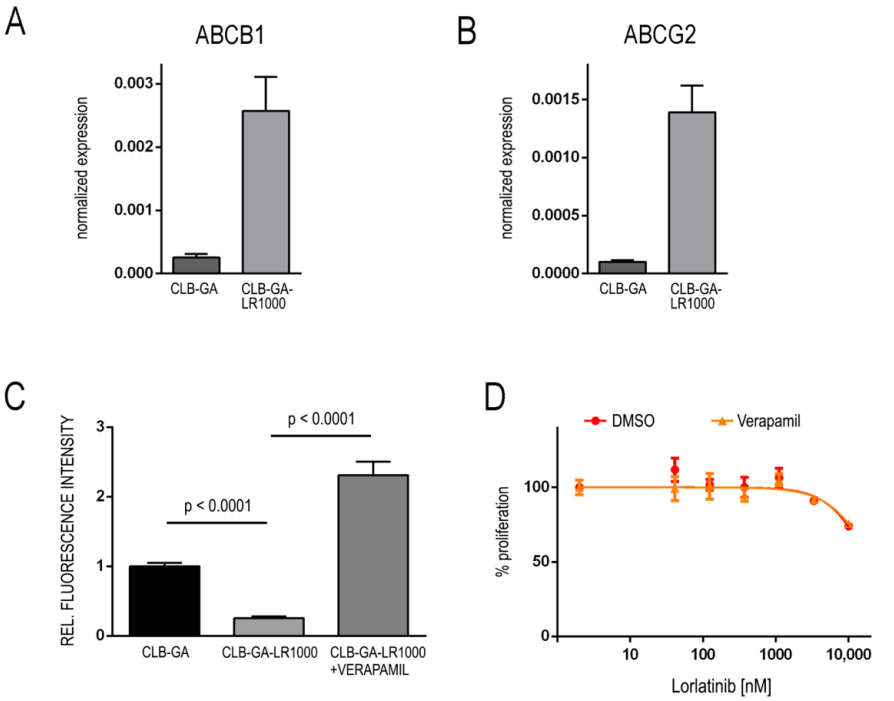
Supplementary Figure S6. *Effect on AS4 cells growth of a three-drug combination of lorlatinib, pictilisib and trametinib, compared to single treatments. The combination produced an additive effect, as predicted by the Bliss independence model.*



Supplementary Figure S7. *Quantitative real-time PCR analysis of ALK (**A**), MYCN (**B**) ERBB4 (**C**) and EGFR (**D**), in CLB-GA-LR1000 relative to parental CLB-GA cells. Mean±SEM from triplicate measures is shown.*



Supplementary Figure S8. *Summary of resistance mechanisms described in this work. In all three disease models, lorlatinib is able to abrogate ALK-driven oncogenic signalling in parental, drug-naïve cells. In* ***ALCL*** *cells, ALK kinase mutations and ALK-independent reactivation of PI3K and MAPK pathways cooperate to induce resistance; concomitant block of all survival pathways is needed to overcome resistance. In* ***NSCLC*** *cells, activation of EGFR opens an alternative pathway that by-passes ALK; accordingly, inhibition of EGFR restores sensitivity to ALK inhibition in H3122 cells. Additional unknown mechanisms (MAPK-mediated?) further by-pass EGFR inhibition in H2228-LR100 cells. In neuroblastoma (****NB****) cells, both upstream (EGFR/ERBB4) and downstream (RAS/MAPK) alterations provide multiple ALK-independent survival signals to the cells. MAPK inhibition appears to be a crucial node of intervention.*



Supplementary Figure S9. *Alteration of multidrug transporters in CLB-GA-LR1000 cells. (**A-B**) Quantitative real-time PCR analysis of ABCB1, also known as P-gp or MDR1 (**A**) and ABCG2, also known as BCRP (**B**), in CLB-GA-LR1000 and in parental CLB-GA cells. Mean±SEM from triplicate measures is shown. (**C**) Functional activity of ABCB1 was assessed by confocal microscopy measuring the intracellular accumulation of the ABCB1 transporter substrate Rhodamine 123 (5 µM), in the presence or absence of the selective transporter inhibitor verapamil (100 µM). Cells were further stained with Hoechst to normalize the signal. Integrated fluorescence intensity for Rhodamine 123 channel was measured with ImageJ (https://imagej.nih.gov/) with a specific designed automated macro and data were analyzed with Graphpad using Student's t-test. (**D**) Dose-response curves of CLB-GA-LR1000 cells treated with lorlatinib alone (DMSO, red) or combined with 30 µM verapamil (orange). Higher concentrations were toxic over a 72-hour course.*

