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Resistance Mechanisms to ALK Tyrosine Kinase Inhibitors (TKIs) in NSCLC

Gonzalo Recondo

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Resistance mechanisms to ALK tyrosine kinase inhibitors in NSCLC

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Thèse de doctorat

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Abstract

The molecular study and classification of lung adenocarcinomas has led to the development of selective targeted therapies aiming to improve disease control and survival in patients.

The anaplastic lymphoma kinase (ALK) is a tyrosine kinase receptor from the insulin tyrosine kinase receptor family, with a physiologic role in neural development. Gene rearrangements involving the ALK kinase domain occur in ~3-6% of patients with lung adenocarcinoma. The fusion protein dimerizes leading to transactivation of the ALK kinase domain in a ligand-independent and constitutive manner.

Lorlatinib is a third generation ALK inhibitor with high potency and selectivity for this kinase in vitro and in vivo, and elevated penetrance in the central nervous system. Lorlatinib can overcome resistance mediated by over 16 secondary kinase domain mutations occurring in 13 residues upon progression to first- and second- generation ALK TKI. In addition, treatment with lorlatinib is effective for patients who have been previously treated with a first and a second generation or a second generation ALK TKI upfront and is currently approved for this indication.

The full spectrum of biological mechanisms driving lorlatinib resistance in patients remains to be elucidated. It has been recently reported that the sequential acquisition of two or more mutations in the kinase domain, also referred as compound mutations, is responsible for disease progression in about 35% of patients treated with lorlatinib, mainly by impairing its binding to the ALK kinase domain. However, the effect of these compound mutations on the sensitivity to the repertoire of ALK inhibitors can vary, and other resistance mechanisms occurring in most patients are unknown.

My PhD thesis aimed at exploring resistance to lorlatinib in patients with ALK-rearranged lung cancer through spatial and temporal tumor biopsies and development of patient-derived models. Within the institutional MATCH-R study

(NCT02517892), we performed high-throughput whole exome, RNA and targeted next-generation sequencing, together with plasma sequencing to identify putative genomic and bypass mechanisms of resistance. We developed patient-derived cell lines and characterized novel mechanisms of resistance and personalized treatment strategies in vitro and in vivo.

We characterized three mechanisms of resistance in five patients with paired biopsies. We studied the induction of epithelial-mesenchymal transition (EMT) by SRC activation in two patient-derived cell lines exposed to lorlatinib. Mesenchymal cells were sensitive to combined SRC and ALK co-inhibition, showing that even in the presence of an aggressive and challenging phenotype, combination strategies can overcome ALK resistance. We identified three novel ALK kinase domain compound mutations, F1174L/G1202R, C1156Y/G1269A, L1196M/D1203N occurring in three patients treated with lorlatinib. We developed Ba/F3 cell models harboring single and compound mutations to study the differential effect of these mutations on lorlatinib resistance. Finally, we characterized a novel mechanism of resistance caused by NF2 loss of function at the time of lorlatinib progression through the development of patients derived PDX and cell lines, and in vitro validation of NF2 knock-out with CRISPR/CAS9 gene editing. Downstream activation of mTOR was found to drive lorlatinib resistance by NF2 loss of function and was overcome by providing treatment with mTOR inhibitors.

This study shows that mechanisms of resistance to lorlatinib are more diverse and complex than anticipated. Our findings also emphasize how longitudinal studies of tumor dynamics allow deciphering TKI resistance and identifying reversing strategies.

Résumé

Les analyses moléculaires et la classification des adénocarcinomes bronchiques ont conduit au développement de thérapies ciblées sélectives visant à améliorer le contrôle de la maladie et la survie des patients. ALK (anaplastic lymphoma kinase) est un récepteur tyrosine kinase de la famille des récepteurs de l'insuline. Des réarrangements chromosomiques impliquant le domaine kinase d'ALK sont présents dans environ 3 à 6% des patients atteints d'un adénocarcinome bronchique. La protéine de fusion provoque une activation du domaine kinase de manière constitutive et indépendante du ligand.

Lorlatinib est un inhibiteur d'ALK de troisième génération avec une efficacité et une sélectivité optimale, ainsi qu'une pénétration élevée vers le système nerveux central. Lorlatinib peut vaincre la résistance induite par plus de 16 mutations secondaires dans le domaine kinase d'ALK acquises lors de la progression aux ALK TKI de première et deuxième générations. Le traitement par lorlatinib est donc efficace chez les patients préalablement traités par un ALK TKI de première ou deuxième génération, et est actuellement approuvé pour cette indication.

Le spectre complet de mécanismes de résistance au lorlatinib chez les patients reste à élucider. Il a récemment été rapporté que l'acquisition séquentielle de deux mutations ou plus dans le domaine kinase, également appelées mutations composées, est responsable de la progression de la maladie chez environ 35% des patients traités par le lorlatinib, principalement en altérant sa liaison au domaine kinase d'ALK. Cependant, l'effet de ces mutations sur la sensibilité aux différents inhibiteurs d'ALK peut varier, et les autres mécanismes de résistance survenant chez la plupart des patients restent inconnus.

Mon travail de thèse avait pour but d'explorer la résistance au lorlatinib chez des patients atteints d'un cancer du poumon ALK réarrangé par la mise en œuvre de biopsies spatiales et temporelles et le développement de modèles dérivés de patients. Dans le cadre de l'étude institutionnelle MATCH-R (NCT02517892), nous avons effectué un séquençage à haut débit de l'exome, de l'ARN et ciblé, ainsi qu'un séquençage des ctDNA afin d'identifier les mécanismes de

résistance. Nous avons établi des lignées cellulaires dérivées de patients et caractérisé de nouveaux mécanismes de résistance et identifiés de nouvelles stratégies thérapeutiques in vitro et in vivo.

Nous avons identifié trois mécanismes de résistance chez cinq patients avec des biopsies appariées. Nous avons étudié l'induction de la transition épithélio-mésenchymateuse (EMT) par l'activation de SRC dans une lignée cellulaire, dérivée de deux patients, exposée au lorlatinib. Les cellules mésenchymateuses étaient sensibles à l'inhibition combinée de SRC et d'ALK, montrant que même en présence d'un phénotype agressif, des stratégies de combinaison peuvent surmonter la résistance aux ALK TKI. Nous avons identifié deux nouvelles mutations composées du domaine kinase d'ALK, F1174L/G1202R, C1156Y/G1269A et L1196M/D1203N survenues chez trois patients traités par le lorlatinib. Nous avons développé des modèles de cellules Ba / F3 exprimant les mutations simples et composées pour étudier leur effet sur la résistance au lorlatinib. Enfin, nous avons caractérisé un nouveau mécanisme de résistance provoqué par la perte de fonction de NF2 au moment de la progression du lorlatinib par l'utilisation de PDX et de lignées cellulaires dérivées de patients, et par CRISPR / CAS9 knock-out de NF2. Nous avons constaté que l'activation de mTOR par la perte de fonction de NF2 provoquait la résistance au lorlatinib et qu'elle pouvait être surmontée par le traitement avec des inhibiteurs de mTOR.

Cette étude montre que les mécanismes de résistance au lorlatinib sont plus divers et complexes que prévu. Nos résultats démontrent également comment les études longitudinales de la dynamique tumorale permettent de déchiffrer la résistance aux TKI et d'identifier des stratégies thérapeutiques.

Synthèse

Au cours des dernières décennies, les progrès des tests moléculaires ont conduit à la découverte de multiples oncogènes du cancer du poumon non à petites cellules et au développement de thérapies ciblées efficaces offrant de nouvelles options de traitement aux patients. La kinase du lymphome anaplasique (ALK) est un récepteur tyrosine kinase qui joue un rôle clé dans la carcinogenèse d'environ 3 à 6% des adénocarcinomes du poumon par réarrangements du gène ALK. En plus du cancer du poumon, des réarrangements d'ALK ont également été rapportés dans d'autres cancers, tels que les lymphomes anaplasiques à grandes cellules, les lymphomes B diffus à grandes cellules, les tumeurs myofibroblastiques inflammatoires de l'enfant et d'autres types de tumeurs. Dans ce contexte, la protéine de fusion ALK se dimérise, conduisant à la transactivation du domaine kinase d'ALK de manière constitutive et indépendante du ligand, qui transmet des signaux à travers des effecteurs en aval tels que la voie PI3K-AKT-mTOR et la voie des MAP kinases.

Le crizotinib, un inhibiteur d'ALK de première génération, et les inhibiteurs d'ALK de deuxième génération, le céritinib, l'alectinib et le brigatinib, sont des options de traitement pour les patients atteints d'un cancer du poumon métastatique ALK réarrangé. Les inhibiteurs d'ALK de deuxième génération ont été conçus pour surmonter les mécanismes de résistance qui se développent sous traitement par crizotinib. Toutefois, la résistance aux inhibiteurs d'ALK de deuxième génération se développe invariablement, la plus courante étant l'acquisition de la mutation G1202R, qui confère une résistance à tous les inhibiteurs d'ALK de première et de deuxième génération.

Lorlatinib est un inhibiteur d'ALK de troisième génération. Il présente une puissance et une sélectivité élevées pour cette kinase in vitro et in vivo et une bonne pénétration dans le système nerveux central. Lorlatinib peut vaincre la résistance induite par toutes les mutations simples du domaine kinase se produisant dans 13 résidus lors de la progression à un ALK ITK (inhibiteur de tyrosine kinase) de première et de deuxième génération, y compris G1202R, et a récemment été approuvé pour le traitement des patients présentant une

progression de la maladie aux inhibiteurs d'ALK de deuxième génération. Ces éléments reposent sur des études précliniques et cliniques, montrant des niveaux d'activité élevés dans le cadre de la résistance aux générations antérieures d'inhibiteurs d'ALK, de la forte pénétration dans le système nerveux central et du manque de spécificité à la glycoprotéine p.

Même lorsque le traitement par le lorlatinib est efficace, la résistance apparaît invariablement. À ce jour, le seul mécanisme connu de résistance au lorlatinib est l'acquisition séquentielle de deux mutations ou plus, présentes en cis, ce qui entraîne une liaison défectueuse du lorlatinib au domaine kinase d'ALK. Cependant, il reste à élucider le spectre complet des mécanismes biologiques à l'origine de la résistance au lorlatinib chez les patients.

Dans la présente thèse, j'ai caractérisé plusieurs mécanismes de résistance apparus chez des patients au moment de la progression de la maladie sous lorlatinib par l'intégration d'un profil moléculaire profond et le développement de modèles dérivés de patients. Dans l'étude institutionnelle MATCH-R (NCT02517892), au moment de la résistance acquise au lorlatinib, un échantillon de tissu tumoral et de plasma a été prélevé. Nous avons effectué un séquençage complet des exomes et de l'ARN ainsi qu'un séquençage plasmatique afin d'identifier les altérations génomiques d'ALK et d'autres gènes pouvant causer la résistance au lorlatinib. Pour étudier de nouvelles mutations composées, nous avons développé des modèles de cellules Ba / F3 portant ces mutations d'intérêt. De plus, pour étudier de nouveaux mécanismes de résistance autres, nous avons développé des modèles dérivés de patients obtenus à la résistance au lorlatinib.

Nous avons caractérisé trois mécanismes de résistance chez cinq patients présentant des biopsies appariées (avant/après lorlatinib). Nous avons étudié l'induction de la transition épithélio-mésenchymateuse (EMT) par l'activation de la kinase SRC dans des lignées cellulaires dérivées de deux patients, dont l'un présentait des signes d'EMT dans la biopsie tumorale. Dans les deux modèles, les cellules mésenchymateuses étaient sensibles à l'inhibition combinée de SRC et d'ALK, montrant un effet synergique et prouvant que l'activation de SRC conduisait à l'EMT et à la résistance au lorlatinib. Nous avons également montré

que le traitement avec des inhibiteurs de SRC seuls pourrait induire une inversion partielle de l'EMT dans les cellules mésenchymateuses.

Nous avons identifié trois nouvelles mutations composées du domaine kinase d'ALK, F1174L / G1202R, C1156Y / G1269A, L1196M / D1203N, survenues chez trois patients traités avec le lorlatinib. Nous avons développé des modèles de cellules Ba / F3 contenant des mutations simples et composées pour étudier l'effet différentiel de ces mutations sur la résistance au lorlatinib. Nous avons montré que les mutations composées peuvent conférer des effets différents sur la liaison au lorlatinib et sur son efficacité dans ces modèles. La C1156Y / G1269A a conféré une sensibilité à la fois au lorlatinib et au brigatinib, la F1174L / G1202R a conféré une résistance au lorlatinib en augmentant l'affinité de la kinase pour l'ATP et en renforçant la liaison du médicament au domaine kinase et la L1196M / D1203N a conféré des niveaux élevés de résistance au lorlatinib en empêchant la liaison du médicament au domaine kinase.

Nous avons aussi caractérisé un nouveau mécanisme de résistance provoquée par la perte de fonction de NF2 en développant des lignées cellulaires et à partir de PDX d'un patient, à partir de sites métastatiques et de temporalité différents. Nous avons montré que la perte de NF2 conférait des niveaux élevés de résistance au lorlatinib en induisant une activation en aval dans le cadre d'une inhibition adéquate d'ALK. La double inhibition de mTOR et d'ALK induit la mort cellulaire par l'apoptose dans ces modèles dérivés de patients. Nous avons effectué une validation in vitro dans des cellules H3122 en inactivant NF2 par édition génique CRISPR / CAS9. En l'absence d'expression de merlin, les cellules H3122 présentaient des niveaux élevés d'activation de mTOR, même lorsqu'elles étaient exposées à des taux élevés de lorlatinib.

En résumé, notre étude démontre que les mécanismes de résistance au lorlatinib sont divers et complexes, y compris avec un effet différentiel des mutations composées, la preuve de l'induction de l'EMT et de nouveaux mécanismes de résistance par activation de voies de contournement. Compte tenu de l'hétérogénéité de la résistance au lorlatinib, une évaluation longitudinale du génotype des tumeurs et du plasma ainsi que le développement de xénogreffes dérivées de patients sont nécessaires pour comprendre la biologie de la

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List of Abbreviations

ALCL	Anaplastic large cell lymphoma
ALKALs	ALK receptor ligands
ATC	Anaplastic thyroid cancer
CAFs	Cancer-associated fibroblasts
CNS	Central nervous system
CRISPR	Clustered regularly interspaced short palindromic repeats
DCR	Disease control rate
DLBCL	Diffuse large B-cell lymphomas
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
ENU	N-ethyl-N-nitrosourea
FFPE	Formalin fixed paraffin embedded
FISH	Fluorescence in situ hybridization
GIST	Gastrointestinal stromal tumors
ICIs	Immune checkpoint inhibitors
IHC	Immunohistochemistry
IMT	Inflammatory myofibroblastic tumors
Ki	Inhibitor constant
KO	Knock-out
MAPK	Mitogen-activated protein kinase
MTD	Maximum tolerated dose
NGS	Next-generation sequencing
NSCLC	Non-small cell lung cancer
NSG	NOD scid gamma
ORR	Overall response rate
OS	Overall survival
PDX	Patient-derived xenografts
PFS	Progression-free survival
PROTACs	Proteolysis targeting chimeras
RNA	Ribonucleic acid
RTK	Tyrosine kinase receptor
SCC	Squamous cell carcinoma
SCLC	Small-cell lung cancer
TDM-1	Trastuzumab emtansine
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitor
TTR	Time to treatment response

List of Genes and Proteins

4EBP	Eukaryotic Translation Initiation Factor 4E Binding Protein 1
ABCB1/MDR1	ATP-binding cassette sub-family B member 1
AKT	AKT Serine/Threonine Kinase 1
ALK	Anaplastic Lymphoma Kinase
APAF1	Apoptotic Protease Activating Factor 1
ATP	Adenosine triphosphate
AXL	AXL Receptor Tyrosine Kinase
BAK	BCL2 Antagonist/Killer
BAX	BCL2 Associated X, Apoptosis Regulator
BCL11A	BAF Chromatin Remodeling Complex Subunit
BCL2	BCL2 Apoptosis Regulator
BH3	BCL2-homology domain 3
BID	BH3 Interacting Domain Death Agonist
BIM	Bcl-2-Like Protein 11
BRAF	V-Raf Murine Sarcoma Viral Oncogene Homolog B
CAS9	CRISPR associated protein 9
CBL	Casitas B-Lineage Lymphoma Proto-Oncogene
CCDC6	Coiled-Coil Domain Containing 6
CD74	CD74 Molecule, Major Histocompatibility Complex
Cdc42	Cell Division Cycle 42
CDH1	E-cadherin
CDH2	N-cadherin
CDKN1B	Cyclin Dependent Kinase Inhibitor 1B
CMTR1	Cap Methyltransferase 1
CPS II	Carbamoyl phosphate synthase II
CUX1	Cut Like Homeobox 1
EGF	Epidermal growth factor
EGFR	Epidermal Growth Factor Receptor
EIF4E	Eukaryotic Translation Initiation Factor 4E
EML4	Echinoderm Microtubule-Associated Protein-Like 4
EPCAM	Epithelial Cell Adhesion Molecule
ERK/MAPK1	Mitogen-Activated Protein Kinase 1
EZR	Ezrin
FAK	Focal Adhesion Kinase
FAM150A	Family With Sequence Similarity 150 Member A
FAM150B	Family With Sequence Similarity 150 Member B
FAS	Fas Cell Surface Death Receptor
FOS	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit
FRS2	Fibroblastic growth factor substrate 2
GCC2	GRIP And Coiled-Coil Domain Containing 2

GDNF	Glial-derived neurotrophic factor
GPX4	Glutathione Peroxidase 4
GRB2	Growth factor bond 2
GTP	Guanosine triphosphate
HDAC	Histone Deacetylase
HELP	Hydrophobic EML protein
HER2	Human Epidermal Growth Factor Receptor 2
HER3	Human Epidermal Growth Factor Receptor 3
HGF	Hepatocyte Growth Factor
HRAS	Harvey Rat Sarcoma Viral Oncogene Homolog
HSP90	Heat Shock Protein 90 Alpha Family Class A Member 1
ICAM	Intercellular Adhesion Molecule 1
IGFR	Insulin Like Growth Factor Receptor
IRS2	Insulin receptor substrate 2
JAK	Janus Kinase 2
JNK	Mitogen-Activated Protein Kinase 8
KDM5A	Lysine Demethylase 5A
KIF5B	Kinesin Family Member 5B
KIT	KIT Proto-Oncogene, Receptor Tyrosine Kinase
KLC1	Kinesin Light Chain 1
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LDLa	Low density lipoprotein class a
LTK	Leukocyte Receptor Tyrosine Kinase
Mcl-1	MCL1 Apoptosis Regulator, BCL2 Family Member
MEK/MAP2K1	Mitogen-Activated Protein Kinase Kinase
MET	Hepatocyte Growth Factor Receptor
mLST8	MTOR Associated Protein, LST8 Homolog
MMP9	Matrix Metallopeptidase 9
mTOR	Mammalian Target Of Rapamycin
MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
NF2	Neurofibromin 2
NOTCH	Notch Receptor 1
NOXA	Phorbol-12-Myristate-13-Acetate-Induced Protein 1
NPM1	Nucleophosmin 1
NRAS	Neuroblastoma RAS Viral (V-Ras) Oncogene Homolog
NRG1	Neuregulin 1
NTRK	Neurotrophic Receptor Tyrosine Kinase
p130CAS	BCAR1 Scaffold Protein, Cas Family Member
PAKS	Serine/threonine p21-activating kinases
PARP	Poly(ADP-Ribose) Polymerase
PD-1	Programmed Cell Death 1
PD-L1	Programmed Cell Death 1 Ligand 1
PDGFR	Platelet Derived Growth Factor Receptor
PDK1	Phosphoinositide-dependent kinase-1
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase

PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PIP2	Phosphatidylinositol-(4,5)-bisphosphate
PIP3	phosphatidylinositol-(3,4,5)-trisphosphate
PLCγ	Phospholipase Cγ
PRAS40	Proline-rich Akt Substrate of 40 kDa
PTPN3	Protein Tyrosine Phosphatase Non-Receptor Type 3
PUMA	Bcl-2-binding component 3
Rac1	Rac Family Small GTPase 1
RAPTOR	Regulatory Associated Protein Of MTOR Complex 1
RET	Rearranged During Transfection
RHEB	Ras Homolog, MTORC1 Binding
RICTOR	Rapamycin insensitive companion of mTOR
ROCK	Rho Associated Coiled-Coil Containing Protein Kinase
ROS1	ROS Proto-Oncogene 1, Receptor Tyrosine Kinase
RSK	Ribosomal Protein S6 Kinase A1
S6K1	Ribosomal Protein S6 Kinase B1
SCF	Stem cell factor
SCLA2	Solute Carrier Family 2
SH2	Src Homology 2
SHC	Src homology and collagen protein
SHP	Src-homology 2 domain (SH2)-containing protein tyrosine phosphatases
SLC34A2	Solute Carrier Family 34 Member 2
SLUG	Snail Family Transcriptional Repressor 2
SMACS	Second Mitochondrial-Derived Activator of Caspases
SMAD	Mothers Against Decapentaplegic Homolog
SNAI1/SNAIL	Snail Family Transcriptional Repressor 1
SRC	V-Src Avian Sarc
STAT	Signal Transducer And Activator Of Transcription
STK11/LKB1	Serine/Threonine Kinase 11
STRN	Striatin
TAPE	Tandem atypical β-propeller
TGFB	Transforming Growth Factor Beta 1
TGFα	Transforming Growth Factor Alpha
TNRF1	Tumor necrosis factor receptor 1
TPM3	Tropomyosin 3
TRAIL	TNF Superfamily Member 10
TRK	Tropomyosin receptor tyrosine kinase
TSC	Tuberous Sclerosis Complex
TWIST	Twist Family BHLH Transcription Factor 1
VEGFR	Vascular endothelial growth factor
VIM	Vimentin
VIT	Vitrin
WNT	Wingless-Type MMTV Integration Site Family
ZEB1	Zinc Finger E-Box Binding Homeobox 1

1. The evolution of lung cancer diagnosis and treatment: the road to personalized medicine

1A. Epidemiology of non-small cell lung cancer

Lung cancer is the leading cause of cancer-related deaths worldwide. In 2018, about 2 million new cases (11.6%) and 1.7 million deaths (18.4%) caused by lung cancer were estimated (1). In France, lung cancer is also the first cause of cancer related deaths in men, and the second in women, constituting a major public health problem (2).

Lung cancer is most frequently detected at advanced stages, usually with clinical and symptomatic evidence of metastatic disease (3). This translates into dismal survival rates, ranging from a 5-year survival rate of 97% for stage IA1 tumors (< 1cm in diameter and no lymph node involvement) to 10% for patients with stage IV (metastatic disease) (4).

Lung cancer is classified per histological features in non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). Both subtypes of cancer have distinct histology, biologic features and treatment strategies. For the purpose of this thesis, the focus is placed on the NSCLC subtype (5,6).

Tobacco exposure is the main risk factor for lung cancer (7,8). Tobacco combustion releases about 70 carcinogens including polycyclic aromatic hydrocarbons, and N-nitrosamines. These compounds inflict DNA damage, resulting in the onset of oncogenic mutations leading to lung carcinogenesis (9). In addition to active smoking, second-hand smoking is also an important factor related to lung carcinogenesis, and is responsible for about 7.330 deaths from lung cancer each year in the United States (10,11).

Importantly, lung cancer is not restricted to patients with a history of tobacco smoking. Non-smokers, defined as individuals smoking less than 100 cigarettes in a lifetime, are also at risk of lung cancer. Worldwide, about 25% of lung cancers occurs in non-smokers, and the incidence of lung cancer in never smokers is particularly higher among Asian population (12–14). These epidemiological differences between smoking status, geographic localization and race are also correlated with significant differences in the molecular profile of patients with NSCLC, particularly in the adenocarcinoma histology, as it will be addressed in detail in following chapters.

There are several environmental factors that have been linked to the development of lung cancer in never smoker population. Residential radon gas exposure is the leading cause of lung cancer in never smokers and second cause of lung cancer after smoking, accounting for about 21.000 deaths annually in the United States (15,16). Other risk factors leading to NSCLC are exposure to asbestos, air pollution and, in lesser extent, germline mutations that result in hereditary lung cancer predisposition syndromes (17–19). However, it is not fully understood what is driving the increase prevalence of lung cancer in non-smoking patients around the world, and specially in Asia. This is an active research area, aiming to identify novel carcinogens and implement politics to avoid and prevent carcinogenic exposure.

1B. Histological classification of non-small cell lung cancer

The evolution of pathology and molecular biology has shed a light to the different subtypes of non-small cell lung cancer in the last decades (20). NSCLC accounts for about 85% of all lung cancers. Diverse histological subtypes of lung cancer convey in the group of tumors classified as NSCLC: lung adenocarcinoma, squamous cell carcinoma, large cell neuroendocrine carcinoma, and sarcomatoid carcinomas, amongst others (21). The classification of NSCLC in different histologic is also supported by the distinct molecular profiles of these tumors (22–24).

Lung adenocarcinoma is the most frequent histological subtype in smokers and non-smoking patients, whereas lung squamous cell carcinoma is the second most common histologic subtype of NSCLC but is rare in patients without history of tobacco exposure.

Classifying NSCLC tumors in histologic subtypes was the first step towards a better distinction of the intrinsic molecular, phenotypical and prognostic features of NSCLC, and became the first approach to develop “personalized” treatment strategy based on the tumor characteristics. The development of molecular biology techniques applied to the study of cancer genomics revealed the complexity of genomic and epigenetic differences between these histologic subtypes, unravelling the existence of potent oncogenic drivers in lung adenocarcinomas and giving rise to the era of targeted therapies for this disease (25).

1C. Molecular classification of lung adenocarcinomas

Lung adenocarcinomas are classified in molecular subtypes according to the presence of well identified and characterized molecular alterations that drive cancer initiation and progression (23). This genomic classification relies on the detection of point mutations, copy number alterations, rearrangements, insertions and deletions in key oncogenes that have an initiating and perpetuating effect on cancer, and whose inhibition can induce cancer cell death.

Most of these oncogenic drivers are tyrosine kinase receptors (RTK) and protein kinases (PK) that regulate intracellular signaling pathways. The rational for this classification is based on the preclinical characterization of molecular alterations in the functionality of these RTK and phosphokinase proteins. In most cases, kinase inhibitors have been successfully developed to target these alterations. The most relevant driver oncogenes and the molecular alterations leading to classification of genomic subtypes of lung adenocarcinoma are the following: *KRAS* mutations, *EGFR* mutations and indels, *ALK* fusions, *ROS1* fusions, *MET* exon 14 skipping mutations and amplification, *BRAF* mutations, *RET* fusions and *NTRK* fusions (26).

The distribution of these alterations varies across geographical regions, race, sex, and the methods used to study tumor genomics. Multiple collaborative efforts have been done to identify these oncogenic drivers and provide evidence on the distribution and frequency of these alterations across patients with lung adenocarcinoma.

The first nationwide effort to characterize the prevalence of oncogenic alterations in advanced lung adenocarcinoma was led by "L'Intergroupe Francophone de Cancérologie Thoracique" (IFCT) in France (27). This comprehensive characterization of lung tumors initially assessed mutations in *KRAS*, *BRAF*, *EGFR*, *PIK3CA* and *HER2* together with rearrangement in *ALK*, across 28 testing centers in France. A total of 18.679 samples from 17.664 patients were studied and included mostly lung adenocarcinoma histology (76%). In this subtype, the prevalence of *KRAS* mutations was 32%, *EGFR* mutations

12%, *BRAF* mutations 2%, *HER2* exon 20 insertions 2% and the prevalence of ***ALK* rearrangements was 5% (Figure 1A)**. Importantly in never smokers (1619 patients), the proportion of *EGFR* mutations rose to 44% and *ALK* rearrangements to 14% and for *KRAS* mutations, it descended to 9% **(Figure 1B)** (27). This highlights the influence of smoking status on the genomic profile of lung adenocarcinomas.

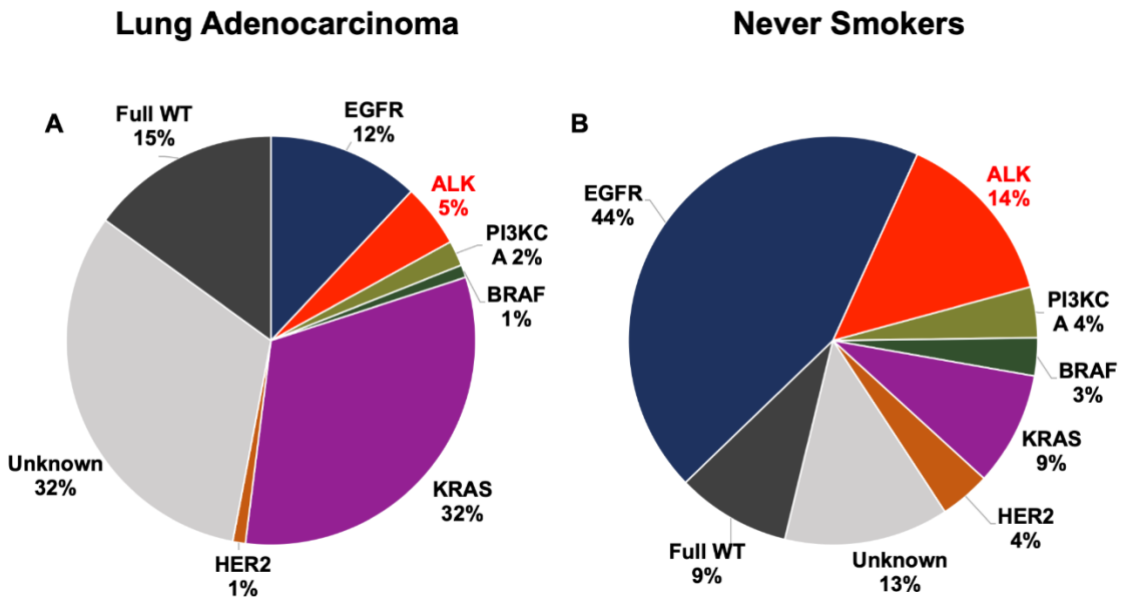


Figure 1. Distribution of molecular alterations in tumors from patients with diagnosis of lung adenocarcinomas (A) and in never-smokers (B) in France. Figure and legend adapted from Barlesi F et al. Lancet Oncology 2016 (27).

Similarly, the Lung Cancer Mutation Consortium (LCMC), pioneered in the molecular characterization of lung adenocarcinomas in 14 academic institutions in the United States (28). In 1.102 eligible patients, *KRAS* mutations were found in 25% of tumors, *EGFR* mutations in 17%, ***ALK* rearrangement in 8%,** *HER2* exon 20 insertions in 3% and *BRAF* mutations in 2% of cases.

The development of high-throughput next generation sequencing (NGS) platforms, has allowed to expand the testing for multiple genes by targeted sequencing using customized gene panels that require small quantities of tumor DNA (29). In addition, whole-exome and RNA sequencing provides a more

comprehensive view of the landscape of molecular alterations in coding regions and gene expression. The Cancer Genome Atlas (TCGA) Program is currently ongoing and has characterized over 20.000 tumor samples from 33 different types of cancer using whole-exome sequencing and RNA sequencing. In the first report on lung adenocarcinomas, mainly from early stage resected specimens, driver alterations were detected in 62% of the samples (23). *KRAS* mutations were found in 32.2% of samples, *EGFR* mutations in 11.3%, *BRAF* mutations in 7%, ***ALK* rearrangements in 1.3%**, *ROS1* fusions in 1.7%, *RET* fusions in 0.9%, and *MET* exon 14 alterations in 4.3%. The differences in the prevalence in *ALK* rearrangements in this data set, mostly from stage I/II resected tumors, suggests that the prevalence of molecular alterations might also be stage-dependent.

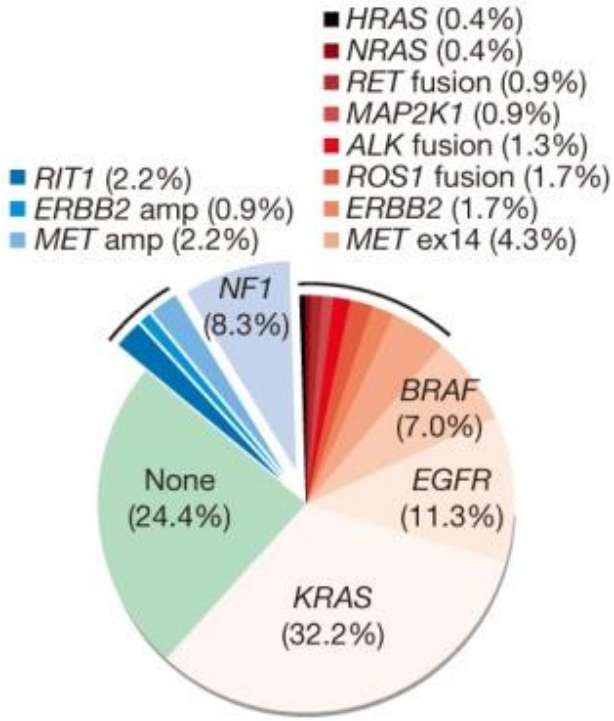


Figure 2. Prevalence of molecular alterations in key oncogenic drivers in lung adenocarcinomas using whole exome sequencing from The Cancer Genome Atlas Project. *Figure and legend adapted from The Cancer Genome Atlas Group, Nature 2014* (23).

As previously mentioned, the prevalence of these oncogenic alterations in western countries, with high proportion of Caucasian populations, differ

significantly to those from Asia. Across Asia Pacific, the prevalence of *EGFR* mutations is significantly higher than in western countries, reaching 49% of lung adenocarcinomas and, inversely, the prevalence of *KRAS* mutations is low in Asia (30). However, the prevalence of *ALK* rearrangements in Chinese population is similar to the observed in Europe and United States (4.2%) (30,31). Currently, there is no scientific explanation for the differences observed among western and Asian populations in the distribution of molecular alterations, though the role of radon exposure and air pollution is being studied (32,33).

1D. Biology of oncogenic drivers and pathways in lung cancer

The well-established drivers of lung adenocarcinoma are either tyrosine kinase receptors (eg: EGFR, ALK, ROS1, MET, RET, NTRK), intracellular G-protein and kinases (eg: BRAF) or GTP-ase proteins (eg: KRAS). There are about 535 protein kinases encoded in the human exome, that compose the human kinome. Protein kinases mediate the activation of protein functionality by catalyzing the phosphorylation of multiple protein substrates in tyrosine, serine or a threonine residues (34). In this chapter, we will review the signaling pathways involved in the biology of oncogene addicted lung cancers, including tyrosine kinase receptors, the MAPK and the PI3K/AKT/mTOR pathways and their regulation, and other oncogenic mediators like SRC and regulation of apoptosis and cell death. This will contextualize the findings of our work on mechanisms of resistance to the ALK inhibitor lorlatinib.

Tyrosine Kinase Receptors

Within the family of protein kinases, several tyrosine kinase receptors are frequently involved in lung adenocarcinoma carcinogenesis. Tyrosine kinase receptors are located in the cell membrane and are composed of an extracellular, a transmembrane and a cytoplasmic region and promote cell proliferation, migration and survival by the binding of different growth factors **(Figure 3)** (35).

The extracellular domain binds to a specific ligand, and this induces conformational changes promoting receptor homodimerization, and in some cases, heterodimerization with other RTK to form a receptor complex. These dimers are formed through different mechanisms: a bivalent ligand can bind to two molecules inducing a “ligand dependent” dimerization but, in most cases, it is not dependent on the ligand but mostly on receptor-receptor interactions (36,37).

The juxtamembrane domain in the cytoplasm maintains the receptor in an autoinhibitory conformation in inactivating conditions (38). Receptor dimerization promotes transphosphorylation of key tyrosine residues in this domain, which disrupts the autoinhibitory conformation and promotes receptor activation. These residues differ among tyrosine kinase receptors. Within the intracellular compartment, the tyrosine kinase domain is the most relevant structure to initiate and sustain receptor signaling. The activation of the tyrosine kinase domain (TKD), in most tyrosine kinase receptors, is also induced by transphosphorylation of tyrosine residues within this stable dimer complex. The phosphorylation of the kinase domain leads to the recruitment of other protein kinases and docking proteins that bind specifically to phosphotyrosines and activate the downstream signaling cascades.

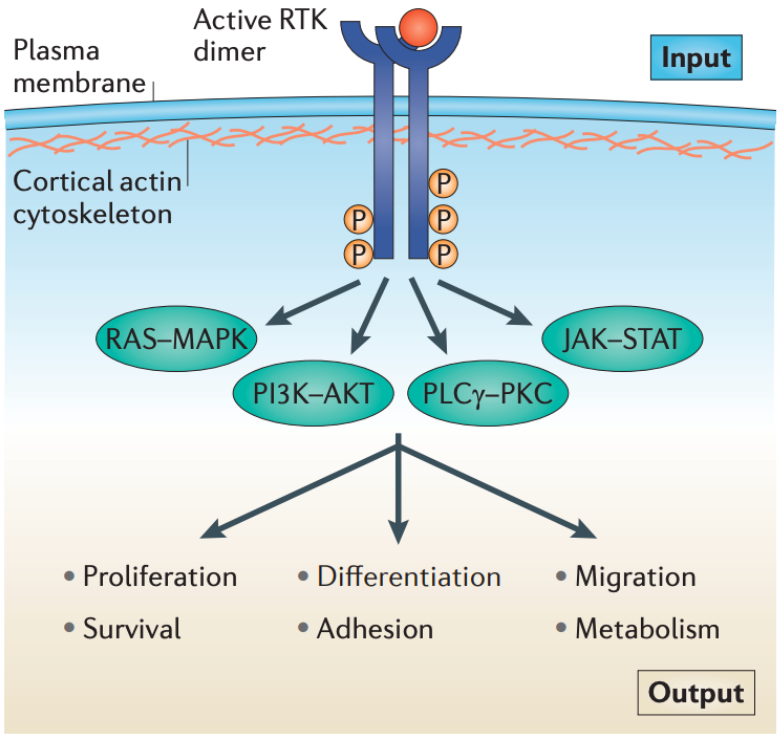


Figure 3. Schematic representation of a tyrosine kinase receptor activation. Upon the growth factor binding to the extracellular domain, the tyrosine kinase receptor adopts a differential conformation leading to dimerization, and transphosphorylation of the tyrosine kinase domain, which in turns promotes the activation of adaptor proteins that lead to the phosphorylation of downstream signaling pathways like the RAS-MAPK, PI3K-AKT, PLCY-PKC and JAK-STAT that convey in promoting multiple biological hallmarks that lead to cell proliferation, differentiation, migration, metabolism, adhesion and survival. *Figure and legend Adapted from Casaletto et al. Nature Reviews in Cancer 2012* (39)

Dysregulation of tyrosine kinase receptor function by oncogenic events like mutations, indels, amplification and rearrangements in the coding genes culminate in enhanced signaling and sustained downstream pathway activation. Relevant dysregulated RTK in lung adenocarcinoma are ALK, EGFR, RET, ROS1, HER2, MET and NTRK (40–45).

The focus of this work is on the anaplastic lymphoma kinase (ALK) tyrosine kinase receptor. For this thesis, the biology and oncogenicity of ALK, together with the development of ALK tyrosine kinase inhibitors, will be reviewed extensively **(Chapter 2)**. To fully understand the implications of the molecular subtypes of lung cancer and the biological rational for targeting selective kinases, the most relevant oncogenic drivers and intracellular oncogenic signaling involved in lung cancer carcinogenesis are described.

EGFR

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of tyrosine kinase receptors. EGFR is a tyrosine kinase receptor and is activated by the binding of its ligand, the epidermal growth factor (EGF) to the receptor extracellular domain (46). Specific *EGFR* mutations in the tyrosine kinase domain (exons 18 to 21), clustering around the active site of the kinase, induce ligand independent kinase domain phosphorylation. The most frequent activating “classic” molecular alterations (~90%) are in-frame deletions in exon 19 and the L858R point mutation in exon 21(36). Other less frequent activating molecular alterations are point mutations in exons 18 and 20 and exon 20 insertions.

As previously mentioned, *EGFR* activating mutations occur in about 12-15% of patients in western countries and ~50% in patients from east Asia (27,30). EGFR TKIs were initially developed for the treatment of lung cancer patients, irrespective of a molecular biomarker selection. The discovery of the predictive role of sensitizing mutations in *EGFR* on the activity of EGFR inhibitors became

a landmark in the development of potent drugs to treat molecularly selected patients with NSCLC (41,47).

Multiple phase III trials comparing the first-generation EGFR TKIs erlotinib, gefitinib, or icotinib, as well as the second-generation TKI afatinib, with platinum-based chemotherapy as frontline therapies showed a benefit in progression free survival (PFS) with these targeted agents (48–51). The third generation EGFR inhibitor osimertinib has shown significant activity in the treatment of patients that acquire the T790M mutations as a resistance mechanism to first and second-generation EGFR TKIs and in upfront in treatment naïve patients (52,53). *EGFR* exon 20 insertions confer, in general, resistance to currently available EGFR inhibitors, though new compounds are being developed to target this alteration (54). Patients with *EGFR* mutant lung cancer can achieve significant benefit when treated with EGFR TKI (55).

HER2

The Epidermal growth factor receptor 2 (HER2) is, as EGFR, a member of the ErbB family of tyrosine kinase receptors. Amplifications in *HER2* occur in about 25% of breast cancers, and this has led to the development of multiple targeted treatments in this field like monoclonal antibodies, tyrosine kinase inhibitors and antibody-drug conjugates (56). In a clear difference with breast cancer, *HER2* amplification is rarely observed in in lung cancer (~1.2%). However, insertions or duplications affecting the kinase domain in exon 20 occur in about 3-4% of patients with metastatic lung adenocarcinoma (57). *HER2* exon 20 insertions induce a rigid active conformation of this receptor together with structural modifications in the drug binding pocket that lead to steric hindrance and resistance to common tyrosine domain inhibitors (54). HER2 inhibitors like lapatinib, neratinib, afatinib and dacomitinib have limited activity in targeting HER2 exon 20 insertions in lung cancer. As with *EGFR* exon 20 insertions, there are currently covalent inhibitors like poziotinib and TAK-788 under development (54). Trastuzumab emtansine (TDM-1) is an antibody-drug conjugate directed

against HER2, with modest clinical activity in this setting. To date, there are no approved drugs for the treatment of patients with tumors that harbor *HER2* exon 20 insertions.

ROS1

The ROS proto-oncogene 1 receptor tyrosine kinase (ROS1) is a tyrosine kinase receptor, found in 1-2% of patients with lung adenocarcinoma (43). The oncogenic effect of ROS1 in lung cancer is mediated by gene rearrangements involving the *ROS1* tyrosine kinase domain in the 3´ with a variety of 5` fusion partners: *CD74*, *SLC34A2*, *EZR*, *FIG*, *TPM3*, *CCDC6*, among others. The most common reported fusion partner in lung cancer is with *CD74* (58). The fusion partner in the aminoterminal portion of the protein contains dimerization domains that, consequently, lead to homodimerization of fusion proteins and tyrosine kinase domain activation. The tyrosine kinase domain of *ROS1* shares significant homology to the *ALK* kinase domain in the ATP binding sites. ROS1 rearrangements can be targeted with ROS1 TKIs like crizotinib, which is currently approved for this indication (59). Next generation ROS1 inhibitors are also potent inhibitors like ceritinib, entrectinib, brigatinib, lorlatinib and repotrectinib, with promising preclinical and clinical activity in this scenario (60–64).

MET

The Mesenchymal-Epithelial Transition (MET) proto-oncogene, encodes for the MET tyrosine kinase receptor. The extracellular portion that binds to its ligand, the hepatocytic growth factor (HGF) (65). The intracellular portion of MET contains the juxtamembrane and kinase domain together with the carboxyterminal multifunctional docking site. The juxtamembrane domain is encoded in exon 14 and regulates MET degradation by the engagement of the tyrosine Y1003 with the casitas B-lineage lymphoma (c-CBL) E3 ubiquitin ligase

(66). Upon HGF binding to the SEMA domain, MET homodimerization results in the phosphorylation of key tyrosine residues within the kinase domain and in the docking site. Multiple biological alterations have been implied in MET oncogenesis across multiple tumor types.

In lung cancer, the two main mechanisms of MET oncogenicity involve molecular alterations in the splicing regulatory sites of exon 14 and/or *MET* amplification. Point mutations or deletions in the splicing regulatory sites of exon 14, result in exon 14 skipping and loss of the juxtamembrane domain, impairing receptor ubiquitination and degradation, with extended receptor signaling (44). *MET* exon 14 mutations occur in ~3% of advanced NSCLC, and can be targeted with MET tyrosine kinase inhibitors like crizotinib, tepotinib, capmatinib, savolitinib or merestinib which are currently under clinical development (67). *De novo MET* amplification is less frequent in lung cancer. However *MET* amplification is frequently acquired during treatment with EGFR TKI in patients with *EGFR* mutant lung cancer, as a bypass mechanism of resistance (68–70). The combination of MET and EGFR TKIs can overcome resistance in this scenario (71).