Supplementary Table 1. *Sequences (5'-3') of primers used in this study for QPCR and mutation analyses.*

Purpose	Gene	Forward Primer	Reverse Primer
QPCR	**NPM-ALK**	AAGGTTGAAGTGTGGTTCAGG	CAGCTCCATCTGCATGGCTTG
QPCR	**ABCB1**	TGGAGGAAGACATGACCAGG	CAAGACCTCTTCAGCTACTGC
QPCR	**GAPDH**	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
QPCR	**GUS**	GAAAATATGTGGTTGGAGAGCTCATT	CCGAGTGAAGATCCCCTTTTTA
Sequencing	**NPM-ALK KD**	TGCATATTAGTGGACAGCAC	GACTCGAACAGAGATCTCTG
Sequencing	**EML4-ALK KD**	CAATCTCTGAAGATCATGTG	GACTCGAACAGAGATCTCTG

Supplementary Table 2. *Sequences (5'-3') of primers used in this study for Sanger confirmation of some variants identified by whole exome-sequencing. The samples where they were found are indicated.*

Gene	Sample	Forward Primer	Reverse Primer
RHOBTB2	BS1, BD1	TGAGTTCCAGCTCTGTGCCTA	CTAGCCAAGGGTCGCCTAGC
ESYT3	BS1, AS4	TGTCACGATGTATAGGCACAC	TTATGACGTGGGCGATGGTG
EP300	CLB-GA-LR1000	GGAGATATTCTGTGCTATTCCCA	GGTGGCTGCATCTTACCTCT
NF1	CLB-GA-LR1000	GTGAACCTCATCAACCATCTCA	GCCTGGCCTAGTTTGCATTT
SMAD5	CLB-GA-LR1000	GATTCAGGGCTCTATAAAACAGC	AGGAGTGTTGTTGGGCTG
POT1	CLB-GA-LR1000	TGCCCTCTCTTAATTATGCTGG	TGAGTAGATGCCCAAACACG

Supplementary Table 3. *List of mutations identified in BD1, BS1 and AS4 cells by whole exome sequencing. Codon and amino acid changes are shown. For each gene, residue numberings relative to all known isoforms are reported.*

BD1		
ALK	AAC->CAC	Asn1178His
BMF	CTG->CTT	Leu146Leu; Leu167Leu; Ala114Ser
BMP2	CAG->AAG	Gln156Lys
DNAJC18	GAG->TAG	Glu346*; Glu284*
DOCK3	CAG->AAG	Gln1396Lys
DSCAML1	GTC->ATC	Val1436Ile
IL17RC	GAC->CAC	Asp29His
LYZL2	AAC->AGC	Asn104Ser
MUC16	GAC->TAC	Asp3437Tyr
NR2F1	CCC->CAC	Pro398His; Pro347His; Pro373His
PTK7	CAG->AAG	Gln512Lys; Gln520Lys
RHOBTB2	GTT->TTT	Val126Phe; Val111Phe; Val104Phe
SLC7A13	AGC->AGA	Ser126Arg
TFR2	GGG->AGG	Gly298Arg; Gly586Arg; Gly757Arg
TTN	CCA->CAA	Pro22180Gln; Pro20539Gln; Pro19612Gln; Pro13307Gln; Pro13240Gln;
ZNF701	CGT->CAT	Arg365His; Arg431His
BS1		
ALK	CTG -> ATG	Leu1196Met
ANGPTL5	AGC->ATC	Ser301Ile
APPL2	GTA->GCA	Val195Ala; Val244Ala; Val238Ala
CCDC37	CAG->AAG	Gln346Lys; Gln347Lys
CNTN4	ATA->GTA	Ile119Val; Ile447Val
DAPK3	CGG->CAG	Arg130Gln; Arg393Gln
ERCC6	CTA->GTA	Leu112Val; Leu142Val; Leu742Val
ESYT3	CTG->ATG	Leu296Met
RADIL	TCC->TGC	Ser25Cys
RHOBTB2	ATA->GTA	Ile158Val
RIMBP3	CAA->GAA	Gln1444Glu
RNF10	CCA->TCA	Pro226Ser; Pro132Ser
ZMYND10	CGC->GGC	Arg404Gly; Arg399Gly
AS4		
ALK	CTG -> ATG	Leu1196Met
CPSF2	TTA->TCA	Leu760Ser
CYP2D7	CGG->TGG	Arg128Trp; Arg168Trp; Arg384Trp; Arg333Trp
ESYT3	CTG->ATG	Leu296Met
HIST1H2BJ	GGC->AGC	Gly27Ser
IL5RA	GAA->CAA	Glu184Gln
ITLN2	ACC->AAC	Thr80Asn; Thr79Asn
UPK2	AGC->AGA	Ser74Arg