RET

The rearranged during transfection proto-oncogene gene (RET) codifies for a tyrosine kinase receptor, and its ligands belong to the glial-derived neurotrophic factor (GDNF) family. *RET* fusions and mutations were initially characterized in thyroid carcinomas and multiple RET tyrosine kinase inhibitors have been developed in this context (72,73). Oncogenic *RET* rearrangements occur in 1-2% of lung adenocarcinoma tumors (74,75). The most common fusion partner is the kinesin family member 5B gene (*KIF5B*), but many other fusion partner have been described (76). As with ROS1 rearrangements, *RET* fusions preserve the RET tyrosine kinase domain which homodimerizes by the interaction of the fusion partner coiled coil domains. Ligand independent RET phosphorylation leads to downstream activation of the JAK/STAT, PI3K and

MAPK pathways. *RET* fusions are mainly diagnosed in the clinical practice by FISH or NGS. To date, several multikinase inhibitors targeting RET like vandetanib, lenvatinib and cabozantinib have been studied in patients with lung cancer showing modest efficacy (76). Alectinib is an ALK inhibitor that is active against RET, and there is retrospective data showing signs of efficacy for this compound (77,78). Novel, selective and potent RET TKIs are currently under clinical development for the treatment of patients with *RET*-rearranged lung cancer (79,80)

NTRK

The neurotrophin kinase (*NTRK*) genes codifies the tropomyosin receptor tyrosine kinases (TRK). *NTRK* rearrangements are involved in a wide variety of tumor types, though it is a rare event in lung cancer, present in less than 1% (81,82). *NTRK3* fusions are pathognomonic in mammary analog secretory carcinoma and infantile fibrosarcoma. *NTRK*-rearranged cancers are highly sensitive to NTRK tyrosine kinase inhibitors. A study of the first generation NTRK inhibitor larotrectinib lead to the first tissue agnostic approval for a tyrosine kinase inhibitors based solely on the molecular alteration (83). New generation NTRK inhibitors have been developed to overcome resistance to larotrectinib by secondary kinase domain mutations (84). Though infrequent (~0.2%), the detection of *NTRK*-rearrangements in patients with lung cancer can lead to substantial benefit with NTRK tyrosine kinase inhibitors.

MAPK signaling pathway

The downstream pathway signaling from tyrosine kinase receptor activation, is mainly catalyzed by multiple cascades of intracellular protein kinases and phosphatases, of which the most relevant are the mitogen-activated protein kinase (MAPK) and the PIK3CA/AKT/mTOR pathways **(Figure 4)**.

The MAPK pathway regulates multiple critical cellular processes and is altered or activated in most cancers. Dysregulation of this pathway, mainly by uncontrolled activation, leads to the survival, propagation and dissemination of cancer cells (85). This pathway begins with the activation of RAS proteins (KRAS, NRAS or HRAS) secondary to the phosphorylation of RTKs in the cell membrane. Activated RAS proteins (in a GTP bound state), interact with various downstream effectors, mainly the RAF family of serine-threonine kinases (A-RAF, B-RAF and C-RAF). RAF protein kinases, mediate MEK1 and MEK2 phosphorylation, which in consequence, phosphorylate ERK1 and ER2K kinases. In its cytoplasmic location, ERK kinases phosphorylate proteins that participate in cell adhesion, mobility and metabolism (86). Phosphorylated ERK also migrates to the nucleus and induces the phosphorylation of transcription factors, mainly CPS II and p90 ribosomal S6 kinase (RSK), which promote cell cycle and mitosis (87). This pathway has several negative regulatory feedbacks, like ERK inhibitory loops directed against C-RAF and B-RAF activation and the induction of dual specific phosphatases (DUSPs) that block pathway overactivation (88).

KRAS

KRAS is an intracellular guanine nucleotide binding protein from the RAS family of GTPases. The active form of KRAS is bound to GTP (KRAS-GTP) (89). KRAS activation is regulated by its intrinsic GTPase activity and by GTPase activating proteins (eg: NF1). Most *KRAS* mutations affect exons 2 and 3, altering the GTPase activity of this protein leading to an active GTP binding state, and

intrinsic activation of its capacity to promote oncogenic signaling (90). *KRAS* is the most common oncogenic driver in lung cancer representing approximately 25-30% of cases (23,26,27). The most common mutations are G12C and G12V. Except for *KRAS* G12D, *KRAS* mutations are more prevalent in patients with a history of smoking. *KRAS* mutations often co-occur with mutations in *TP53* (40%) and *STK11*/*LKB1* (32%), with relevant implications in the response of co-mutant tumors to immunotherapy (91). There are no clinically approved targeted therapies against KRAS in *KRAS* mutant lung cancer, though compounds directed against the KRAS G12C mutant protein are currently under development (90,92).

BRAF

The RAF family of kinases is composed by ARAF, BRAF and CRAF and are activated by GTP- bound RAS proteins. The binding of RAS proteins results in the development of RAF homodimers (BRAF-BRAF) or heterodimers (CRAF-BRAF) leading to RAF activation (85). Once activated, RAF proteins bind and phosphorylate MEK. *BRAF* is the second most common mutated gene in the MAPK pathway after *KRAS*, driving its oncogenic potency by the occurrence of point mutations. *BRAF* mutations occur in about 3% of lung adenocarcinomas, being the most common mutation the substitution of a valine for a glutamic acid in codon 600 (V600E). This class I mutation confers constitutive activation of BRAF to signal as a monomer, leading to high levels of phosphorylated ERK (93). Class II (signaling through mutant dimer) and III BRAF mutations (signaling through mutant and wild type RAF heterodimers) include mutations in G469, G466, G596 and K601 residues. Current available BRAF inhibitor are only effective against *BRAF* V600X mutations by binding to activated BRAF monomers. Dabrafenib (BRAF inhibitor) in combination with Trametinib (MEK inhibitor) is a currently approved regimen for patients with BRAF V600E mutant lung cancer (94).

PIK3/AKT/mTOR pathway

The PIK3/AKT/mTOR pathway is a relevant downstream signaling pathway in lung cancer activated by RTK, G-coupled receptors and activated RAS proteins (95) **(Figure 4)**. Upon receptor phosphorylation, PI3K catalyzes the phosphorylation of cell membrane bound phosphatidylinositol-(4,5)-bisphosphate (PIP2) to phosphatidylinositol-(3,4,5)-trisphosphate (PIP3). PIP3 is a potent secondary messenger and activates phosphoinositide-dependent kinase-1 (PDK1). Upon PIK3 phosphorylation, AKT translocates to the inner membrane and is phosphorylated by PDK1 in its activation loop. AKT, in turn, indirectly activates the mammalian target of rapamycin complex 1 (mTORC1) by phosphorylation of the proline-rich Akt substrate of 40 kDa (PRAS40) and inhibiting the tuberous sclerosis complex (TSC) (96). The mTOR protein is a serine-threonine kinase that forms the catalytic subunit of the mTORC1 and mTORC2 complexes. The mTORC1 complex is composed by mTOR, Raptor (regulatory protein associated with mTOR), and mLST8. Instead of forming a complex with Raptor, mTORC2 binds to Rictor (rapamycin insensitive companion of mTOR). The rapamycin-FKBP12 complex can directly inhibit mTORC1 but not mTORC2. However, prolonged rapaymicin treatment eventually impairs mTORC2 binding to mTORC1.

Activated mTORC1 mediates its oncogenic role through the phosphorylation of its main effectors: p70 S6 Kinase 1 (S6K1) and eIF4E Binding Protein (4EBP) (97). S6K1 phosphorylates S6 which, in turn, stimulates the transcription of key genes in cancer survival by the activation of transcription factors. 4EBP, a key translation repressor protein, inhibits the eukaryotic translation initiation factor 4E (eIF4E) (98). mTOR phosphorylates 4EBP, which in turn liberates eIF4E which forms a complex that induces mRNA transcription of cyclin D1 and c-myc that promote cell proliferation.

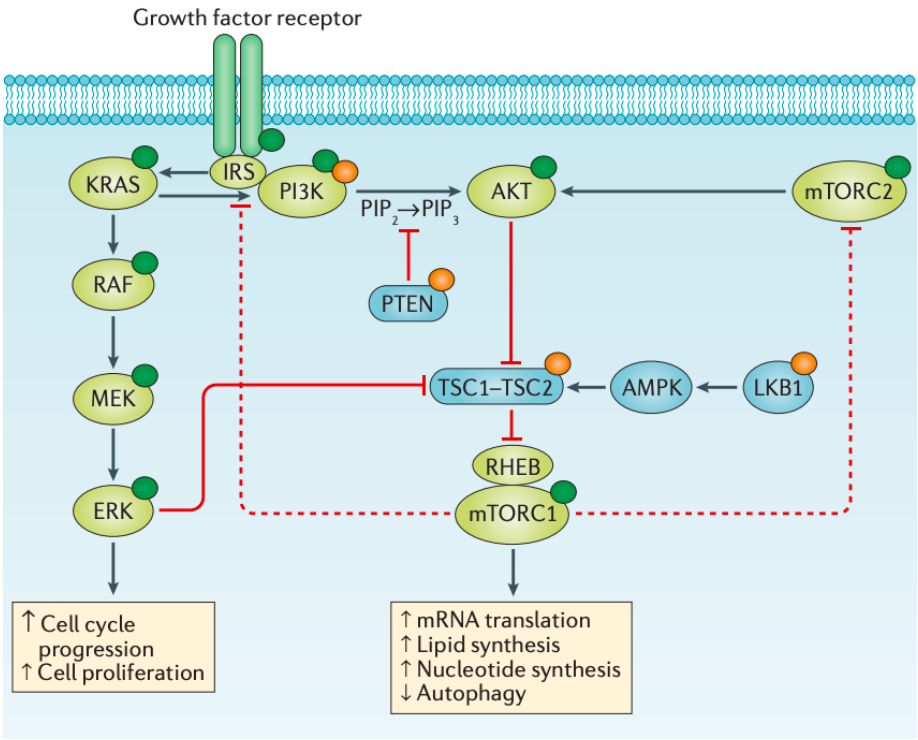


Figure 4. MAPK and PIK3/AKT/mTOR patway activation by tyrosine kinase receptors. Activation of the growth factor receptor tyrosine kinases and G protein-coupled receptors induces KRAS–RAF– MEK–ERK signaling. The MAPK pathway can also be activated constitutively by gain-of-function alterations in the component kinases (eg. KRAS, BRAF, MEK, ERK) (green circles). The class I PI3K proteins are recruited to the plasma membrane by adaptor proteins, leading to phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 is a second messenger that activates the AKT kinases, which are able to phosphorylate tuberous sclerosis protein 1 (TSC1) and TSC2, and thereby dissociate the TSC1–TSC2 complex. resulting in the activation of mTOR complex 1 (mTORC1). mTORC1 is involved in a negative feedback loop to prevent the overactivation of AKT (dashed red lines). The PI3K–AKT–mTOR pathway can be upregulated by activating molecular alterations in the PI3K, AKT, and mTOR (green circles), or by loss-of-function alterations in regulatory subunits, PTEN, TSC1, TSC2, and LKB1 (STK11) (orange circles). ERK phosphorylation can further contribute to mTORC1 activation through dissociation of the TSC1–TSC2 complex promoting crosstalk of these signaling pathways. *Figure and legend adapted from Janku et al. Nature Reviews in Clinical Oncology 2018* (99)

mTOR activation is negatively regulated by several proteins and complexes (100). The major regulator of mTOR is the tuberous sclerosis complex (TSC1 and TSC2), that mediates mTOR activation through RHEB (101). The tuberous sclerosis complex functions as a GTPase activating protein for the

GTPase RHEB, that binds and activates mTOR. When the PIK3/AKT/mTOR pathway is activated, AKT mediates TSC phosphorylation, uncoupling it from RHEB, allowing RHEB to become activated and, in consequence, induce mTOR activation. LKB1/STK11 is another important regulator of mTOR activation and is frequently altered in lung adenocarcinoma (23). LKB1 negatively regulates mTOR by activation of the AMP-activated protein kinase (AMPK) and mTOR inhibition through TSC2 (102).

There are several points of cross-talk between the MAPK and the PIK3CA/AKT/mTOR pathways. Both pathways can negatively regulate or cross-activate each other. For example, RAS-GTP can bind and activate PI3K, and ERK can activate mTOR through TSC phosphorylation. In addition, both pathways converge in the expression of common genes with antiapoptotic effects like *MYC* and *FOXO* genes (103). Both pathways also mediate survival by promoting the expression and functionality of anti-apoptotic proteins (from the Bcl-2 family) and the degradation of pro-apoptotic mediators.

NF2 regulates mTOR signaling

Merlin is a membrane-cytoskeleton linker, encoded by the *NF2* gene, that regulates adherent junctions and numerous pathways including Rho GTPases, (Rac1 and Cdc42), RTK, RAS, FAK-Src, PI3K and the Hippo pathway (104). Merlin has been identified to negatively regulate mTORC1, through the study of type II neurofibromatosis **(Figure 5)** (105). Mutations and deletions in *NF2* cause the Neurofibromatosis type syndrome, characterized by the formation of nervous system tumors like vestibular schwannomas, peripheral schwannomas, meningiomas and ependymomas. These tumors can also form by sporadic mutations in *NF2*.

Merlin is a negative mTOR regulator by mTORC1 inhibition (105). In *NF2* deficient tumors, mTOR has been shown to be constitutively active, and re-expression of the wild-type *NF2* gene represses mTOR signaling (106). Pharmacological mTOR inhibition affects NF2-shwannomas by inhibiting S6 and

4E-BP1 phosphorylation (107) This has also been observed in mesothelioma cells with *NF2* inactivation, where loss of merlin results in integrin-dependent mTOR activation, promoting cell cycle proliferation (108). The oncogenic effect of *NF2* loss can be also explained by its regulatory function as a regulator of the Rho GTPase Rac1 and Cdc42 (105). These effectors can signal through serine/threonine p21-activating kinases (PAKS) and the MAPK effectors JNK and c-Jun, enhancing both cyclin D1 expression and the transcriptional activity of E2F proteins. Merlin indirectly inhibits activation of RAC1 through the GDP-GTP exchange by Rac1-associated guanine exchange factors. Merlin can also prevent the interaction of Cdc42 with its downstream effectors. In addition, Merlin can also inhibit RAS and hence, also regulate cell signaling through the MAPK pathway.

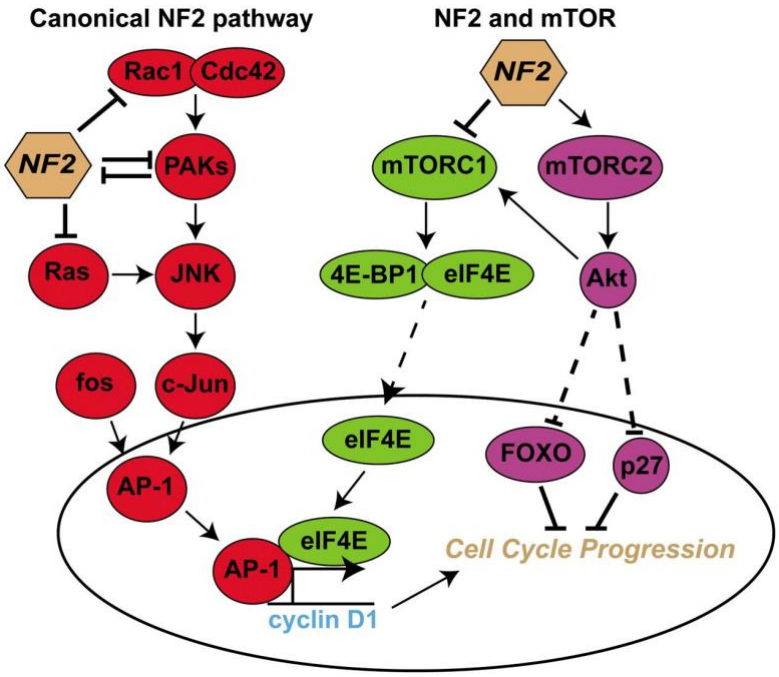


Figure 5. Role of NF2/Merlin in cell cycle regulation via its canonical pathway (Rac1, Cdc42) and via mTOR. In the cytoplasm, NF2 disrupts the downstream signaling of Rac1 and Cdc42 it can also inhibit activation of PAKs, and Ras. Through this, NF2 prevents the activation of JNK and c Jun. Interestingly, NF2 has differential effects on mTORC1 (inhibition) and mTORC2 (activation). In the context of NF2 loss, mTORC1 leads to the dissociation of transcription factors, 4E-BP1 and eIF4E, resulting in the transcriptional activation of cyclin D1. *Figure and legend adapted from Beltrami et al. Anticancer Cancer 2013* (105)

SRC family of protein kinases

c-Src is a cytoplasmic tyrosine kinase, with well-known oncogenic properties, that interacts with multiple RTK and intracellular protein complexes by enhancing signal transduction (109). There are nine members of the Src-family kinases that have Src homology domains.

Activation of RTK like EGFR, VEGFR, PDGFR, IGFR-1 and ALK leads to the activation of c-Src via its SH2 domain. Src can directly activate the MAPK and the PI3K/AKT/mTOR pathways by direct cooperation with RTK activation. PI3K contains an SH2 domain in its regulatory domain (p85), where it can be activated by SRC and FAK. Src also regulate cell to cell adhesions by interacting with p130, paxillin and focal adhesion kinases (FAK) to induce cell migration through dissociation of cell-cell junctions and the activation of matrix metalloproteases. Activated Src also regulates angiogenesis through the expression of the vascular endothelial growth factor (VEGF).

Apoptosis inhibition by oncogenic signaling

Oncogenic signaling pathways culminate in the transcription of multiple genes and the induction of mediators of cell proliferation, migration, invasion and survival. In this scenario, cell survival is also sustained by the inhibition of apoptosis (110).

The apoptotic process is mainly executed by caspases, a family of endoproteases that trigger signaling pathways that eventually lead to cell death. Caspases can by activated in apoptosis by two main pathways: the extrinsic and intrinsic pathways (111). The extrinsic pathway requires the activation of ¨death receptors¨ in the cell membrane like TRAIL, FAS or TNRF1. The intrinsic pathway is the most regulated in cancer and is activated by the result of intracellular stimuli including metabolic alterations, DNA damage and endoplasmic reticulum stress. In this pathway, BH3-only protein (PUMA, BID and BIM) activates the pro-

apoptotic proteins BAX and BAK, which in turn mediate the permeabilization of the outer mitochondrial membrane. Consequently, cytochrome C and second mitochondrial-derived activator of caspases (SMACS) are released from the mitochondria to in the cytoplasm. Cytochrome C interacts with apoptotic protease activating factor 1 (APAF1) to form the apoptosome complex which activates caspase-9. This event leads to the activation of caspase-3 and caspase-7, and ultimately to apoptosis. The anti-apoptotic BCL-2 family of proteins inhibit the permeabilization of the mitochondria outer membrane by binding and inhibiting BH3-only proteins and by binding to activated BAK and BAX.

Oncogenic signaling through the MAPK and PI3K/AKT/mTOR pathways enhances antiapoptotic signaling. One of the mechanisms of cell survival is the induction of proteasome-mediated degradation of the proapoptotic BH3-only protein BIM by phosphorylated ERK (112). Another mechanisms involves Mcl-1, an antiapoptotic protein from the Bcl-2 family that binds and sequestrates proapoptotic proteins like BAX, BAK, NOXA and BIM and is positively regulated by mTOR activation (113). The inhibition of RTKs and intracellular kinases in oncogene-addicted lung cancer induces BIM upregulation and apoptosis mainly through ERK inhibition (114). The dependency on BIM to induce apoptosis upon TKI treatment is reflected by the induction of treatment resistance by silencing BIM expression and by the lower levels of apoptosis observed with BIM deletion polymorphisms in *EGFR* mutant lung cancer cells (115).

1E. Targeted therapies in lung adenocarcinoma

Genomic testing in lung adenocarcinoma tumors is key to guide the treatment strategy for patients with advanced disease. The landscape of targetable oncogenic alterations has led to the development of a large list of compounds directed to inhibit abnormal activated kinases in lung cancer. These targeted therapies are mostly tyrosine kinase inhibitors and serine-threonine kinase inhibitors.

Tyrosine kinase inhibitors are a group of small molecules designed to inhibit the transfer of the terminal phosphatase of ATP to a tyrosine residue in the tyrosine kinase domain by irrupting the kinase ATP binding site. TKIs can be classified according to their mechanisms of action. Type I inhibitors bind to the active conformation of the kinase in the ATP pocket at the catalytic site, type II inhibitors bind to the catalytic site in the inactive (unphosphorylated) confirmation of the kinase (DFG-out) and type III are non-ATP competitive inhibitor (allosteric inhibitors) (116). Allosteric inhibitors were further classified as class III if the binding of the molecule is within the cleft between the small and large lobes adjacent to the ATP binding pocket and type IV inhibitors if the drug binding occurs outside of the cleft. Allosteric inhibitors induce conformational changes that make the protein inactive. A recent classification has included type I ½ kinases which bind to protein kinases in a DFG-Asp in and C-helix out conformation (117). TKIs inhibit the phosphorylation of the kinase domain and, in consequence, blocks the receptor downstream signaling pathways, removing the main growth and survival stimuli in abnormally activated kinases, resulting in apoptosis and cancer cell death.

Currently, there are multiple kinase inhibitors approved for the treatment of patients with *ALK*-rearranged, *EGFR* mutant, *BRAF* V600E mutant, *ROS1* and *NTRK*-rearranged lung cancer based on enhanced response rates and disease control **(Table 1)**. There are also several kinase inhibitors in development targeting RET, HER2/EGFR exon 20 insertions, KRAS, MET and a wide variety of drugs targeting DNA damage response elements, cell-cycle checkpoint

kinases and epigenetic modulators in lung cancer. In addition to selecting patients for targeted therapies based on the tumor biology, the identification of molecular actionable targets is necessary to select patients for treatment with novel compounds in the setting of clinical trials.

Gene	Molecular Alteration	Clinical Approved Kinase inhibitors	Kinase inhibitors in Development
ALK	Rearrangements	Crizotinib, Ceritinib Alectinib Brigatinib Lorlatinib	Ensartinib Entrectinib PLB1003
EGFR	L858R mutations and exon 19 deletions.	Erlotinib Gefitinib Afatinib Dacomitinib, Osimertinib	-
	Exon 18 and 20 point mutations	Afatinib	Osimertinib
	Exon 20 insertions	None	Poziotinib, TAK-788
	T790M resistance (post 1st-2nd Generation EGFR TKI)	Osimertinib	Nazartinib Abivertinib
ROS1	Rearrangement	Crizotinib	Ceritinib Brigatinib Lorlatinib Repotrectinib, Entrectinib
BRAF	V600E mutations	Dabrafenib/Trametinib	
NTRK	Rearrangements	Larotrectinib	LOXO-195 Entrectinib Repotrectinib
MET	Exon 14 skipping, amplification	None	Crizotinib Capmatinib Tepotinib Savolitinib Merestinib
KRAS	G12C	None	AMG-510 MRTX849
RET	Rearrangements	None	LOXO-292 BLU-667 Alectinib Lenvatinib
HER2	Exon 20 insertions	None	Poziotinib TAK-788

Table 1. Targetable Oncogenic drivers in non-small cell lung cancer. Genes, molecular alterations that lead to oncogenic activation and targeted therapies with kinase inhibitor approved and in development.

While the exploration of new oncogenic targets is far from ending, a new chapter in the treatment of lung cancer is being written with the development of immune-checkpoint inhibitors (ICIs) like PD-1 and PD-L1 monoclonal antibodies (118). These compounds have shown to be efficacious in the treatment of patients with NSCLC in the metastatic setting alone and in combination with chemotherapy, and as consolidation treatment for patients with stage III disease after concurrent chemoradiation therapy (119–121). However, clinical studies suggest that the role of immunotherapy on patients with *EGFR* mutant, *ALK* rearranged lung cancer is limited (122). In addition, early trials studying the combination of anti EGFR or ALK TKI with ICIs have shown significant toxicities without signs of added benefit (123–125). This further validates the role of targeted therapies as the standard and preferred treatment for patients with EGFR and ALK-driven tumors.

2. Targeting the Anaplastic Lymphoma Kinase (ALK) in lung cancer: from discovery to treatment with second-generation ALK inhibitors

2A. The ALK tyrosine kinase receptor

The Anaplastic Lymphoma Kinase (*ALK*) gene is located in chromosome 2p23.1 and encodes for the Anaplastic Lymphoma Kinase tyrosine kinase receptor. *ALK* was first discovered in the year 1994 through the study of t(2;5)(p23;q35) translocated anaplastic large cell lymphomas. Morris and colleague's discovered that the product of this translocation was the fusion between ALK and nucleopasmin 1 (NPM1), and disclosed the high homology of *ALK* to other members of the insulin tyrosine kinase receptor family like the leukocyte tyrosine kinase (LTK)(126). Since then, the murine and human full ALK receptors have been cloned and characterized (127,128).

ALK contains 29 exons that encode a protein of 1620 amino acids **(Figure 6)**. The ALK tyrosine kinase receptor is composed of an extracellular domain, a transmembrane domain and an intracellular kinase domain (129,130). The extracellular domain is constituted by 1038 amino acids and contains an external signal peptide, two MAM segments (meprin, A5 protein, PTPmu), and a LDLa (low density lipoprotein class a) domain between both MAM segments. The MAM segments are thought to participate in cell-cell adhesion and the role of the LDLa domain is unknown (131).

The extracellular domain is the binding site for the ALK receptor ligands (ALKALs). JEB (jelly belly) has been identified to activate ALK in *Drosophila melanogaster,* and Hen-1 (hesitation 1) is the ligand in *Caenorhabditis elegans* (132). FAM150A (AUGβ) and FAM150B (AUGα) have been recently discovered as ALK ligands in vertebrate organisms (133,134). These secretory proteins are potent ALK and LTK ligands capable of activating ALK *in vitro* in the picomolar

range (135). Conditioned medium containing FAM150A or FAM150B potently activated PC12 cells expressing full length *ALK*. In addition ligand-dependent ALK phosphorylation can be potently inhibited with crizotinib, a first generation ALK TKI (136). Furthermore, the role of these ALK ligand were confirmed *in vivo* in vertebrate models using the zebrafish Danio rerio. The Danio rerio LTK has high homology to the human ALK in sequence and domain structure. This RTK controls the development of the neural crest-derived pigment cells iridophores. Overexpression of the FAM150 proteins cause ectopic iridophore development and, loss of function mutations in these genes, result in lack of iridophore formation in these models.

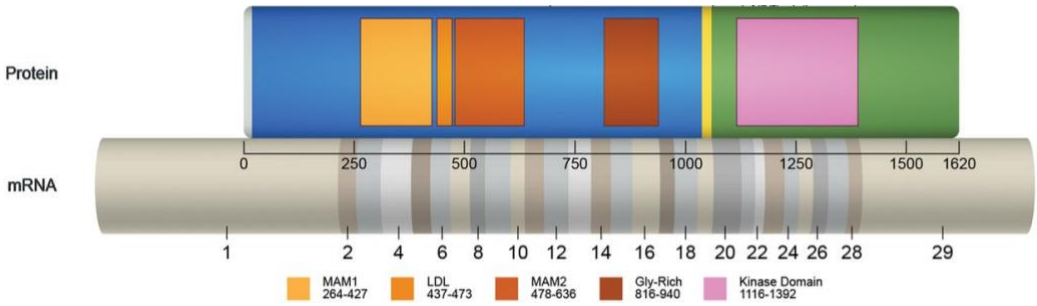


Figure 6. ALK mRNA and protein sequence with key domains. mRNA sequence depicts full reference sequence of ALK (NM_004304) with exon numbers marked. ALK protein sequence (0–1620 amino acids) shows different functional domains (MAM1, LDL, MAM2, Gly-rich, and kinase domain) with starting and ending amino acid numbers (UniProt). *Figure and legend adapted from Holla et al. Cold Spring Harbor Molecular Case Studies 2017*(137)

The ALK transmembrane domain connects the extracellular domain to the receptor juxtamembrane domain in the intracellular portion of the receptor (residues 1060-1620). The ALK kinase domain is encoded in exons 20 to 28 and is composed by an amino-terminal lobe and a carboxyterminal lobe linked to a hinge region that forms the binding pocket for ATP, where the catalytic action of the kinase takes part (138). The ALK kinase domain contains two hydrophobic motifs called the catalytic and regulatory spines (139). The catalytic spine contains the adenine ring of bound ATP that is conformed in an activated state.

In the kinase domain, the catalytic activity is regulated by several essential segments, including a catalytic loop, the activation loop, the αC-helix and glycine-rich loop (129). The crucial residues in the kinase domain include E1167 in the αC-helix, the HRD residues H1247, R1248 and D1249 within the catalytic loop, the K1150 residue within the N-lobe, and the DFG residues D1270, F1271 and G1272 within the activation segment. The spatial structure promotes and regulates the activation state of the kinase.

ALK receptor dimerization results in transphosphorylation of residues Y1278, Y1282, and Y1283 in the activation loop (140). Following this, multiple tyrosine residues in the kinase domain become phosphorylated (1507, 1584, 1586, 1604, 1139, 1358, 1385 and 1401). Most of these phosphorylated residues serve as docking sites for adaptor proteins that initiate signaling pathway: SHC (Src homology and collagen protein), FRS2 (fibroblastic growth factor substrate 2), IRS2 (insulin receptor substrate 2), GRB2 (growth factor bond 2), amongst others (141). This triggers the activation of previously described oncogenic signaling pathways including the RAS/RAF/MEK/ERK, PI3K/AKT/mTOR, JAK/STAT, JNK, PLCγ (phospholipase Cγ) and SRC signaling. The Src-homology 2 domain (SH2)-containing protein tyrosine phosphatases, SHP1 and SHP2, also play a role in cell proliferation in ALK-driven cancers (142,143).

The study of ALK downstream signaling pathway has been mainly performed on oncogenic NPM-ALK and EML4-ALK rearranged cancer cell models. These pathways ultimately culminate in the activation and expression of several oncogenic proteins that regulate cell proliferation (FOS, JUN, MYC, CDKN1B, cyclin D2, Cdc42), cell motility (p130CAS, MMP9) and inhibition of apoptosis (BIM inhibition) **(Figure 7)**.

The physiological role of ALK in humans is not fully understood, but multiple *in vivo* studies in other species suggest that ALK participates in neural development and behavior. In mice, high levels of *ALK* expression were seen on neonatal brain and spinal cord. Interestingly, *ALK* knockout mice experience basal hippocampal progenitor proliferation, increase dopamine levels in the basal cortex and alterations in behavioral tests (144). In humans, ALK inhibition with the highly brain penetrant TKI lorlatinib can provoke psychiatric alterations

including cognitive function, mood, and speech as adverse events with this drug (145). Though the biological rational behind this unique adverse event has not been explored, it could provide new hypothesis to the role of ALK in the human brain and guide further research on this matter.

The involvement of ALK in a variety of human cancers has been well characterized over the past 25 years. The two main biological mechanisms involved in ALK carcinogenesis are gene rearrangements involving the *ALK* kinase domain in the 3' end and point mutations in the ALK tyrosine kinase domain. *ALK* rearrangements are most commonly distributed among the different tumor types driven by *ALK* alterations.

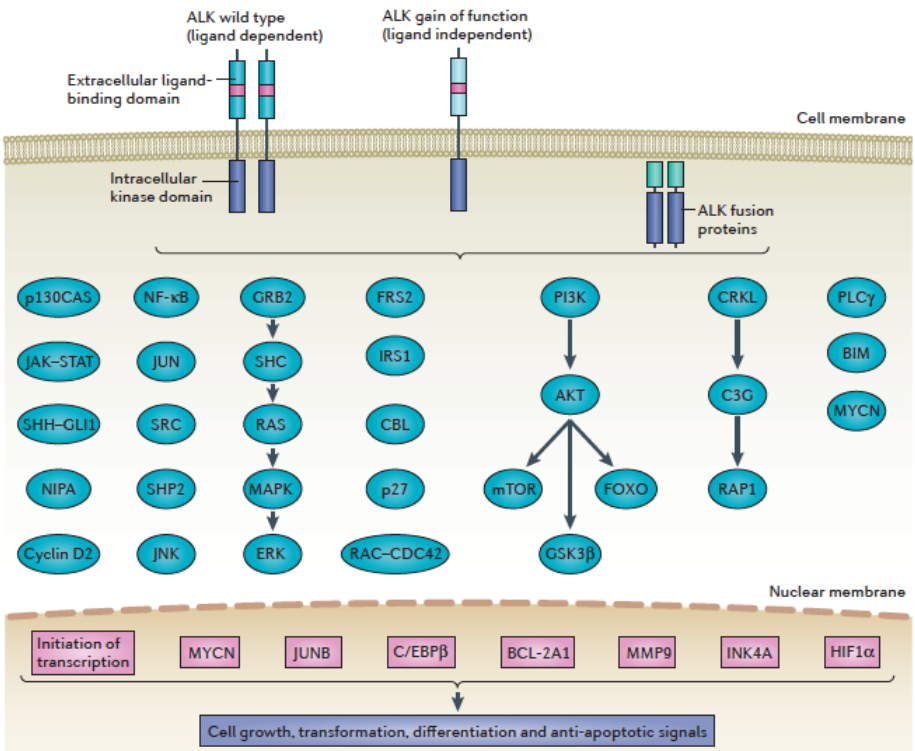


Figure 7 Molecular mechanisms of ALK oncogenic activation and downstream signaling. *Wild type ALK signals by receptor dimerization after binding to its ligand. Gain of function kinase domain mutations (eg. F1174L and F1245C) and rearrangements involving the ALK kinase domain (eg. EML4-ALK) confer ligand independent activation. Signaling pathways implied in ALK oncogenicity are the RAS–MAPK, PI3K–mTOR, PLCγ, RAP1, Janus kinase (JAK)–signal transducer and activator of transcription (STAT) and JUN pathways. Adaptor proteins like IRS1, SHC, GRB2, SHP2, C3G, CBL, CRKL and FRS2 are activated in the kinase domain and mediate downstream signaling. This finally conveys in the regulation of the transcription of several genes that promote cancer*

proliferation and survival. Figure and legend adapted from Hallberg et al. Nature Reviews in Clinical Oncology 2013 (141).

Since the discovery of the *NPM-ALK* rearrangement, over 30 *ALK* fusion partners have been identified in a wide variety of cancer including: lung cancer, inflammatory myofibroblastic tumors (IMT), anaplastic thyroid cancer (ATC), esophageal, breast, colon, leiomyomas/sarcomas, renal cell carcinomas, large B-cell lymphomas (LBCL), endometrial cancer, histiocytosis and gliomas (129). **(Table 2)**.

ALK rearrangements are transduced in fusion proteins composed by the amino-terminal portion of the fusion partner and the carboxi-terminal portion of *ALK*, containing the complete kinase domain. The most common *ALK* breakpoints occur between exons 19-20 or 20-21. When rearranged, the subcellular localization of ALK is determined by the unique characteristics of the partner proteins, which can reside in the nucleus, in the cytoplasm, and in intracellular organelles. In gene rearrangements, the transcription of the fusion gene is regulated by the promoter of the fusion partner. At the protein level, the fusion partner homodimerizes leading to trans-autophosphorylation of the tyrosine kinase domain and activation of downstream signaling pathways. It is unclear if the different fusion partners may confer differential oncogenic properties, but recent evidence suggests that the fusion partner protein may impact the potency of ALK tyrosine kinase inhibitors (146).

ALK rearrangements are common in inflammatory myofibroblastic tumors (IMT), the most common pediatric form of primary lung tumors in children. About 50% of IMT harbor *ALK* rearrangements resulting in constitutive ALK activation **(Table 2)** (147)**.** Less frequently, other oncogenic rearrangements are involved in the pathogenesis of this disease including *ROS1*, *RET* and *NTRK3*. ALK+ IMT cancers are susceptible to treatment with ALK TKIs, with about 80% of patients achieving responses with crizotinib.

ALK-rearranged diffuse large B cell lymphomas (DLBCL) is a rare and aggressive form of cancer. Patients with *ALK*-driven DLCBC have a dismal prognosis compared to non-rearranged DLBCL. Anecdotal response to ALK TKIs have been reported in the context of chemotherapy refractory disease. *ALK*

rearrangements have been detected in other solid tumors, but the clinical relevance of these infrequent findings needs to be further explored **(Table 2)**

Cancer Type	*ALK* Rearrangements / Fusion partner
ALCL	*NPM, ATIC, RNF213, TPM3, TPM4, TRAF1, MSN, TFG, MYH9, CLTC, ALO17*
NSCLC	*EML4, KIF5B, KLC1, PTPN3, STRN, SLC2A, CMTR1, VIT, GCC2, CUX1, BCL11A, KLC1.*
IMT	*TPM3, TPM4, CLTC, CARS, ATIC, SEC31, PPFIBP-1, RANBP2, NUMA1, THSBS1, IGFBP5, HNRNPA-1, A2M,*
Anaplastic Thyroid Cancer	*STRN, EML4, GFPT1, TFG*
Breast Cancer	*EML4*
Esophageal Cancer	*TPM4*
Renal Cell Carcinoma	*VCL, TPM3, EML4, STRN, HOOK1*
Colorectal	*CAD, EML4, CDorf44*
Leiomyosarcoma	*ACTG2*
Glioma	*PP1CB*
Endometrial cancer	*EML4*
Ovarian Cancer	*FN1-ALK*
LBCL	*CLTC, NPM, SQSTM1, SEC31A, GORASP2*
Epithelioid histiocytoma	*PRKAR2A, MLPH*
Cancer Type	***ALK* kinase domain mutations**
Neuroblastoma	R1275Q/L (43%), F1174L/I/C/S/V (30%), F1245C/L/V (12%)
Anaplastic Thyroid Carcinoma	L1198F

Table 2. Summary of the different ALK- dependent cancers and molecular alterations. *ALK-rearrangements occur in a variety of tumor types, being the most frequently observed in inflammatory myofibroblastic tumors (IMT), non-small cell lung cancer (NSCLC) and anaplastic large cell lymphomas (ALCL).*

A less common mechanism of oncogenic ALK activation is by the point mutations in the *ALK* kinase domain, mainly described in neuroblastomas. Neuroblastoma is a pediatric cancer arising from the sympathetic nervous system, most commonly in the adrenal medulla. MYC amplification is observed in about 30% of cases and is associated with poor survival (148). Activating point mutations in the *ALK* kinase domain are present in about 7-8% of neuroblastoma tumors (139). Oncogenic *ALK* kinase domain mutations in the following residues F1174, F1245, F1275, G1128, M1166, I1170, I1171, R1192, L1196, L1240 and Y1278 confer constitutive activation of ALK kinase domain in neuroblastoma. The most common mutations detected in neuroblastomas are the F1174L and R1275Q and are usually somatic, but in rare occasion, germline *ALK* mutations are found in familial neuroblastoma (149). Interestingly, some of neuroblastoma activating mutations like F1174L and L1196M also confer resistance to the first generation ALK inhibitor crizotinib by increasing the ATP affinity of the kinase or by blocking the binding of the ALK TKI to the kinase domain (150,151). Preclinical models and case reports support the use of ALK inhibitors in the treatment of ALK mutant neuroblastomas, promoting the need for the clinical development of targeted therapies in this disease (152).

2B. The role of ALK in lung cancer

ALK rearrangements occur in about 3-6% of lung adenocarcinomas. The activation of ALK by the complex formation of fused proteins is the sole mechanism of ALK oncogenicity in lung cancer. Multiple fusion partners have been described in lung cancer including *EML4, KIF5B*, *KLC1*, *PTPN3*, *STRN*, *SLC2A*, *CMTR1*, *VIT*, *GCC2*, *CUX1*, *BCL11A* and *KLC1.* Nevertheless, *EML4-ALK* rearrangements are the most common fusions in patients with *ALK*-rearranged lung cancer (153).

Soda and colleagues published the first report and characterization of an *EML4-ALK* rearrangement in lung cancer in 2007 (40). They identified a 3.926 base-pair cDNA, for a 1.059 amino acid protein with an amino-terminal sequence identical to *EML4* and a carboxi-terminal sequence identical to *ALK*. This fusion protein EML4-ALK (variant 1) was the product of a disrupted *EML4* in exon 13 with *ALK* in exon 20, including the full *ALK* kinase domain in the 3´extreme.

Echinoderm microtubule like proteins (EML) are family of proteins that participate in microtubule regulation. Humans express six different subtypes of EML proteins (154). EML4 is a member of this family and is composed of an amino-terminal coiled-coil domain and a carboxi-terminal domain that contains a hydrophobic EML protein (HELP) domain. The coiled-coil region of EML4 is necessary for oligomerization, this region is also called trimeric dimerization (TD). The carboxyterminal region also contains a tandem atypical β-propeller in EML protein (TAPE) domain that also participates in the hydrophobic core of this protein. The hydrophobic core of EML4 mediates the binding to tubulin in human cells.

EML4-ALK proteins form homodimers through the coiled-coil domain in EML4, inducing transphosphorylation of the kinase domain and activation of oncogenic downstream pathway signaling, as previously mentioned. Both *EML4* and *ALK* are oriented in opposite directions within the short arm of chromosome 2 and *EML4-ALK* fusions are produced by paracentric inversions [inv(2)(p21p23)]

in this locus. Different breaking points in *EML4* give origin to a spectrum of *EML4-ALK* fusion variants (155). At least 15 different EML4-ALK variants have been identified, and the most common EML4 break points occur in exon 13 (variant 1) in 43% of cases, exon 20 (variant 2) in 6%, and exon 6 (variant 3) in 40% **(Figure 8)** (156). The EML4-ALK variant 3 was identified in 2008 by Choi and colleagues, and is comprised by the isoforms 3a and 3b resulting from alternative splicing (157). All three EML4-ALK variants conferred transforming properties in vitro, and phosphorylated EML4-ALK can be successfully inhibited with ALK TKI inhibitors (158). The EML4-ALK variants 3a/b and 5a/b are the only variants that lack the EML4 TAPE domain. Expression of the EML4 TAPE domain confers instability to the fusion protein and might explain higher levels of sensitivity of TAPE containing variant to ALK and HSP90 inhibitors (159). Shorter variants that do not contain the TAPE domain like variant 3 are more stable.

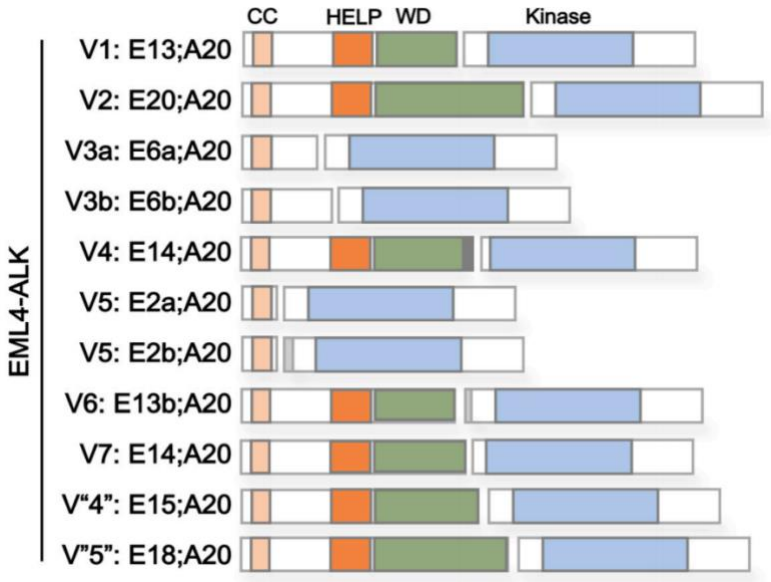


Figure 8. Schematic display of the most frequent EML4-ALK rearrangements. *The variant number is followed by the breakpoint locus in EML4 and ALK. The representation of EML4 includes the coil coiled domain (CC), the hydrophobic EML protein (HELP) domain and the variable tryptophan-aspartic acid (WD) repeats. Variants 3a, 3b and 5 do not contain the EML4 HELP and WD domains. All the EML4-ALK variants include the entire ALK kinase domain. Figure and legend adapted from Wu et al. Cancers (Basel) 2017 (*(160)

In addition the variant type also determines sub-cellular localization of the fusion protein, variant 1 is detected in the cell cytoplasm while variant 3 EML4-ALK fusion proteins are localized in microtubules and nucleus (161). Clinical studies did not show a significant impact of the different variant types in the response to ALK TKI in patients with ALK rearranged lung cancer but this subject is currently being studied (156,162). However, in the setting of resistance to tyrosine kinase inhibitors, there is initial evidence showing associations between the type of variants and specific mechanisms of resistance (156).

Most *ALK* rearranged lung cancers are lung adenocarcinomas. Infrequently, ALK rearranged squamous cell carcinomas and large cell carcinomas have been reported, but these were found in non-smoker patients (163). Adenocarcinomas with *ALK* fusions may present signet cell features under pathology revision, and this might be associated with a poorer prognosis (164). ALK rearrangements can be diagnosed in pathologic specimens of lung cancers in different ways: by immunohistochemistry (IHC), fluorescence in situ hybridization (FISH) and next-generation sequencing (NGS) (165).