Supplementary Table 4. *Mutations identified in CLB-GA-LR1000 cells by WES. PROVEAN score predicts the effects of a sequence variation on protein function (https://provean.jcvi.org/index.php).*

Gene	Codon Change	Aminoacid Change	CLB-GA-LR1000 mut (%)	CLB-GA mut (%)	P-value	PROVEAN
EP300	AAA->GAA	K1558E	49.51	0	0.0002	Deleterious
NF1	GGA->TGA	G2392*	49.32	0	0.0001	Truncating
SMAD5	AAT->AGT	N145S	45.59	0	0.0001	Neutral
POT1	CAA->GAA	Q87E	44.12	0	0.0002	Neutral

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CHAPTER III

Summary, conclusions and future perspectives

1. SUMMARY

ALK tumors exemplifies the potential of personalized medicine. ALK-dependent tumors can be specifically targeted with ALK TKIs leading to tumor regression with milder side effects in comparison to traditional cytotoxic chemotherapies. Since the identification of ALK fusion protein; EML4-ALK in NSCLC, patients' outcomes have been significantly improved with the use of ALK TKIs. A number of ALK inhibitors have been approved in the last few years by the FDA for the treatment of ALK+ NSCLC patients. Despite the remarkable responses achieved, the full potential of ALK TKIs is not realized due to the inevitable emergence of resistance. Most of the patients only experience short-term response and relapse. One third of the resistance can be attributed to the development of secondary point mutations in the ALK kinase domain while the rest of the resistance mechanisms are diverse involving bypass signalling pathways, other genetic aberrations as well as unknown intrinsic resistance mechanisms.

Lorlatinib, a third generation ALK inhibitor, has been shown to have superior potency, anti-tumor activity, efficacy and CNS activity in comparison to the existing ALK TKIs[1] . However, resistance also arises

against lorlatinib[2]. The present work was conceptualized to better understand the routes of lorlatinib resistance in ALK disease models, *in vitro* and *in vivo*. Diverse resistance mechanisms were identified in different ALK tumors that assisted in escaping the lorlatinib mediated targeted inhibition.

In ALCL models, lorlatinib-resistant cells accumulated compound mutations either from the beginning, as in the case of SUPM2-LR100 cells carrying the C1156F/L1198F mutation or progressively with increasing drug concentration as observed in K299-LR1000 cells carrying G1269/G1202R mutation. The G1269A and G1202R mutations were also recurrently observed *in vivo,* reaffirming their role in conferring resistance against lorlatinib in ALCL setting. An interesting observation was the identification of a N1178H NPM-ALK mutant *in vivo* in 50% of mice (5/10) which did not confer high resistance in Ba/F3 cellular models. Further characterization of the NPM-ALK^{N1178H} mutant revealed a preferential cytoplasmic localization of the NPM-ALK protein. Since the cytoplasmic fraction of NPM-ALK is considered to be oncogenic, we hypothesize that the cytoplasmic localization of NPM-ALK^{N1178H} mutant functionally mimics overexpression of the fusion protein. In certain cases where the identified mutations (e.g. L1196M and N1178H) did not explain the resistance behaviour completely, a global NGS approach was used to discern potential ALK-independent resistance mechanisms. Indeed, PI3K/AKT/mTOR and RAS/MAPK signaling were found to be commonly altered in the lorlatinib-resistant xenografts. These findings were validated by the reversal of resistant phenotype with the use of pan-PI3K and MEK inhibitors. Taken together, we observed that in

ALCL models, resistance to lorlatinib can be acquired through several mechanisms coexisting in the same cell, each attributing part of the resistant phenotype.