ALK-rearranged lung cancer tends to occur at younger age and can be detected in about 13% of patients with less than 50 years old with lung adenocarcinoma (166–168). There is a strong association between this molecular subtype and a history of light (<10 pack/years) or never smoking and its prevalence is higher in women. Most patients are diagnosed with ALK-rearranged lung cancer at advanced stages, and this molecular subtype of lung cancer is characterized by a high tropism for the central nervous system (CNS). Brain metastasis occur in about 20% of patients at the time of diagnosis but the incidence of brain metastasis increases during the course of treatment with ALK tyrosine kinase inhibitors rising up to 60%, which conveys significant morbidity and mortality (169,170). For these reasons, effective and highly CNS penetrant tyrosine kinase inhibitors have been developed to achieve both intracranial and extracranial disease control.

Currently, there are five effective ALK targeted agents available for the treatment of patients with lung cancer, with proven efficacy and capable of

conferring prolonged disease control and survival when appropriately indicated and managed.

The implementation of rapid ALK testing with IHC and the biological and chemical development of ALK tyrosine kinase inhibitors, together with an effective clinical trial development, led to the approval of the first-generation ALK TKI crizotinib in 2011, just 4-years after the discovery of the EML4-ALK rearrangement in lung cancer. In the following 7-years, the arsenal of ALK TKIs expanded significantly to adapt drug design to deliver more potent and CNS penetrant ALK TKIs, capable of overcoming resistance to the first-generation ALK inhibitor crizotinib.

2C. ALK Tyrosine Kinase Inhibitors in the treatment of ALK+ NSCLC: first and second generation ALK TKI

Currently, there are five ALK tyrosine kinase inhibitors available for the treatment of patients with ALK-rearranged lung cancer: the first generation ALK TKI crizotinib, second generation ceritinib, alectinib and brigatinib and the third generation ALK TKI lorlatinib **(Table 3)**. Treatment with ALK directed tyrosine kinase inhibitors has dramatically improved the survival and quality of life of patients with ALK rearranged lung cancer. This has been achieved by the sequential use of first-, second- and third-generation ALK inhibitors.

Drug	Generation	Clinical Setting	Approval
Crizotinib	First	First Line	EMA/FDA
Ceritinib	Second	First Line Second after crizotinib	EMA/FDA EMA/FDA
Alectinib	Second	First Line Second after crizotinib	EMA/FDA EMA/FDA
Brigatinib	Second	First Line Second after crizotinib	No EMA/FDA
Lorlatinib	Third	After two lines of ALK TKIs including crizotinib or 2nd line after a second generation ALK TKI.	EMA/FDA

Table 3. Summary of available ALK tyrosine kinase inhibitors. The table summarizes the clinical setting in which the ALK tyrosine kinase inhibitors have been tested and the approval status by the European Medicines Agency (EMA) and the Food and Drug Administration of the United States (FDA).

The original approach for the treatment of patients with ALK+ lung cancer consisted of providing first-line treatment with crizotinib and switching to second or third generation ALK TKIs after progression. Patients treated with crizotinib in the first line setting can achieve responses in about 74% of cases, with median progression-free survival (PFS) of 10.9 month **(Figure 9)** (171). Second generation ALK TKIs can overcome resistance by most of the on-target

resistance mutations developing with crizotinib. Treatment with ceritinib, alectinib or brigatinib in the second or further lines after disease progression with crizotinib conveys response rates ranging from 37 to 73% of patients and median progression-free survival durations between 5.4 and 12.9 months (172). The third generation ALK inhibitor, lorlatinib, was then develop to overcome resistance mechanisms to first and second-generation ALK inhibitors, in particular the development of highly resistant mutations like the solvent-front G1202R mutations (172). Recent reports from the phase I/II study of lorlatinib show that patients with treated with > 2 previous lines of ALK TKI, lorlatinib elicited responses in 39% of patients and median PFS duration in this group was 6.9 months (173).

In the past years, this paradigm shifted to place more potent and selective second generation ALK TKI in the first line setting. This has been supported by improved PFS observed with ceritinib, alectinib and brigatinib in the first line (162,174–176).

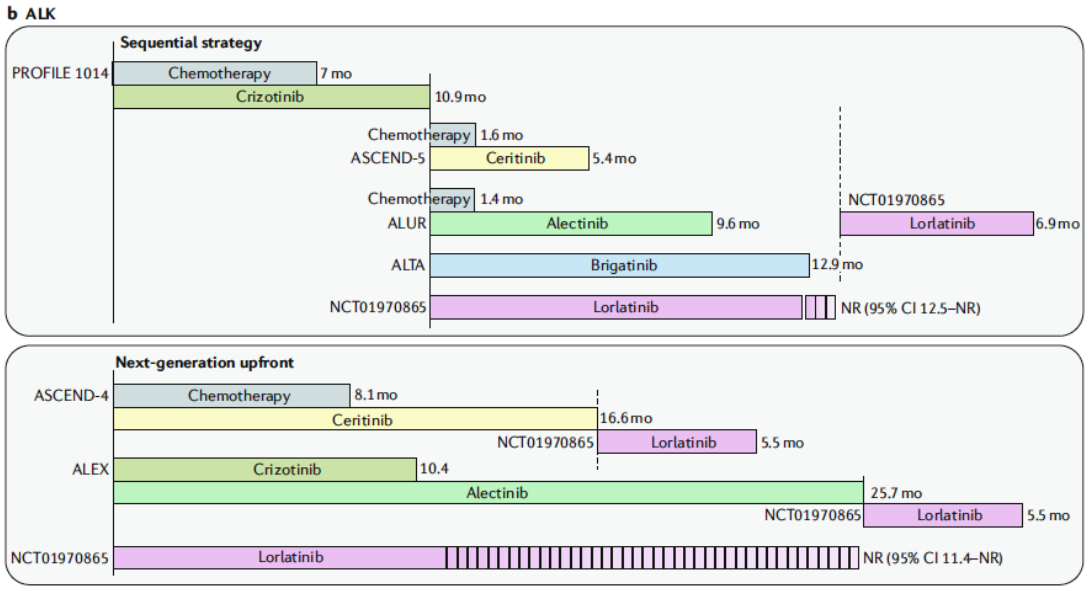


Figure 9. Treatment strategies with ALK inhibitors in NSCLC. *Sum of progression-free survival (PFS) durations in different trials of frontline ALK tyrosine kinase inhibitors in a sequential strategy (top) with first line treatment with crizotinib and the strategy with first line second generation ALK TKIs ceritinib and alectinib. Abbreviations: mo, months; NR , not reported.*

Crizotinib

Crizotinib (PF-2341066) is an orally available ATP-competitive inhibitor of ALK, MET and ROS1 (177). The early preclinical studies showed that crizotinib is a potent ALK inhibitor in Karpas299 or SU-DHL-1 ALCL cells (IC50: 24 nmol/L). ALK inhibition by crizotinib results in G1-S–phase cell cycle arrest and induction of apoptosis in these cell lines (178). Crizotinib is also a potent inhibitor of ALK rearranged lung cancer, as seen by inhibitory properties the EML4-ALK rearranged H3122 cells in vitro (179). The H3122 cell line is composed of lung cancer cells that harbor an EML4-ALK variant 1 rearrangement and have been extensively used to characterize ALK inhibitors.

Four clinical trials were conducted showing the efficacy of crizotinib in the treatment of patients with ALK+ lung cancer **(Table 4)**. The most relevant study in this context was the PROFILE 1014, a phase III trial that compared the efficacy of crizotinib to chemotherapy in the first line setting (171,180). Treatment with crizotinib resulted in increased response rate (74% vs 45%), prolonged PFS (10.9 months vs. 7 months) and impressive survival in patients, reaching 4-year survival rates of 56.6% with crizotinib (180,181).

Crizotinib can penetrate the brain barrier, and can confer higher intracranial disease control compared to chemotherapy (DCR 85% vs 45%) (182). However, in patients with baseline treated brain metastasis, about 43% of patients experienced intracranial disease progression and 22% of patients without baseline brain metastasis developed central nervous system (CNS) progression. This propelled the need for the development of enhanced brain penetrating ALK inhibitors to confer better CNS disease control. Based on this evidence, crizotinib was widely adopted and approved in 2011 for the treatment of patients with advanced ALK rearranged lung cancer as a first line or subsequent line of treatment.

Trial	Trial design (phase, primary end point, and treatment arms,	Median follow-up (months)	Outcomes ∗ ORR, ∗ median PFS and ∗ median OS
PROFILE 1001 (REFS (183,184)**)**	I ORR, DOR, TTR, PFS, 6–12 mo OS, safety profile Crizotinib (*n* = 149)	16.3	∗ 60.8% ∗ 9.7 Mo ∗ 1-year OS 74·8%
PROFILE 1005 (REF. (185)**)**	II ORR Crizotinib (*n* = 1069)	NA	∗ 54% ∗ 8.4 mo ∗ 21.8 mo
PROFILE 1007 (REF. (186)**)**	III PFS Crizotinib (*n* = 173) vs pemetrexed or docetaxel (*n* = 174)	12.2 mo (crizotinib) and 12.1 mo (chemotherapy)	∗ 65% vs 20% ∗ 7.7 mo vs 3.0 mo (HR 0.49; *P* <0.001) ∗ 20.3 mo vs 22.8 mo (HR 1.02; *P* = 0.54)
PROFILE 1014 (REF (171,187)**)**	III PFS Crizotinib (*n* = 172) vs platinum + pemetrexed (*n* = 171)	46 mo	∗ 74% vs 45% ∗ 10.9 mo vs 7.0 mo (HR 0.45; P<0.001) ∗ NR (45.8 mo–NR) vs 47.5 mo (32.2 mo–NR; HR 0.76; *P* = 0.048)

Table 4. Summary of clinical trials with crizotinib. *ORR: overall response rate; DOR: duration of response; TTR: time to treatment response; PFS: progression free survival; OS: overall survival. Adapted from Recondo et al. Nature Reviews Clinical Oncology 2018* (172)

Ceritinib

Ceritinib (LDK375) is a second generation, ATP competitive ALK inhibitor. Besides ALK, ceritinib can also inhibit the Insulin Growth Factor Receptor (IGFR) (188). Ceritinib was designed to overcome resistance to some of the most frequent resistance mutations occurring at disease progression with crizotinib, like the L1196M gatekeeper mutation (189).

Ceritinib was the first second-generation ALK TKI tested in the context of resistance to crizotinib, showing response rates in about 40% of patients with median PFS durations of 6 months (190–193) **(Table 5)**.

In the first-line setting, ceritinib was superior to chemotherapy, eliciting higher response rates (72.5% vs. 26.7%) and PFS (16.6 months vs. 8.1 months) (175). Hence, ceritinib is currently an upfront treatment option for patients with ALK-rearranged lung cancer. However, given the development of other potent and better tolerated second generation ALK inhibitors like alectinib and brigatinib, the current role of ceritinib in the second- or first-line treatment seems to be declining.

Trial	Trial design (phase, primary end point, and treatment arms,	Median follow-up (months)	Outcomes ∗ ORR, ∗ median PFS ∗ median OS
ASCEND-1 (REFS (190,191)**)**	I MTD Ceritinib (*n* = 246) First line (33%) or second line after crizotinib (66%)	11.1 mo	∗ 72% or 56% ∗ 18.4 mo or 6.9 mo ∗ NR or 16.7 mo
ASCEND-2 (REF. (192)**)**	II ORR Ceritinib (*n* = 140) Second line after crizotinib	11.3 mo	∗ 38.6% ∗ 5.7 mo ∗ 14.9 mo
ASCEND-3 (REF. (194)**)**	II ORR Ceritinib (*n* = 124) First line	8.3 mo	∗ 63.7% ∗ 11.1 mo ∗ NA
ASCEND-4 (REF. (175)**)**	III PFS Ceritinib (*n* = 189) vs platinum + pemetrexed (*n* = 187) First line	19.7 mo	∗ 72.5% vs 26.7% ∗ 16.6 mo vs 8.1 mo (HR 0.55; *P* <0.00001) ∗ NE (29.3 mo–NE) vs 26.2 mo (22.8 mo–NR ; HR 0.73; *P* = 0.056)
ASCEND-5 (REF. (193)**)**	III PFS Ceritinib (*n* = 115) vs pemetrexed or docetaxel (*n* = 116) Second line after crizotinib	16.5 mo	∗ 39.1% vs 6.9% 5∗ .4 mo vs 1.6 mo (HR 0.49; *P* <0.0001) ∗ 18.1 mo vs 20.1 mo (HR 1.00; *P* = 0.5)

Table 5. Summary of clinical trials with ceritinib. *ORR: overall response rate; DOR: duration of response; TTR: time to treatment response; PFS: progression free survival; OS: overall survival. Adapted from Recondo et al. Nature Reviews in Clinical Oncology 2018* (172)

Alectinib

Alectinib is a benzo[b]carbazole derivative developed to be highly potent, with an IC50 in cell-free assays of 1.9 nM and confers high levels of ALK inhibition in EML4-ALK rearranged cell lines *in vitro* and *in vivo* (195). Alectinib was firstly clinically developed as a second line regimen for patients previously treated with crizotinib **(Table 6)**. The phase III study ALUR, compared alectinib to single agent docetaxel or pemetrexed in patients who had previously progressed on treatment with crizotinib, and like what was observed with ceritinib, treatment with alectinib conferred higher response rates than chemotherapy (37.5% vs 2.9%) and prolonged PFS (9.6 vs. 1.4 months) (196). Based on these results alectinib was granted approval for the treatment of patients who had experienced disease progression after treatment with crizotinib.

Differently to crizotinib and ceritinib, alectinib is not a substrate of the P-glycoprotein (P-gp), also known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1) (197). P-gp is a key efflux transporter present in the capillary endothelial cells of the blood brain barrier, and by this mean, can reduce the bioavailability of drugs in different body compartments, like the brain. As alectinib is not a substrate for this transporter and is a highly penetrant drug, it can achieve high concentrations in the CNS (198). A pooled analysis of phase II studies revealed that in patients with measurable brain metastasis, the intracranial ORR with alectinib was 64%, with a median intracranial duration of response of 10.8 months (199).

The phase III trial J-ALEX, conducted in Japan, and the international ALEX trial compared the efficacy of first-line treatment with alectinib to crizotinib (200,201). Alectinib was associated with a significant PFS benefit as well as a more favorable toxicity profile than crizotinib, achieving a median duration of PFS of 34.8 months. Intracranial response rates were superior with alectinib, (81% vs 50%) together with improved duration of responses and lower incidence of brain metastases compared to crizotinib. Based on the results of the ALEX and J-ALEX trials, alectinib has become the standard choice for the first line treatment of patients with ALK rearranged lung cancer in many regions of the world.

Trial	Trial design (phase, primary end point, and treatment arms)	Median follow-up (months)	Outcomes ∗ ORR, ∗ median PFS ∗ median OS
AF-001JP (REF (200,201)**)**	I/II DLT and MTD (phase I) or ORR (phase II) Alectinib (*n* = 46) First line	36 mo[b]	∗ 93.5% ∗ NR; 3-year PFS: 62% ∗ NE; 3-year OS: 78%
AF-002JG (REF (202)**)**	I/II Recommended phase II dose Alectinib (*n* = 47) Second line after crizotinib	4.2 mo	∗ 55% ∗ NA ∗ NA
NP28761/ NP28673 (REF. (203,204)**)**	II ORR (*n* = 225; *n* = 189 evaluable for response) Second line after crizotinib	92.3 weeks	∗ 51.3% ∗ 8.3 mo ∗ 29.1 mo
ALUR (REF (196)**)**	III PFS Alectinib (*n* = 72) vs docetaxel or pemetrexed (*n* = 35) Second line after crizotinib	6.5 mo	∗ 37.5% vs 2.9% ∗ 9.6 mo vs 1.4 mo (HR 0.15; *P* <0.001) ∗ 12.6 mo (9.7 mo–NR) vs NR (NR–NR; HR 0.89)
AF-001JP (REF (200,201)**)**	I/II DLT and MTD (phase I) or ORR (phase II) Alectinib (*n* = 46) First line	36 mo[b]	∗ 93.5% ∗ NR; 3-year PFS: 62% ∗ NE; 3-year OS: 78%
J-ALEX (REF (205)**)**	III IRC-assessed PFS Alectinib (*n* = 103; 300 mg BID) vs crizotinib (*n* = 104)	12 mo (alectinib) and 12.2 mo (crizotinib)	∗ 92% vs 79% ∗ NR (95% CI 20.3 mo–NE) vs 10.2 mo (95% CI 8.2–12.0 mo; HR 0.34; *P* <0.0001) ∗ NA (immature data)
ALEX (REF (162,174)**)**	III Investigator-assessed PFS Alectinib (*n* = 152; 600 mg BID) vs crizotinib (*n* = 151)	22.8 (alectinib) and 27.8 (crizotinib)	∗ 82.9% vs 75.5% ∗34.8 mo vs 10.9 mo (HR 0.43) ∗ 1-year OS 84.3% vs 82.5% (HR 0.76; *P* = 0.24)

Table 6. Summary of clinical trials with alectinib. *ORR: overall response rate; DOR: duration of response; TTR: time to treatment response; PFS: progression free survival; OS: overall survival. Adapted from Recondo et al. Nat Rev Clin Onc 2018* (172)

Brigatinib

Brigatinib (AP26113) is a second generation ALK inhibitor. Brigatinib was originally developed as an ALK inhibitor that could potentially overcome resistance by the solvent front mutation G1202R which is a known mechanism of resistance to first and second generation ALK TKIs. Brigatinib inhibits the kinase activity of wild-type ALK with an IC50 0.6 nmol/L and is active *in vitro* against multiple ALK resistance mutations like C1156Y (0,6 nmol/L), F1174L (1.4 nmol/L), L1196M (1.7 nmol/L), and G1202R (4.9 nmol/L). However, the *in vitro* activity of this drug for the G1202R mutation was not replicated *in vivo* nor in patients. Importantly, brigatinib has modest activity against mutant EGFR, especially in presence of the T790M mutation (206). In addition to kinase inhibition, pre-clinical models of brigatinib also revealed that this drug has high CNS penetration, as proven by significant tumor reductions in orthotopic mouse brain tumor models.

In the clinical setting, three clinical trials support the efficacy of brigatinib given after crizotinib progression **(Table 7)**. In this setting, the phase II study ALTA trial showed that treatment with brigatinib resulted in 56% response rate, with a median PFS of 15.6 months. Therefore, brigatinib was approved as a second line treatment option for patients previously treated with crizotinib.

In the similar context than the ALEX trial for alectinib, brigatinib was compared to crizotinib in the first line setting in a phase III randomized study, the ALTA1L trial (176). Treatment with brigatinib resulted in improved response rates (71% vs 60%) and disease control (176). In concordance with the observations with alectinib in the first line, brigatinib conferred higher intracranial response rates, (78% vs. 29%) and lower incidence of brain metastasis during treatment.

Trial	Trial design (phase, primary end point, and treatment arms)	Median follow-up (months)	Outcomes ∗ ORR, ∗ median PFS and ∗ median OS
NCT01449461 (REFS (207,208)**)**	Recommended phase II dose (phase I) or ORR (phase II) Brigatinib (*n* = 79) First line (10%, *n* = 8), second line after crizotinib (85%, *n* = 68), or third line after crizotinib and ceritinib (5%, *n* = 3)	>31 mo[b]	First-line brigatinib (n = 8) ∗ 100% ∗ 34.2 mo ∗ NR (2-year OS 100%) Brigatinib after crizotinib (n = 71) ∗ 73% ∗ 13.2 mo ∗ 30.1 mo (2-year OS 61%)
ALTA (REFS (209,210)**)**	II ORR Brigatinib 90 mg daily (*n* = 112) vs brigatinib 180mg with a 7-day lead in of 90mg/d. (*n* = 110) Second line after crizotinib	19.6 mo (90 mg daily) 24.3 mo (standard dose)	∗ 46% vs 56% ∗ 9.2 mo vs 15.6 mo ∗ 29.5 mo (18.2 mo–NR) vs 34.1 mo (27.7 mo–NR
ALTA1L (REFS (176)**)**	III PFS First Line: Brigatinib 180mg with a 7-day lead in of 90mg/d (137) or crizotinib 250mg BID. (138)	11.0 months in the brigatinib group and 9.3 months in the crizotinib group	∗ 71% vs 60% ∗ 12-month PFS: 67% (95% CI, 56 to 75) vs. 43% (95% CI, 32 to 53) ∗ 12-month OS: 85% (95% CI, 76 to 91) vs. 86% (95% CI, 77 to 91).

Table 7. Summary of clinical trials with brigatinib. *ORR: overall response rate; DOR: duration of response; TTR: time to treatment response; PFS: progression free survival; OS: overall survival. Adapted from Recondo et al. Nat Rev Clin Onc 2018* (172)

As previously mentioned, the rational for developing newer generation ALK inhibitors is to overcome acquired resistance by cancer cells. But the study of mechanisms of resistance to ALK inhibitors has not been fully integrated in the clinical practice and are not routinely used for clinical decision making. However, it is key to comprehend the different implications of the biology driving tumor resistance and progression to ALK targeted therapies.

3. Biologic Mechanisms of Resistance to first- and second-generation ALK TKI: rational for lorlatinib development.

3A. Resistance mechanisms to first- and second-generation ALK tyrosine kinase inhibitors in lung cancer

As we previously reviewed, tyrosine kinase inhibitors are effective for the treatment of patients with lung cancer. Nevertheless, even when high response rates and prolonged disease control can be achieved, cancer cells invariable adapt and develop complex biological mechanisms that drive resistance to tyrosine kinase inhibitors **(Figure 10)**.

The landscape of acquired or primary resistance mechanisms to tyrosine kinase inhibitors can be grouped into 4 main categories:

1) On target mechanisms of resistance: when resistance occurs by acquired molecular alterations in the driver oncogene, mainly kinase domain mutations and gene amplification. In this context, there is sustained phosphorylation of the driver kinase domain, even in the presence of the tyrosine kinase inhibitor.
2) Bypass track mechanisms of resistance: occurs in the context of successful inhibition of the targeted driver, by the activation of other tyrosine kinase receptors (parallel resistance) of intracellular effectors of key oncogenic signaling pathways (eg: MAPK pathway, PI3K/AKT/mTOR pathway, SRC, etc.)
3) Histologic transformation: resistance is mediated by phenotypical changes that derive from genomic or epigenetic modifications of tumor cells like epithelial-mesenchymal transition, small cell transformation from a non-

small cell lung cancer cell, or adenocarcinoma cells transforming into a squamous cell carcinoma type.

4) Miscellaneous mechanisms of resistance including apoptotic defects and epigenetic modifications.

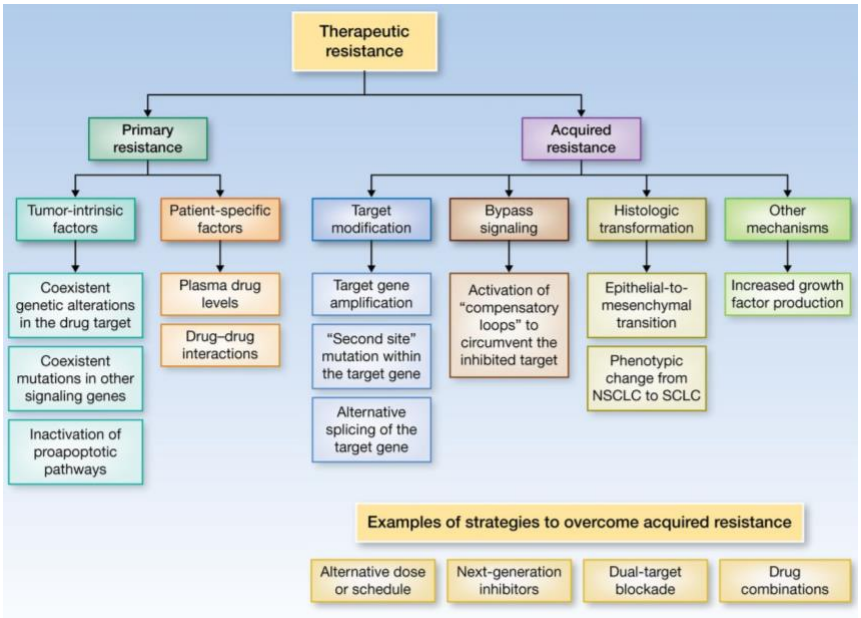


Figure 10. Mechanisms of resistance to kinase inhibitors. *The figure summarizes the causes of primary resistance (left) and acquired resistance (right). Figure and legend adapted from Lovely et al. Clinical Cancer Research 2014* (211)

On Target Resistance: resistant kinase domain mutations and gene amplifications

On target mechanisms of resistance occur in ~30% of tumors upon progression with crizotinib, of which 20% are secondary resistance mutations and 8-10% of cases, *ALK* amplification. ALK kinase domain mutations translate into aminoacidic substitutions in key residues that can affect drug binding, increase the affinity of the kinase for ATP and modify functional regulatory sites (212). Depending on the type of mutation and its effect on kinase-drug interactions, they can be divided into: gatekeeper mutations, solvent-front mutations, covalent binding site mutations and other types.

Gatekeeper residues are located between the amino and carboxyterminal lobes, within the kinase domain. The aminoacidic change in gatekeeper mutation partially of fully block deep hydrophobic regions within the ATP binding pocket of the kinase domain (213). The gatekeeper residue is important for kinase selectivity. The most common gatekeeper residues are occupied by a glycine, valine, alanine, or threonine. Gatekeeper mutations are responsible for resistance to a variety of kinase inhibitors. In ALK, the gatekeeper residue is the L1196 amino acid. L1196M is the most common resistance mutation to crizotinib in patients with *ALK*-rearranged lung cancer.

Solvent front mutations most commonly involve glycine residues in the solvent exposed helix of the kinase and affect kinase inhibitor binding by steric hindrance. The most relevant solvent front mutation in *ALK* is the G1202R, and it confers resistance to all the first- and second-generation ALK TKI: crizotinib, ceritinib, alectinib and brigatinib (214). The sole available drug capable of overcoming resistance to this mutation is the third generation ALK inhibitor lorlatinib, that was specially designed for this purpose.

In vitro models are useful to study the effect of secondary kinase domain mutations on different kinase inhibitors. Patient-derived cell lines and the establishment of Ba/F3 cell models harboring the specific gene and mutations can be used to study the biological effect of this mutation on the kinase and to design and test novel compounds to overcome resistance (215). In addition, N-ethyl-N-nitrosourea (ENU) mutagenesis screens using Ba/F3 cells harboring the gene of interest can help predict the occurrence of new resistance mutations that can be later validated in vitro (216).

Unlike resistance to other oncogenic drivers in lung cancer, the spectrum of acquired resistance mutations causing resistance to ALK TKIs is varied. Mutations that cause resistance to the first-generation ALK TKI crizotinib include: L1196M, G1269A, C1156Y, I1171T, L1152P, F1174L/C/V, E1210K and the solvent front mutations G1202R, D1203N and S1206Y/C (150,151,214,217–219). The most common crizotinib resistant mutations are the gatekeeper L1196M, and the G1269A mutation. The G1269A mutation lies in the ATP binding pocket, and impedes crizotinib binding (220). Mutations in the N-terminal region

of the kinase domain like L1152P, C1156Y, F1174C/L do not directly affect the crizotinib binding but might influence the αC-helix mobility, destabilizing this helix to promote the active conformation of the catalytic domain, enhancing the ATP affinity of the kinase (150,189). The solvent front mutations G1202R, G1202del, D1203N, and S1206Y/C confer high levels of resistance to crizotinib by inducing steric hindrance. Most of the secondary kinase domain mutations responsible for crizotinib resistance can be targeted with ceritinib, alectinib or brigatinib, except for the G1202R mutation, which confers resistance to all second-generation ALK inhibitors **(Figure 11).**

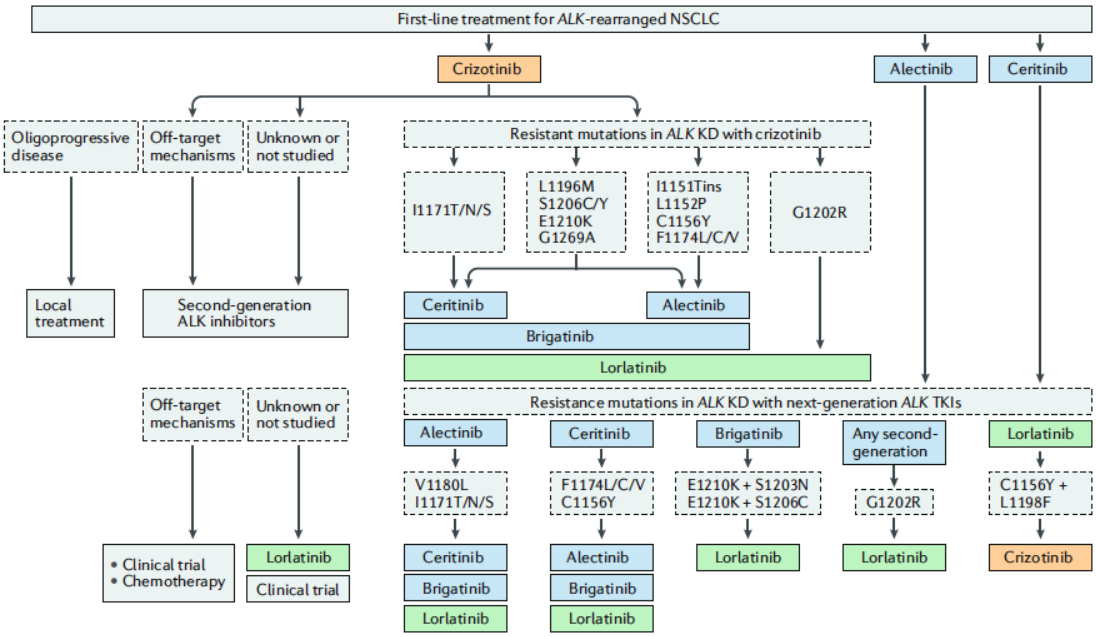


Figure 11. Biomarker integration in the management of patients with NSCLC. *The optimal sequencing strategies for the treatment sequence with tyrosine-kinase inhibitors (TKIs; either first generation or next generation), based on the type of acquired mechanisms of resistance in patients with NSCLC harboring ALK rearrangements. In first-line treatment with crizotinib, secondary kinase domain mutations can select for specific second generation ALK TKIs based on the differential sensitivity to these compounds. This is repeatedly the case in the second line setting, were for the exception of the G1202R mutation, other secondary kinase domain mutations could be overcome by switching to another second generation TKI. In the presence of the G1202R, the sole effective ALK directed treatment is lorlatinib. Off target mechanism in resistance to crizotinib can be overcome with second generation TKI. If resistance mutations are not identified at progression with a second generation TKI, lorlatinib is still an option, but in the presence of a known bypass mechanisms, a potential benefit could be obtained with clinical trials of combination therapies. KD: kinase domain. Figure and legend adapted from Recondo et al. Nature Reviews Clinical Oncology 2018* (172)

Ceritinib, can effectively inhibit ALK in the presence of most acquired resistance mutations with the exception of E1151T, L1152P, C1156Y and F1174C, which confer resistance to ceritinib (189). However, cancer cells harboring these mutations remain sensitive to alectinib and brigatinib. Among the crizotinib resistant mutations, alectinib is effective against most mutations with the exception of the I1171T/N/S mutations, that confer resistance to this compound but can be targeted with ceritinib (189,221). Together with this mutation, the V1180L gatekeeper mutation also confers resistance to alectinib, but both I1171T and V1180L mutations remain highly sensitive to brigatinib (206,222).

Resistance mechanism to brigatinib have been less explored than for other second-generation ALK TKI, mainly due to the later development of this compound. Two compound mutations (when two kinase domain mutations are present in cis) were reported to cause resistance to brigatinib, E1210K + S1203N and E1210K + S1206C, in addition to the G1202R mutation.

In the context of disease progression to first- and second-generation ALK TKI, on-target mechanisms of resistance can be detected in about 50-70% of cases. In this scenario, the most common acquired resistance mutation is the solvent front G1202R mutations, which is detected in approximately in 30-40% of cases (214). This is significantly higher than the 2% detection rate of G1202R mutations at crizotinib resistance.

The EML4-ALK variant type seems to influence the acquisition of G1202R mutations, as this mutation seems to occur exclusively in variant 3 EML4-ALK rearrangements. In a recent study, the G1202R mutation was detected in 37% of variant 3 and none of the variant 1 EML4-ALK rearranged lung cancers (156). Other non-G1202R ALK mutations also occur more commonly in variant 3 EML4-ALK rearrangements after progression to crizotinib and after progression to second-generation ALK TKI. For the moment it is unclear why the G1202R mutation occurs mainly in V3 EML4-ALK rearrangements, but it has been hypothesized that it could be related to the stability of this variant due to the absence of the TAPE domain, resulting in a shorter and more stable fusion protein.

On-target gene amplifications constitutes another resistance mechanism to treatment with crizotinib, resulting in the overexpression of the target protein affecting the capacity of the kinase inhibitor to full target ALK. *ALK* amplification has been reported in ~8% of patients treated with crizotinib (214). *ALK* amplification does not overlap with secondary crizotinib resistance kinas domain mutations. It can be overcome with more potent and selective ALK TKI, and has not been detected at resistance with second-generation TKI (214).

Bypass mechanisms of resistance

In this scenario, resistance is commanded by the parallel or downstream activation of an oncogenic protein different from the original driver. In the context of a bypass mechanisms of resistance, the targeted protein kinase is inhibited by the tyrosine kinase inhibitor. However, downstream oncogenic signaling pathways remain highly activated **(Figure 12)**.

There are multiple tyrosine kinase receptors and phosphokinases that have been implied in resistance through this mechanism. To overcome bypass mechanisms of resistance, effective drug combinations targeting the original driver and the acquired activated effector are required. Some bypass resistance mechanisms like gene amplifications, mutations or copy loss can be identified in patients using NGS and other molecular diagnostic methods. In many cases, however, bypass mechanisms of resistance are due to aberrant oncogenic activation of a non-mutated or amplified driver (eg: SRC, AXL) (223,224).

To study genomic and non-genomic bypass mechanisms of resistance, patient-derived models, like patient-derived xenografts (PDX) or patient-derived cell lines, are necessary to identify the biological processes driving resistance and to screen for new combination strategies to overcome it (223). Drug screens using multiple compounds alone and in combination with the drug to which the tumor acquired resistance, can identify “hits” in cell viability assays that can provide useful information on combinatorial treatment strategies but also on the underlying mechanism of resistance. Another way to identify aberrant protein

kinase activation is with “protein kinase arrays”, including tyrosine kinase receptor arrays and phosphokinase arrays. These immunoblotting screens, have been specifically designed to profile the level of protein kinase phosphorylation of multiple proteins in one experiment using cell lysates (225). CRISPR/Cas9 gene editing is also used to study resistance, mainly by the introduction of activating molecular alterations in gene (knock-in) or by modifying gene integrity that results in gene loss of function (knock-out). This is of particular interest to study the role of deleterious events in tumor suppressor genes in resistance to tyrosine kinase inhibitors (226). Given the high biologic diversity of off-target mechanisms of resistance, it is important to develop patient derived cell models and establish effective screening strategies to identify and target these alterations.

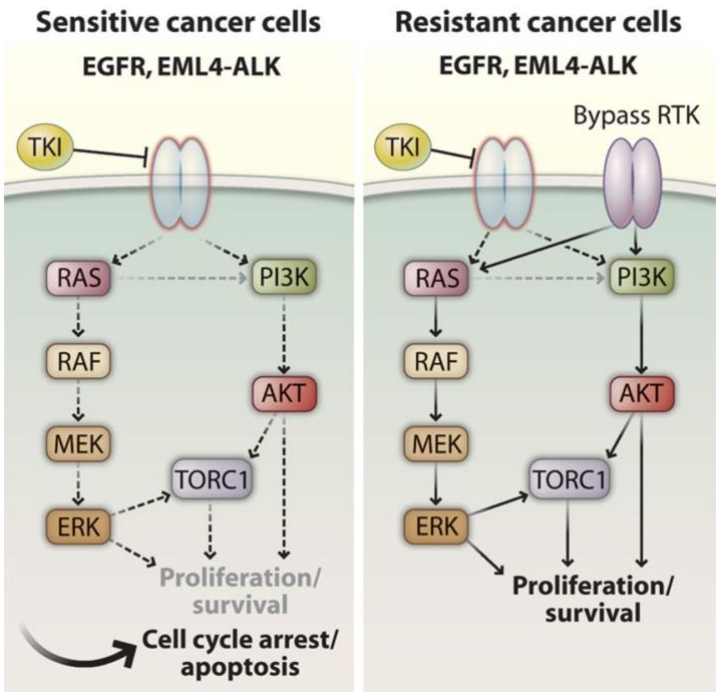


Figure 12. Bypass mechanisms of resistance to kinase inhibitors. *In sensitive cells (left), effective tyrosine kinase inhibitors bind and inhibit the receptor, and consequently downstream signaling cascades. In receptor bypass resistance, other activated RTK o phosphokinase maintains downstream signaling even in the context of effective inhibition of the original driver. RTK: tyrosine kinase receptor. Figure and legend adapted from Niederst et al. Science Signaling 2013* (227)

Several bypass mechanisms have been shown to drive resistance to ALK TKIs in lung cancer involving the insulin growth factor receptor 1 (IGFR1), SRC, PI3K, MEK, EGFR, HER2, HER3 and MET. It is unclear which proportion of tumors develop bypass mechanisms of resistance because most of the described biological mechanisms are not due to mutations in genes that can be detected by NGS. These are mainly mediated by overactivation, and overexpression of these tyrosine kinase receptors and intracellular phosphokinases.

Through the development of patient-derived cell lines and by performing drug screen assays, Crystal and colleagues reported that an acquired MEK K57N mutation conveyed resistance to the ALK inhibitor ceritinib and that combination treatment of ceritinib with the MEK inhibitor selumetinib (AZD6244) could overcome resistance in vitro and in vivo (223). The cytotoxic effect was only observed with the combination of the ALK and MEK inhibitor, and though MEK is downstream of ALK, full suppression of oncogenic signaling through the PI3K/AKT/mTOR pathway by ALK inhibition is also required to induce high levels of apoptosis. The same group also reported a second bypass mechanism by induction of SRC activation through ALK inhibition. In several patient derived models, ALK inhibition with crizotinib induced upregulation of SRC signaling. In this context, the combination of an ALK TKI with the SRC inhibitor saracatinib (AZD0530) was effective in reverting SRC mediated resistance. This was also observed in vitro mediating ceritinib resistance with the H3122 cell line (228).

Activation of the ErbB family of receptors including EGFR, HER2 and HER3 has also been implied in resistance to ALK inhibitors through receptor bypass signaling (217,229,230). EGFR activation is frequently observed at low levels in ALK rearranged NSCLC cell lines, contributing to the maintenance of downstream signaling (217). It has been proposed that EGFR can amplify downstream signaling by the fusion kinase, transactivate fusion kinases, and mediate signaling through adaptor proteins like GRB (229). In the setting of resistance, EGFR signaling can be enhanced and treatment with EGFR and ALK inhibitors can restore sensitivity and induce apoptosis *in vitro* (231). Neuregulin 1, the endogenous ligand for HER3, can be induced by ALK inhibition. HER3 can form heterodimers with HER2 and induce activation of this latter receptor, and

promoting oncogenic bypass signaling. ALK dependent cell lines become resistance by exposure to NRG1 and this can be reverted by the combination of ALK TKI with lapatinib, a HER2 tyrosine kinase inhibitor (232).

MET amplification is one of the most common mechanisms of resistance to EGFR inhibitors in lung cancer. However, in ALK rearranged lung cancer, there is scarce information regarding this resistance mechanism, possible because crizotinib is a dual ALK and MET inhibitors. However this has been reported in a patient treated with ALK inhibitor that do not target MET like alectinib (233). In addition to *MET* amplification, activation of MET by paracrine secretion of HGF has been reported to reduce sensitivity in vitro to ALK TKI (234).

The KIT proto-oncogene receptor tyrosine kinase (*KIT*, CD117) is involved in the development of gastrointestinal stromal tumors (GIST) and it´s pathogenicity is mediated by activating mutations in the *KIT* kinase domain. It´s ligand is the stem cell factor (SCF) cytokine. *KIT* amplification and SCF overexpression were detected in a tumor from a patient with acquired resistance to crizotinib (235). H3122 cells overexpressing *KIT* were sensitive to crizotinib treatment in the absence of SCF. However, these cells showed high levels of resistance in the presence of SCF, which was reversed by treatment with the KIT inhibitor imatinib.

The potency and efficacy of second generation ALK TKIs has been observed in the clinical setting and by inducing cell death in crizotinib resistance models that do not harbor secondary kinase domain mutations nor gene amplification (189). This suggests that in the setting of modest bypass signaling activation, where oncogenic dependency is still influenced by ALK activation, full and potent inhibition of ALK can abolish the role of the bypass mechanism. However, bypass mechanisms driving resistance to second generation ALK TKIs, in the absence of secondary resistance mutation, lorlatinib does not revert resistance *in vitro* (214).

As mentioned, the activation of different phosphokinases can mediate off-target resistance, and identification of common activated pathways is necessary. It has been recently reported the activation of the SHP2 (PTPN11), can serve as a common signaling activator of the RAS/MAPK pathway in ALK bypass resistant

models (143). In addition, combined SHP2 and ALK inhibition conveyed an antiproliferative effect in ceritinib resistant cell lines, by greater suppression of ERK phosphorylation and KRAS-GTP loading in these models. SHP2 inhibitors are currently undergoing clinical developed, and might have a broad spectrum of activity to include KRAS mutant cancer among others (236).

Identifying putative bypass mechanisms of resistance can be challenging, and often requires the development of patient-derived models, but as treatment options for patients with tumors that acquire bypass mechanisms of resistance are scant, this can further guide the development of drug combinations or the inhibition of common activation pathways to tackle this problematic.

Histologic Transformation:

One of the less understood mechanisms of resistance is the shift in histologic phenotype that tumors can experience upon exposure to tyrosine kinase inhibitors in lung cancer. The main types of histologic transformation are: epithelial-mesenchymal transition (EMT), small-cell lung cancer (SCLC) transformation, and squamous cell carcinoma (SCC) transformation from originally lung adenocarcinoma tumors.

Epithelial-Mesenchymal Transition

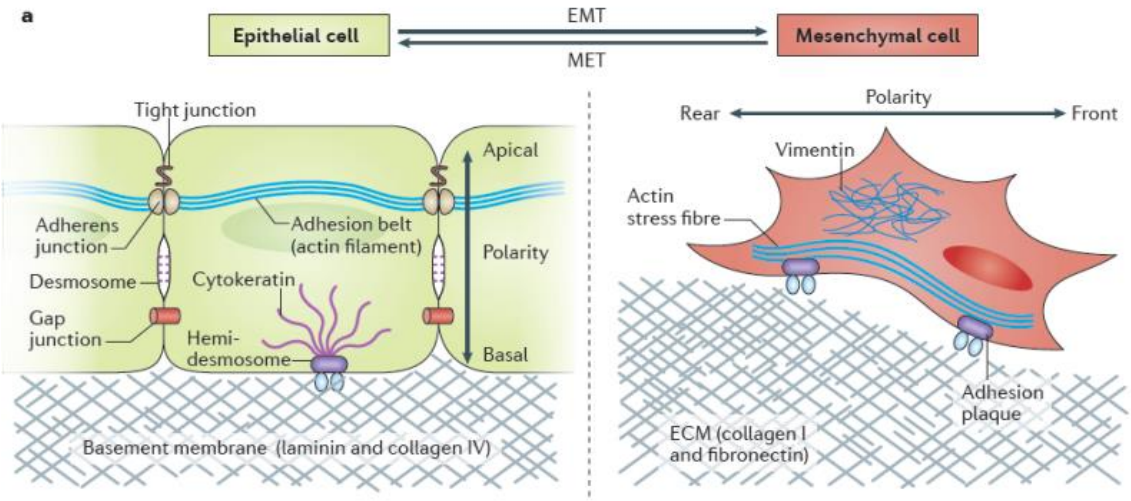
Epithelial-Mesenchymal transition is a dynamic and usually reversible process that consists in the transient acquisition of mesenchymal features from epithelial cells (237). Cells can shift from an epithelial to a partial mesenchymal or full mesenchymal state and backwards. In physiological conditions, this process is key during embryogenesis and in wound healing in adulthood. However, in cancer, EMT is involved early in the course of the disease, favorizing cell migration and metastasis and can also be induced by treatment exposure and trigger resistance to chemotherapy and tyrosine kinase inhibitors. Epithelial cells usually display apico-basal polarity and are in contact with each other

through lateral cell-cell junctions like adherent and tight junctions by multiple proteins including cadherin molecules like E-cadherin. In EMT the expression of E-cadherin in epithelial cells is repressed by transcription factors like SNAIL, SLUG, ZEB1, TWIST1/2, while N-cadherin, vimentin and fibronectin expression is induced. By this mean, tumor cells lose this apico-basal polarity and polygonal shape and induce the degradation of the basal cell membrane. In result, tumor cells acquire a mesenchymal phenotype, as they become more elongated and spread in tissue 2D cell culture, shifting their polarity to rear-front conformation acquiring higher capacity to invade and metastasize **(Figure 13)**.