On the other hand, we did not find *ALK* secondary point mutations or *ALK*-dependent resistance in NSCLC and NB models. Rather, they acquired resistance through activation of bypass signaling pathways. For example, evidence of EGFR and MAPK pathway activation were found in lorlatinib-resistant NSCLC cell lines. A triple combination of lorlatinib, erlotinib (EGFRi) and trametinib (MAPKi) resulted in remarkable growth inhibition of resistant cells. In lorlatinib-resistant NB cell lines, phospho-RTK array analysis revealed hyperactivation of EGFR and ERBB4 kinases. In addition, a heterozygous truncating mutation in the *NF1* gene was found in the resistant NB cells which is predicted to cause aberrant activation of RAS/MAPK pathway. However, combination treatment experiments indicated that the contribution of EGFR/ErbB4 pathway in mediating resistance was limited. Nonetheless, treatment with trametinib restored the sensitivity of resistant NB cells to lorlatinib.

2. CONCLUSIONS

Treatment of ALK+ cancer patients has dramatically changed since the introduction of ALK TKIs. Advancement in our understanding and technology has led to the identification of molecular mechanisms as well as resistance mechanisms involved in ALK+ cancers. Several ALK TKIs are in the clinic for the treatment of ALK+

NSCLC patients. Third generation ALK inhibitor, lorlatinib has shown impressive results in ALK+ NSCLC patients with ALK mutations. However, resistance is expected against lorlatinib.

The work presented in this thesis elucidates various molecular mechanisms by which ALK+ cancer cells might acquire resistance against the new ALK inhibitor, lorlatinib. Our findings indicate that in ALCL, resistance can be acquired primarily through ALK kinase domain compound mutations, however, ALK-independent mechanisms might also come into play. In contrast, our results from the NSCLC and NB cell models suggest the involvement of bypass signaling pathways to achieve resistance against lorlatinib.

One of the limitations of our work is the lack of patient data, however, compound mutations were recently reported in patients upon lorlatinib failure[2], providing clinical relevance to our findings. We and others have found that compound rather than single mutations arise against lorlatinib. Given that lorlatinib is the most potent ALKi at present, it remains to see how lorlatinib resistant patients could be treated.

In case of some compound mutations perhaps switching to other ALK TKIs could help in overcoming lorlatinib resistance as previously reported[3]. Although, the use of other survival signaling pathways by the resistant cells shows the complexity and intricate molecular networks employed in acquiring resistance. Despite the potent anti-tumor activity of lorlatinib against wild-type as well as mutated ALK fusion proteins, it is not effective against off-target resistance mechanisms.

3. FUTURE PERSPECTIVES

Our findings point towards the presence of multiple resistance mechanisms that coexist in tumors, rendering the selection of second line therapies a difficult decision. Perhaps devising strategies to prevent rather than treat resistance, is a more logical approach in the continuously evolving tumor landscape such as in ALK+ cancers. One way to achieve that would be to use combination therapies; targeting more than one target or different forms of one target. Combinatorial approach has been shown to overcome resistance in other disease models such as in CML where use of allosteric inhibitors of BCR-ABL protein; GNF-2/5, in combination with ATP-competitive BCR-ABL inhibitors; imatinib or nilotinib led to a reduction in the number of resistant clones upon continued exposure to single pharmacological agent[4].

Given the different structural profiles and activity of different ALKi, using two ALK TKIs effective against two different mutations might overcome resistance. However, more studies are needed to characterize the various aspects of combining two TKIs, for example, their toxicity profiles. Small molecule inhibitors of alternate pathways could also be used in combination with lorlatinib to overcome ALK-independent resistance. As a proof of concept, we demonstrated in this work that a combination of ALK and EGFR inhibitor prevents the onset of resistance in NSCLC cells. Few *in vitro* studies have demonstrated the efficacy of using combination treatment in ALK+ cancers[5,6] and a number of clinical trials are underway to evaluate the efficacy of ALK inhibitors in combination with other targeted agents such as

NCT03087448 (ceritinib + trametinib; MEKi), NCT03202940 (alectinib + cobimetinib; MEKi), NCT02321501 (ceritinib + everolimus; mTORi), NCT02292550 (ceritinib + LEE011; CDK4/6i)[7]. It is expected that the results of these clinical trials and further research refining the molecular mechanisms behind drug resistance will advance our understanding of ALK+ cancers and help in devising better treatment strategies for patients.

Based on our results, we propose that combinatorial approach could be the future to overcome lorlatinib resistance with either combining ALK TKIs or ALK TKI with bypass signalling pathway inhibitors.

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