Transcription factors that initiate and propagate EMT are activated through several signaling pathways including the TGFB, WNT, NOTCH, SRC, AXL and MET (238–243). The Transforming growth factor beta (TGFB) pathway is frequently involved in the development of EMT. Upon binding of soluble TGFB to the TGFB receptor, its activation triggers the downstream phosphorylation of SMAD proteins that form SMAD complexes and induce the transcription of EMT related genes like SNAIL, SLUG, TWIST, ZEB1 that finally inhibit the expression of E-cadherin and induce the differentiation to a mesenchymal phenotype. SRC is also a key determinant of EMT by localizing to peripheral cell-substrate adhesions, regulating its disassembly through phosphorylation of focal adhesion kinase (FAK) and promoting the degradation of cell-adhesion components (244). In addition it can also suppress the function of e-cadherin (241).

EMT is an epigenetic driven process and it is not detected by DNA-based NGS. In addition, it is not diagnosed nor studied in routine clinical care, as there are no robust therapeutic strategies developed to overcome EMT-mediated resistance mechanisms. EMT can be inferred in fixed tissue biopsies by studying the expression of vimentin, n-cadherin by IHC, and by the lack of expression of E-cadherin (214). In addition, RNA sequencing can also provide significant insight on the transcriptomic level, by studying the expression of genes associated with an EMT phenotype (245). Furthermore, the disposition of actin filaments in cells in culture can be studied to infer this phenomenon. In epithelial cells, actin filaments adopt a ring structure in relationship with other cytoskeletal proteins like myosin (246). In mesenchymal cells, the presence of actin stress

fibers is characteristic and plays a role in the invasiveness and migration of these cells (247).



Tight junctions, adherens junctions, gap junctions, and desmosomes	**Cell–cell junctions**	Minimal
• Actin filaments are aligned with adherens junctions (adhesion belt) • Cytokeratin intermediate filaments	**Cytoskeleton**	• Actin stress fibres • Vimentin intermediate filaments
Via integrin $\alpha_6\beta_4$ at hemidesmosomes; linked to cytokeratins	**Interactions with ECM**	Via β_1-containing or β_3-containing integrins at adhesion plaques; linked to actin stress fibres
Apical–basal polarity	**Polarity**	Front–rear polarity

Figure 13. Morphological changes associated with epithelial-mesenchymal transition (EMT). *A Morphology of epithelial cells with apico-basal polarity, E-cadherin expression and cell to cell adhesion, and intact basal membrane. B Activation of the EMT program induces loss of cell-cell junctions, loss of E-cadherin and induction of vimentin expression, actin stress fibers, front-rear polarity and disruption of the extracellular matrix (ECM), and cell polarity. Figure and legend adapted from Shibue et al. Nature Reviews Clinical Oncology* (248)

However, even after identifying which patients experience disease progression by EMT, targeting this complex biologic process is difficult. Depending on the mechanism of induction of EMT, resistance to TKIs can be potentially reversed *in vitro* by combining the TKI targeting the primary oncogenic driver and a second drug targeting the EMT pathway activation. This has been reported in cell lines harboring EGFR activating mutations, resistant to EGFR TKI by SRC activation, where dual inhibition using EGFR TKI and dasatinib (a SRC inhibitor) could overcome resistance (249). HDAC inhibitors have also shown to

restore E-cadherin expression in vitro and to revert resistance in ALK-rearranged models resistant to crizotinib (250,251). In addition, the induction of EMT through TGFBR in EGFR mutant cells can be prevented in vitro by MEK inhibition (252). Nevertheless, this has not translated into clinical development, and there is an unmet need to design effective strategies to diagnose and target EMT as a resistance mechanism to targeted therapies in lung cancer.

In ALK-rearranges lung cancer, the onset of EMT seems to be frequently involved ALK TKI resistance. Phenotypical characteristic of mesenchymal differentiation have been reported in about 40% of tissue samples from twelve ceritinib resistant tumors with IHC staining showing loss of E-cadherin and gain of vimentin expression (214). In addition, EMT has been reported to mediate resistance to crizotinib in H3122 cells. In this study, the induction of miR-200c expression by HDAC inhibitors restored sensitivity to crizotinib by EMT reversion (251). This was validated in mouse models, were pretreatment with the HDAC inhibitor quisinostat resensitized EMT tumors to ALK inhibition with crizotinib and alectinib. This shows that EMT is a potential targetable resistance mechanism in ALK-driven lung cancers.

Small-Cell Lung Cancer and Squamous Cell Carcinoma transformation

Histological transformation from non-small cell lung cancer, most commonly lung adenocarcinoma, to the high grade neuroendocrine small-cell lung cancer (SCLC) phenotype has been extensively reported at resistance to TKIs in lung cancer, mainly in resistance to EGFR targeted therapies (70,253,254). Next-generation sequencing in samples from EGFR-mutant tumors that underwent SCLC reveal that tumors show biallelic inactivation of RB1 and TP53, and it might be a predisposing factor to develop SCLC transformation when detected previous to TKI treatment (255). Clonal evolution studies revealed that SCLC transformed cells are present at early phases of the disease and can emerge as a consequence of selective pressure of the TKI over NSCLC cells sensitive to the inhibitor. In a SCLC phenotype, cancer cells loose EGFR dependency and the treatment of patients is the same than for primary SCLC patients, with platinum and etoposide combined regimens.

The prognosis of patients in this scenario is dismal, with median overall survival durations from the time of SCLC transformation were reported to be about 11 months (255,256). It is unclear in these cases, whether maintaining the treatment with a TKI in addition to chemotherapy may confer greater benefit for patients and is mainly driven by the lack of effective SCLC transformation preclinical models.

SCLC transformation has been scantly reported in the setting of resistance to ALK inhibitors in lung cancer, and it seems to be a rare event. Single cases of histologic transformation to ALK inhibitors have been reported including small cell lung cancer transformation in the context of treatment with crizotinib, ceritinib, alectinib and lorlatinib (257–260)

Transformation from lung adenocarcinoma to squamous cell carcinoma histology has been recently reported as a mechanisms of resistance to EGFR and ALK TKIs in lung cancer (257,260,261). Squamous cell differentiation has been reported at resistance to alectinib in a single case (257). The biological bases of resistance to TKI in this context is still unknown and together with the incidence of SCLC transformation in ALK TKI resistant tumors.

Other mechanisms of resistance

There are miscellaneous mechanisms of resistance to TKIs that include apoptotic defects, epigenetic, metabolic o tumor microenvironment alterations. Genomic polymorphisms in the pro-apoptotic effector BIM, specifically by an intronic deletion that confers alternative splicing and skipping of the pro-apoptotic BCL2-homology domain 3 (BH3), confers intrinsic resistance *in vitro* to EGFR TKI by impairing apoptosis, but could be reverted with BH3 mimetic drugs (262). However, in a retrospective study of patients treated with first generation EGFR TKIs, carriers of BIM polymorphisms (15% of the population) had similar response rates and clinical outcomes than controls, suggesting that the clinical impact of this polymorphism needs further validation (263).

A common denominator of acquired resistance to TKI is the fact that it is driven by a group of cancer cells that persist in the presence of drug, even when impressive clinical responses can be achieved with these agents. Several research groups have been focused on studying the adaptive mechanisms of “persister” cells, a small subpopulation of tumor cells (<5%) that remain alive and are reprogrammed into a drug-tolerant state in the presence of a TKI in cell culture (264–268). Initial studies have suggested that epigenetic reprogramming of TKI-persister cells involves the histone demethylase KDM5A and thus could be selectively targeted by histone deacetylase inhibitors (264). Persister cells can later proliferate by the acquisition of resistance mechanisms, such as secondary mutations or activation of bypass signaling (265,267). Persister cells display a defective apoptotic response to TKI, and treatment with inhibitors of the BCL-2 family anti-apoptotic proteins is a potentially effective therapeutic strategy. In addition, recent evidence suggests that persister cells have specific dependency on the lipid hydroperoxidase GPX4 (268,269). These epigenetic, apoptotic and metabolic mechanisms involved in drug tolerant persister states contribute to maintain this quiescent state that later can result in the acquisition of secondary mutations and the emergence of bypass mechanisms of resistance and EMT.

The interaction between tumor cells and other cell types that conform the tumor microenvironment can condition the response to TKI therapy and trigger biological mechanisms of resistance (234,270,271). Co-culture of EGFR-mutant cells with cancer-associated fibroblasts (CAFs) can induce EMT by the secretion of multiple paracrine-acting factors including HGF and AXL. Similarly, the secretion of EGF, TGFα, other factors by endothelial cell induce EGFR bypass track activation and resistance to ALK inhibitors.

In addition to intrinsic mechanisms involving cancer cells, drug pharmacokinetic properties may also influence response and progression to treatment and should be considered. As previously mentioned, the ABC family of transporter proteins, including the MDR1 transporter (p-glycoprotein), can confer resistance to chemotherapy drugs and kinase inhibitors, mainly by affecting the bioavailability of the drug by efflux. This is especially relevant in the blood-brain barrier, where high levels of p-glycoprotein can affect drug concentrations in the CNS. In addition, overexpression of p-glycoprotein was reported to confer

resistance to ceritinib and crizotinib in ALK-rearranged lung cancer patients, but could be overcome in vivo by combining ALK TKIs with p-glycoprotein inhibitors (197). Novel ALK inhibitors like alectinib and lorlatinib are not substrates of this transporters.

In summary, there are distinct biologic mechanisms that drive resistance to first- and second-generation ALK inhibitors in tumors harboring ALK rearrangements. Most importantly, the high incidence of kinase domain mutations causing resistance to second generation ALK TKIs, including the solvent front G1202R mutation has prompted the development of the third-generation ALK inhibitor lorlatinib. This inhibitor can target all single kinase domain mutations including G1202R and is the last effective ALK inhibitory strategy available in patients with ALK rearranged lung cancer.

4. Lorlatinib, drug development and current evidence on resistance mechanism to the third generation ALK TKI.

4A. Lorlatinib is a potent ALK TKI designed to overcome resistance to first- and second-generation inhibitors.

Lorlatinib (PF-06463922, Pfizer) is the most novel and the sole third generation ALK inhibitor currently available in the clinical setting for the treatment of patients. Lorlatinib is a potent, reversible and ATP competitive ALK and ROS1 inhibitor, designed to inhibit ALK in the presence of all known single resistant kinase domain mutations (272). In biochemical assays, lorlatinib inhibits the catalytic activity of ALK with mean Ki of <0.07 nM. In addition to the potency of lorlatinib, its macrocyclic structure, which is unique compared to all other ALK inhibitors, confers different conformational binding properties to the ALK kinase domain, remaining unaffected by aminoacidic changes that occur due to single known secondary resistance mutations **(Figure 14)** (273). In addition, the lipophilic properties of lorlatinib and its low susceptibility to P-glycoprotein efflux, confers high levels of CNS penetration and intracranial activity.

In preclinical studies, lorlatinib showed elevated potency in ALK suppression and cell death in Ba/F3 cells expressing wild-type ALK and mutant forms. This included the wide spectrum of resistance mutations that occur with first- and second-generation ALK inhibitors: L1196M, I1171T, L1152R, 1151Tins, C1156Y, G1269A, F1174L, S1206Y and the solvent front mutation G1202R (272). Compared to other first and second-generation ALK TKI, lorlatinib was also a more potent ALK inhibitor in its non-mutant form. In addition, lorlatinib showed significant activity in G1202R mutant H3122 cells *in vivo*. The IC50 of lorlatinib in Ba/F3 cells expressing the EML4-ALK rearrangement with the G1202R mutation is about 50 nanomol/L, and the IC50 values for all other single mutations range from this value down to 4.6 nanomol/L. Lorlatinib also yielded important tumor

responses in brain orthotopic mice models and prolonged survival of mice bearing patient-derived tumors (272). In these models, pharmacokinetic analysis showed that the free fraction of lorlatinib in the brain in reference to plasma was 4-fold higher with lorlatinib compared to crizotinib.

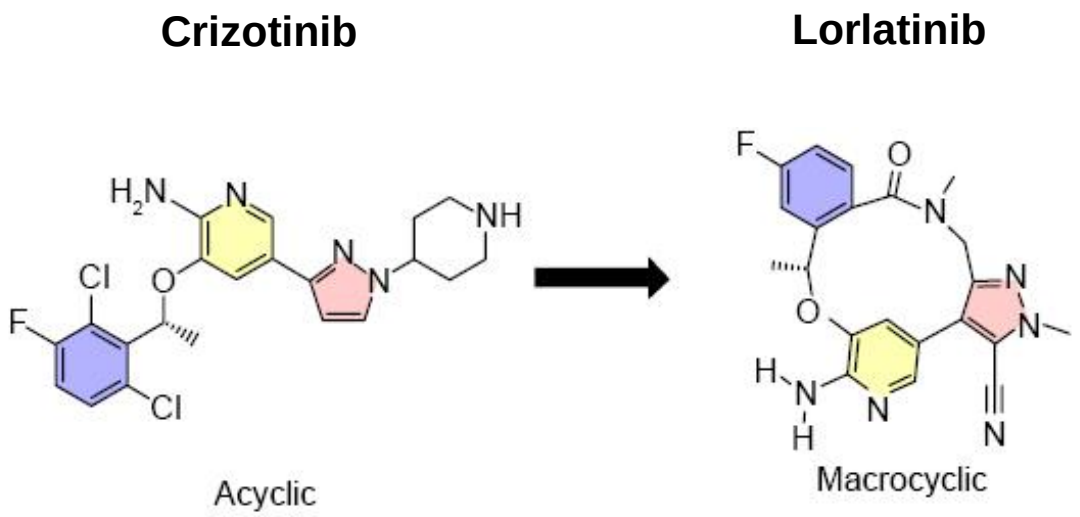


Figure 14, Structure of crizotinib (acyclic) and lorlatinib (macrocyclic). *Lorlatinib was developed from crizotinib using a structure-based drug design approach to overcome ALK mutant resistance and high P-gp efflux Figure and legend adapted from Akamine et al. OncoTarget and Therapy 2018* (274)

Lorlatinib is orally bioavailable and the established dose is 100mg/ daily based on the safety and the estimated plasma concentration needed to target the G1202R mutation. The time to maximum plasma concentration is between 1-2 hours and its half-life extends from 19 to 28.8 hours (63). The phase I trial enrolled 41 patients with heavily pretreated ALK-rearranged NSCLC, among which 72% of patients had brain metastasis at the time of enrollment, 12 of whom had not been treated with radiation therapy. The mean lorlatinib cerebral-spinal fluid (CSF) concentrations to plasma was 0.75, correspond to 75% of unbound plasma concentrations, proving high levels of blood brain barrier penetration.

In this study, the response rate with lorlatinib was 46%, 57% among patients treated with one previous line of ALK TKI and 42% amongst patients

treated with two or more lines of ALK TKIs. In patients with measurable and non-measurable brain metastasis, a partial or complete response was observed in 31% of cases, of whom 50% had been previously treated with two or more lines of ALK inhibitors, including second-generation ALK TKIs. The efficacy of lorlatinib in the treatment of brain metastasis is relevant in the context of elevated levels of CNS progression with first- and second-generation ALT TKI.

The multicohort phase II study of lorlatinib included patients with treatment naïve disease (EXP1, N = 30), patients that had receive crizotinib without a second generation ALK TKI (EXP2, N = 27) or with previous chemotherapy (EXP3A, N = 32), patients who had received one non-crizotinib ALK TKI with or without chemotherapy (EXP3B, N = 28), and patients that had previously received two (EXP4, N = 66) or three (EXP5, N = 46) lines of ALK TKI. About 60-70% of patients previously treated with an ALK TKI had baseline brain metastasis. The response, progression free survival and CNS activity for all cohorts are depicted in **Table 8**. In only crizotinib pre-treated patients, the ORR was 69% and the median PFS was not reached with the lower limit of the confidence interval reaching almost one year, and intracranial responses were observed in 87% of cases. In patient treated with second generation ALK TKI alone and in patient previously treated with two or three ALK TKIs, response rates ranged between 32-39% with median PFS durations were 5.5 months and 6.9 months. Enhanced intracranial activity was observed in heavily pretreated patients, with ~55% of intracranial responses and a median duration of response reaching 14·5 months (95% CI, 6·9–14·5). In addition, the phase II study also provided a hint on the activity of this drug in treatment naïve patients, with 90% response rates and long progression free survival durations. In accordance with the shift in the treatment paradigm for ALK positive patients of moving second generation ALK TKIs in the first line setting, there is an ongoing phase III study comparing first line treatment with lorlatinib to crizotinib (CROWN trial, NCT03052608). In preclinical models, first line treatment with lorlatinib was highly efficacious.

Cohort	Previous ALK TKI	N	ORR	Median PFS months(95% CI)	IC ORR
EXP 1	None	30	90%	NR (11·4–NR)	66.7%
EXP 2	Crizotinib no chemotherapy	27	69.5%	NR (12·5–NR)	87%
EXP 3A	Crizotinib and chemotherapy	32			
EXP 3B	Second-generation ALK TKI +/- chemotherapy	28	32.1%	5·5 (2·7–9·0)	55.6%
EXP 4	Two lines of ALK TKI	66	38.7%	6·9 (5·4–9·5)	53.1%
EXP 5	Three lines ALK TKI	46			

Table 8. Expansion (EXP) cohorts from the phase I/II study of lorlatinib. *ORR: overall response rate; PFS: progression free survival; IC: intracranial.* (173)

Lorlatinib was highly active in patients with detectable *ALK* resistant mutations to earlier generation ALK inhibitors but was also active in patients with undetectable kinase domain mutations. Among patient previously treated with crizotinib there were no differences in response rates among patients with detectable ALK resistance mutations compared to patients without detectable resistance mutations (ORR: 73% versus 75%) and median PFS was similar between groups [HR, 1.03 (95% CI, 0.39 to 2.69)] (275). This is concordant with responses observed with second generation ALK TKI after progression to crizotinib, showing that crizotinib resistant tumors still may have high ALK dependency in the absence of resistance mutations, probably due to weak bypass activation (214). In contrast, after progression to second generation ALK TKI, the response rate with lorlatinib in patients with detectable ALK resistance mutations was 62% compared to 32% for patients with undetectable ALK resistance mutations in plasma NGS. Similar results were observed when NGS was performed in tissue samples (69% versus 27%). In addition, median PFS was significantly longer in patient with detectable *ALK* mutations in tissue (11 versus 5.3 months), as was median duration of response (24.4 versus 4.3

months), reflecting that patients with undetectable on-target resistance to previous ALK TKI have a lesser benefit with lorlatinib than patients whose tumors develop on-target resistance mechanisms. Importantly, in the clinical setting, lorlatinib was highly effective in patients with detectable solvent front *ALK* G1202R/del mutations, with response rates of 57% and median duration of PFS of 8.2 months (275).

Based on the clinical efficacy and safety, lorlatinib received approval from the United States Food and Drug administration (FDA) in November 2018 and the European Medicine Agency (EMA) in May 2019 for the treatment of patients who have experienced disease progression with crizotinib and a second-generation ALK TKI, or to first-line treatment with alectinib or ceritinib. However, like with other targeted agents, resistance to lorlatinib invariably leads to disease progression in patients. In the frontier of ALK targeted treatments, the understanding of the biologic mechanisms driving lorlatinib resistance are crucial to develop strategies to prevent and overcome resistance to lorlatinib, and to provide patients with new effective treatment options.

4B. Current preclinical and clinical evidence on resistance mechanisms to lorlatinib treatment

Unlike crizotinib and the second-generation ALK TKI, mechanisms of resistance to lorlatinib still need to be intensively explored. The first report on resistance to lorlatinib was derived from the molecular study of a patient´s tumor at the time of lorlatinib progression in the context of the phase I study by Shaw AT, Friboulet L. and colleagues at the Mass General Hospital (276). The patient had been treated with crizotinib in the first line setting, and upon progression to this drug a resistant *ALK* C1156Y mutation was detected. The C1156Y mutation confers resistance to crizotinib and ceritinib. The patient was treated sequentially with ceritinib and a HSP90 inhibitor without benefit and fourth line chemotherapy with a total duration of response of 6 months. The patient then received lorlatinib and achieved a partial response lasting for 8 months. At the time of disease progression, a liver biopsy was performed and NGS analysis of the tumor sample revealed two *ALK* mutations, the previously crizotinib resistant C1156Y and a new L1198F mutation. The two mutations were present in the same allele (compound mutation), and clonal analysis using whole exome sequencing data showed that the cancer cells containing the compound mutation at lorlatinib resistance were subclones derived from tumor cells that had acquired the C1156Y mutation with crizotinib. In crystallography modelling of ALK, the substitution of a leucine for a phenylalanine in position 1198 leads to a steric clash with lorlatinib, affecting the resulting in unfavorable binding. The binding affinity, the ALK L1198F and L1198F+C1156Y mutant ALK was lower with lorlatinib and most second generation ALK inhibitors. Interestingly, the L1198F mutation does not clash with crizotinib, and in fact improves crizotinib binding, leading to increased affinity for ALK. The patient was treated with crizotinib, experiencing a significant response and proving that this compound mutation resensitized this cancer to crizotinib.

In the context of this compound mutation, the increase affinity for crizotinib binding, induced by the presence of the phenylalanine counteracted the negative

effect of C1156Y. The induction of resistance to lorlatinib and the resensitization to crizotinib by the sequential acquisition of the C1156Y and the L1198F mutation was also demonstrated *in vitro* in Ba/F3 models. This study was highly relevant because it revealed for the first time that compound mutations in ALK could drive resistance to lorlatinib, and that the presence of the L1198F mutation in this context could be targeted with crizotinib. Compound mutations acquired sequentially with first and second generation ALK TKI had also been reported to drive resistance to brigatinib, like the D1203N + E1210K, but these compound mutations remain sensitive to lorlatinib inhibition (214). These initial observations suggested that compound mutations had differential activity on resistance to ALK TKI.

The same research group further explored the role of compound mutations in lorlatinib resistance by performing an ENU mutagenesis screen in Ba/F3 cells expressing non-mutant EML4-ALK rearrangements and Ba/F3 harboring EML4-ALK rearrangement with known single resistance mutations to first- or second generation ALK TKIs. After exposure to ENU, Ba/F3 cells were treated with crizotinib and lorlatinib. In EML4-ALK non-mutant cells, there was a lack of resistant clones arising to lorlatinib treatment, but as expected, multiple clones emerged with crizotinib. This suggested that no single *ALK* mutation conferred resistance to lorlatinib at physiological doses achieved in patients. This was further proved in vivo, by implanting H3122 cells in mice and treating them with lorlatinib. After tumor regrowth, none of the resistance cancer cells harbored a single ALK mutation.

To study the effect of the sequential acquisition of resistance mutations, Ba/F3 harboring the common resistance mutations to first- and second-generation TKIs (C1156Y, F1174C, L1196M, G1202R, and G1269A) underwent ENU mutagenesis screen with lorlatinib. Multiple compound mutations were identified in lorlatinib resistant clones**.** A functional validation was done by developing Ba/F3 cell models harboring EML4-ALK and the following compound mutations G1202R+L1196M, G1202R+L1198F and L1196M+L1198F. In concordance, these cells and were highly resistant to lorlatinib. In hand with previous findings, compound mutations containing the L1198F mutation were sensitive to crizotinib. In patients, the following compound mutations were

identified at lorlatinib resistance: I1171N + L1198F, I1171N + D1203N, G1202R + G1269A, G1202R + L1196M; including to “triple mutant” G1202R + L1204V + G1269A and E1210K + D1203N + G1269A. This study further demonstrates that the consecutive acquisition of kinase domain mutations after exposure to crizotinib and/or second generation ALK TKI and lorlatinib can induce conformational changes in the kinase domain that hinder lorlatinib binding and can also result in increased kinase ATP affinity in about 35% of cases.

In a subsequent study by Okada and colleagues, the authors again performed an ENU mutagenesis screen on G1202R and I1171N mutant EML4-ALK Ba/F3 cells, and showed similar findings (216). In total, 13 ALK compound mutations involving G1202R and I1171N were identified including a novel compound mutation that caused lorlatinib resistance but remained targetable with alectinib (L1256F). This group also identified a G1202R + G1269A compound mutation in patient derived cell line resistant to lorlatinib. It is clear with both studies, that ENU mutagenesis screen is a useful tool to predict potential mutations to lorlatinib, but even when most compound mutations will cause resistance to all available ALK inhibitors, in few selected cases, resistance can be overcome with an earlier generation ALK TKI.

Off-target mechanisms of resistance in ALK rearranged NSCLC cell lines have been characterized *in vitro* by exposing commercially available H3122 and H2228 cell to increasing lorlatinib concentrations. The lorlatinib resistant cell lines, showed overactivation of EGFR as a bypass mechanism to ALK inhibition *in vitro* (277). This has been previously shown for crizotinib in H3122 cell lines, suggesting that EGFR activation might be a recurring mechanism of resistance in this cell line (278). In neuroblastoma cell lines harboring full length ALK with R1275Q mutation (CLB-GA) exposed to lorlatinib, resistant clones harbored a truncating mutation in NF1 emerged. Combined treatment with trametinib and lorlatinib could overcome resistance in this *in vitro* model. To date, there is lack of information regarding the type of bypass mechanisms of resistance with lorlatinib treatment from patients. From the largest reported series of patients with lorlatinib resistance, 65% of tumor samples did not harbor compound mutations that could explain resistance, suggesting that bypass mechanisms or histologic

transformation could cause resistance in a significant proportion of patients. (279). A recent case report of neuroendocrine transformation at the time of resistance to lorlatinib with a concomitant L1196M mutation, demonstrates that histologic transformation, like in EGFR mutant lung cancer, can occur independently of acquired resistance mutations, and that relying solely on liquid biopsies without tissue analysis can lead to underdiagnoses of histological transformations.

In summary, molecular targeted therapies in lung cancer are consider a standard and prioritized treatment option for patients with tumors harboring sensitizing molecular alterations. ALK-rearrangements confer constitutive activation of the ALK kinase domain, leading to cancer initiation and propagation. Effective ALK tyrosine kinase inhibitors have been developed to target ALK-rearranged lung cancer cells and constitute the main treatment for these patients. Mechanisms of resistance arise during the treatment with ALK TKI, mainly by the acquisition of secondary kinase domain mutations or ALK amplification, the emergence of bypass mechanisms and histologic transformation. Lorlatinib, is a third generation ALK TKI, capable of overcoming resistance mediated by single kinase domain mutations including the G1202R mutation. Lorlatinib is currently approved for the treatment of patients with ALK-rearranged lung cancer who have previously progressed on treatment with crizotinib and a second generation ALK TKI or a second generation ALK TKI given upfront. There is scarce scientific evidence on the biological mechanisms of resistance to this compound in the clinical setting. To date, only the sequential acquisition of specific compound mutations has been shown to confer resistance to lorlatinib. During my PhD thesis, we studied novel biological mechanisms of resistance to lorlatinib occurring in patients treated at the Institut Gustave Roussy

Part II. Results

1. Resistance mechanisms to the third-generation ALK inhibitor lorlatinib in ALK-rearranged lung cancer

1A. Presentation and objectives

Treatment with lorlatinib after disease progression with first- and second-generation ALK tyrosine kinase inhibitors for patients with ALK rearranged NSCL is effective. Nevertheless, even when clinical benefit can be observed with this drug, cancer cells invariably develop resistance to lorlatinib, leading to cancer proliferation and disease progression. Besides the emergence of compound mutations causing resistance to lorlatinib, the spectrum of biologic mechanisms that drive resistance to lorlatinib in patients with ALK rearranged lung cancer remains unknown. In this scenario, through longitudinal tumor and plasma sampling from patients experiencing disease progression to lorlatinib and other ALK inhibitors, we aimed to study and elucidate the biologic mechanisms of resistance to lorlatinib. In the context of the multidisciplinary and institutional MATCH-R study at Institut Gustave Roussy, we developed patient derived models from patient tumor samples at the time of resistance to lorlatinib. Simultaneously, targeted next-generation sequencing, whole exome sequencing and RNA sequencing were performed on lorlatinib resistant biopsies, and the results of the genomic and transcriptomic analysis were integrated with the study and development of *in vitro* and *in vivo* patient-derived tumor models. The primary objective of our work was to unravel the mechanism of resistance to lorlatinib using this translational approach, including the acquisition of novel ALK genomic alterations, the emergence of unknown bypass mechanisms of resistance and the role of histologic transformation; and to develop novel therapeutic strategies to overcome resistance to lorlatinib.

1B. The MATCH-R trial: systematic and integrated study of resistance to targeted therapies and immunotherapy

The MATCH-R trial (NCT02517892) is a prospective, single institution study held at Gustave Roussy Cancer Campus. The study aim is to characterize molecular mechanisms of resistance to targeted therapies and immunotherapies across different tumor types. Patients who experienced a partial or complete response or stable disease as best response for at least 6 months with a targeted therapy or immunotherapy and are candidates to undergo a tumor biopsy are eligible to participate in the study. A tumor biopsy of the most representative and accessible progressive lesion is performed. Targeted, whole exome and RNA sequencing are performed in the tumor samples upon resistance and, if available, on pre-treatment biopsies. In addition to providing tissue for NGS, selected tumor samples are processed to establish patient-derived xenografts (PDX) and cell lines.

Between January 1st 2015 and as of June 15th 2018, a total of 333 patients were included and 303 patients underwent a tumor biopsy. In total, 163 tumors from patients were engrafted into immune-deficient mice, and 54 patient-derived models were established, with a global success rate of 33%. In total, 18 PDX models derive from lung cancer specimens. The complete feasibility study of the MATCH-R trial is displayed in **Annex #1**.

Among the 303 patients who underwent a successful tumor biopsy, 14 patients had diagnosis of an ALK rearranged cancer and had experienced disease progression with an ALK TKI. Among the 14 biopsies obtained, 10 underwent complete molecular testing, and 8 corresponded to ALK-rearranged lung cancers. Four patients in this group had disease progression while on treatment with lorlatinib. Four patient-derived cell lines were established from 3 patients treated with lorlatinib. At the time of thesis submission, the biological mechanisms of resistance to lorlatinib were identified in three out of the four cases.

1C. Epithelial-mesenchymal transition mediates lorlatinib resistance through SRC activation

A patient-derived xenograft and cell line was developed from a patient (MR57) with ALK rearranged lung cancer, who had been with lorlatinib for 7 months. Tumor NGS confirmed the presence of an EML4-ALK variant 3 rearrangement and reported two ALK kinase domain mutations *in cis*: ALK C1156Y and G1269A. The derived cell line was initially sensitive to lorlatinib. After treatment with incremental lorlatinib concentrations, a resistant cell line was established, acquiring a mesenchymal phenotype.

We developed Ba/F3 cells harboring the compound C1156Y+G1269A, validating that this compound mutation did not confer resistance to lorlatinib. We hypothesized that MR57 resistant (MR57-R) cells had undergone epithelial-mesenchymal transition in the presence of lorlatinib, conveying high levels of resistance. MR57-R cells lacked E-cadherin expression and expressed vimentin, N-cadherin and the EMT-promoting transcription factor SNAIL, while MR57 sensitive (MR57-S) cells maintained an epithelial phenotype. MR57-R cells had sustained AKT/S6 and ERK phosphorylation at high lorlatinib concentrations. We also derived a second cell line from another patient (MR210), that was resistant to lorlatinib, and with evidence of EMT features in the tumor biopsy.

We identified the SRC inhibitor saracatinib (AZD0530) as a potent hit by multi-drug screen in both cell lines. We further confirmed that MR57-R mesenchymal cells showed high levels of SRC activation, and that dual ALK and SRC inhibition induced cell death in mesenchymal cells, inhibiting downstream effectors dependent on SRC signaling and resulting in enhanced apoptosis. In addition to a direct cytotoxic effect by kinase inhibition, we further hypothesized that this lethal effect could be due to EMT reversal from a mesenchymal to an epithelial “sensitive” state. Long-term exposure of mesenchymal cells to saracatinib, resulted in a mild re-expression of E-cadherin and partial EMT reversal.

In summary, resistance to lorlatinib mediated by SRC-driven EMT can be overcome by SRC inhibitors with lorlatinib in vitro.

1D. Characterization of novel ALK compound mutations at lorlatinib resistance

We characterized a novel ALK compound mutation (G1202R + F1174L) observed in a tumor biopsy of a patient at the time of lorlatinib resistance. The patient had received treatment with crizotinib in the first line, and after disease progression, she underwent a tumor biopsy showing the presence of an ALK G1202R mutation and a novel ALK E1154K variant (MR144). The patient continued to receive treatment with the second generation ALK TKIs ceritinib and brigatinib, and after a rapid progression, the G1202R mutation was solely detected at higher allelic fractions. The treatment was switched to lorlatinib, and the patient experienced a rapid response followed by a short interval of response, with disease progression at 4 months.

Tumor and plasma samples at the time of resistance evidenced an ALK G1202R+F1174L compound mutation, and several polyclonal ALK mutations were detected with plasma NGS. Using TOPO-TA cloning from the patient´s tumor RNA/DNA, we noticed that the G1202R and E1154K mutations observed at crizotinib resistance occurred in separate alleles and the G1202R and F11174L observed at lorlatinib resistance were present in the same allele. This was further supported by clonal evolution analysis using whole exome sequencing. Ba/F3 cells harboring F1174L+G1202R mutations showed a mild shift towards resistance to lorlatinib compared to G1202R mutant cells. Comparative immunoblotting analysis showed that G1202R+F1174L mutant Ba/F3 cells had higher baseline ALK phosphorylation levels compared to single mutant or non-mutant EML4-ALK cells, suggesting that this compound mutation could increase the kinase ATP affinity, impacting the efficacy of lorlatinib.

In a second case (MR347), the ALK compound mutations L1196M/D1203N emerged after treatment with crizotinib and ceritinib, and Ba/F3 cell models of these mutations showed high levels of lorlatinib resistance, in concordance with previous reports of compound mutations that impede drug binding to the kinase domain (216,279). Altogether, the differential effect of the compound mutations on lorlatinib resistance shows that the biologic implications of these mutations can be heterogeneous, while some retain sensitivity to lorlatinib (like C1156Y/G1269A), other can confer resistance by different mechanisms.

1E. NF2 loss of function mediates resistance to lorlatinib and can be overcome with mTOR inhibition

In a third case, a patient with ALK-rearranged lung cancer treated with lorlatinib experienced disease progression in a single lung metastasis after an initial clinical response with lorlatinib. A tumor biopsy was performed, and a lorlatinib-resistant patient derived cell line was developed (MR135-R1). NGS of the tumor sample showed two deleterious events in *NF2*, a non-sense mutation (S288*) and a pathogenic 9 base pair exon 10 skipping, secondary to an intronic splicing site mutation (NM_000268,3:c.886-1G>A). The patient derived cell line only harbored the splicing site mutation, with no evidence of the wild-type allele in cDNA sequencing, suggesting loss of heterozygosity. The patient underwent treatment with stereotactic radiation therapy to the oligoprogressive site and continued treatment with lorlatinib and, after 8 months on treatment, systemic disease progression occurred. A second tumor biopsy from the adrenal gland was done, and a second lorlatinib resistance cell line was established (MR135-R2). NGS from the tumor sample again showed the inframe 9 base pair *NF2* exon 10 skipping and a new pathogenic *NF2* K543N mutation. Both mutations were detected in MR135-R2 cells, again in a pattern compatible with loss of heterozygosity. Both cell lines harbored an EML4-ALK variant 3 rearrangement and no ALK resistance mutations were detected. In a 66-compound drug screen, the dual mTORC1 and mTORC2 inhibitor vistusertib (AZD2014) showed potent inhibition alone and a mild additive effect when combined with lorlatinib. This effect was further validated with the rapamycin analogue everolimus. The cytotoxic effect of vistusertib was reproduced in MR135-R2 cell. mTOR inhibition alone and in combination with lorlatinib induced cell death, enhancing apoptosis in MR135-R1 cells, confirming the downstream activation of mTOR as the mechanisms of lorlatinib resistance. We validated this by treating immunodeficient mice engrafted with MR135-R2 resistant cells, showing a synergistic effect of the mTOR and ALK combination in tumor growth suppression. We hypothesized that NF2 loss of function mutations impaired mTOR inhibition by merlin. To validate this, we performed NF2 knockout using CRISPR-CAS9 gene editing in H3122 cells (EML4-ALK variant 1 cell line). NF2 knockout resulted in lack of merlin expression and resistance to lorlatinib in vitro. We demonstrated that NF2 knock out cells maintained high levels of S6 phosphorylation even in the presence of high concentrations of lorlatinib and effective ALK inhibition. Hence, we demonstrated that deleterious NF2 mutations can drive lorlatinib resistance through mTOR

overactivation, constituting a novel bypass mechanism of resistance to lorlatinib. In addition, our results suggest that mTOR inhibition can resensitize NF2 deficient cells to ALK inhibition.

1D. Conclusions

The study of mechanisms of resistance to lorlatinib is central in the current context of ALK treatment strategies, in which lorlatinib has become the last line of available ALK kinase inhibition for patients with ALK-rearranged lung cancer. Through the longitudinal study of lorlatinib resistance within the MATCH-R trial, by developing patient derived models and performing next-generation sequencing, we have studied the biological process driving resistance to lorlatinib and designed novel treatment strategies to overcome it.

In our study, we characterized the role of SRC-mediated EMT in lorlatinib resistance in vitro and we showed that combined SRC and ALK inhibition can induce cell death in mesenchymal cells. By this mean, we provided new treatment strategies to target EMT. This is highly relevant in current context, as EMT features have been found in about 40% of tumors at resistance to second generation ALK TKI in a small cohort (214). EMT is difficult to target as it can involve the activation of multiple and various oncogenic pathways. In addition to targeted therapies, EMT can drive resistance to different anticancer agents like chemotherapy and immunotherapy (280,281). However, in this study, the identification of SRC as the main kinase driving EMT mediated resistance to lorlatinib, led to successful targeting of EMT cells by SRC and ALK inhibition.

Secondly, we studied the functional role of two novel compound mutations observed at the time of lorlatinib resistance. In the case of MR57, EML4-ALK Ba/F3 cells harboring the C1156Y+G1269A compound mutation remained sensitive to lorlatinib and brigatinib inhibition, proving that not all ALK compound mutations mediate resistance to lorlatinib, emphasizing the need of in vitro characterization of these mutations. In addition, we studied the biologic effect of the G1202R+F1174L compound mutations, confirming the effect on lorlatinib resistance, but also showing that higher concentrations of lorlatinib can convey effective ALK inhibition. In addition, we characterized a novel highly resistant compound mutation L1196M+D1203N mutation.

To date there are no next-generation inhibitors to overcome resistance to lorlatinib in the presence of compound mutations. The sole exceptions where earlier generation ALK TKI can overcome resistance to lorlatinib is in the context of compound mutations harboring the F1198L mutation, sensitive to crizotinib, or the L1256F, sensitive to alectinib.

Thirdly, in our study, we reported the acquisition of NF2 deleterious mutations as a novel bypass mechanism of resistance to lorlatinib occurring in a patient. This is the first report of a bypass mechanism causing resistance to lorlatinib. We demonstrated that NF2 loss of function resulted in overactivation of mTOR and that dual inhibition of mTOR and ALK could overcome resistance in vivo. Identifying and characterizing novel mechanisms of resistance to lorlatinib is necessary to develop combinatorial strategies. Our work provides additional evidence of the role of mTOR inhibition in tumors with NF2 alterations.

In conclusion, in the cohort of patients with ALK-rearranged lung cancer experiencing disease progression with lorlatinib in the MATCH-R study, the mechanisms of resistance are diverse and varied. Combination strategies can overcome resistance in vitro in the context of bypass mechanisms of resistance and SRC activation and EMT induction. With this study we hope to provide further preclinical evidence to support the design of new treatment strategies.

2. Article 1: "Diverse resistance mechanisms to the third-generation ALK inhibitor lorlatinib in ALK-rearranged lung cancer" Clinical Cancer Research, considered with revisions (May 2019).

Diverse resistance mechanisms to the third-generation ALK inhibitor lorlatinib in ALK-rearranged lung cancer

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Running Title: Resistance to lorlatinib in ALK-rearranged lung cancer

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Statement of significance

Diverse resistance mechanisms were identified using next-generation sequencing and cell lines established from patients with ALK-rearranged NSCLC treated with lorlatinib. These mechanisms include epithelial-mesenchymal transition (EMT) susceptible to combined ALK/SRC inhibition, ALK compound mutations, and a novel bypass mechanism, mediated by NF2 loss and overcome by mTOR inhibition. This study provides further evidence on the complexity of lorlatinib resistance and new treatment strategies to overcome resistance in selected scenarios.

Conflict of interest statement:

L.M. Consulting, advisory role: Roche Diagnostics. Lectures and educational activities: Bristol-Myers Squibb, Tecnofarma, Roche, AstraZeneca. Travel, Accommodations, Expenses: Chugai.

D.P. Consulting, advisory role or lectures: AstraZeneca, Bristol-Myers Squibb, Boehringer Ingelheim, Celgene, Daiichi Sankyo, Eli Lilly, Merck, MedImmune, Novartis, Pfizer, prIME Oncology, Peer CME, Roche. Honoraria: AstraZeneca, Bristol-Myers Squibb, Boehringer Ingelheim, Celgene, Eli Lilly, Merck, Novartis, Pfizer, prIME Oncology, Peer CME, Roche. Clinical trials research as principal or co-investigator (Institutional financial interests): AstraZeneca, Bristol-Myers Squibb, Boehringer Ingelheim, Eli Lilly, Merck, Novartis, Pfizer, Roche, Medimmun, Sanofi-Aventis, Taiho Pharma, Novocure, Daiichi Sanky. Travel, Accommodations, Expenses: AstraZeneca, Roche, Novartis, prIME Oncology, Pfizer

A.G. Received travel accommodations, congress registration expenses from Boehringer Ingelheim, Novartis, Pfizer, Roche. Consultant/Expert role for Novartis.Principal/sub-Investigator of Clinical Trials for Aduro Biotech, Agios Pharmaceuticals, Amgen, Argen-X Bvba, Arno Therapeutics, Astex Pharmaceuticals, Astra Zeneca, Aveo, Bayer Healthcare Ag, Bbb Technologies Bv, Beigene, Bioalliance Pharma, Biontech Ag, Blueprint Medicines, Boehringer Ingelheim, Bristol Myers Squibb, Ca, Celgene Corporation, Chugai Pharmaceutical Co., Clovis Oncology, Daiichi Sankyo, Debiopharm S.A., Eisai, Exelixis, Forma, Gamamabs, Genentech, Inc., Gilead Sciences, Inc, Glaxosmithkline, Glenmark Pharmaceuticals, H3 Biomedicine, Inc, Hoffmann La Roche Ag, Incyte Corporation, Innate Pharma, Iris Servier, Janssen , Kura Oncology, Kyowa Kirin Pharm, Lilly, Loxo Oncology, Lytix Biopharma As, Medimmune, Menarini Ricerche, Merck Sharp & Dohme Chibret, Merrimack Pharmaceuticals, Merus, Millennium Pharmaceuticals, Nanobiotix, Nektar Therapeutics, Novartis Pharma, Octimet Oncology Nv, Oncoethix, Oncomed, Oncopeptides, Onyx Therapeutics, Orion Pharma, Oryzon Genomics, Pfizer, Pharma Mar, Pierre Fabre , Rigontec Gmbh, Roche, Sanofi Aventis, Sierra Oncology, Taiho Pharma, Tesaro, Inc, Tioma Therapeutics, Inc., Xencor. Research Grants from Astrazeneca, BMS, Boehringer Ingelheim, Janssen Cilag, Merck, Novartis, Pfizer, Roche, Sanofi. Non-financial support (drug supplied) from Astrazeneca, Bayer, BMS, Boringher Ingelheim, Johnson & Johnson, Lilly, Medimmune, Merck, NH TherAGuiX, Pfizer, Roche

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A.H. Consultant/Advisory role for Amgen, Spectrum Pharmaceuticals, Lilly. Invitations to national or international congresses from Servier, Amgen, Lilly Courses, trainings for Bayer.

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E.A. Consulting or Advisory Role: Merck Sharp & Dohme, GlaxoSmithKline, Celgene Research, MedImmune. Travel, Accommodations, Expenses: AbbVie, Roche, Sanofi, Pfizer, MedImmune. Principal/sub-Investigator of Clinical Trials (Inst.) for Abbvie, Aduro, Agios, Amgen, Argen-x, Astex, AstraZeneca, Aveo pharmaceuticals, Bayer, Beigene, Blueprint, BMS, Boeringer Ingelheim, Celgene, Chugai, Clovis, Daiichi Sankyo, Debiopharm, Eisai, Eos, Exelixis, Forma, Gamamabs, Genentech, Gortec, GSK, H3 biomedecine, Incyte, Innate Pharma, Janssen, Kura Oncology, Kyowa, Lilly, Loxo, Lysarc, Lytix Biopharma, Medimmune, Menarini, Merus, MSD, Nanobiotix, Nektar Therapeutics, Novartis, Octimet, Oncoethix, Oncopeptides AB, Orion, Pfizer, Pharmamar, Pierre Fabre, Roche, Sanofi, Servier, Sierra Oncology, Taiho, Takeda, Tesaro, Xencor.

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D.S. Full-time employee of Life Chemicals Inc.

All other authors declare no competing interests

Abstract

Purpose: Lorlatinib is a third-generation ALK tyrosine kinase inhibitor with proven efficacy in patients with ALK-rearranged lung cancer previously treated with first and second-generation ALK inhibitors. Beside compound mutations in the *ALK* kinase domain, other resistance mechanisms driving lorlatinib resistance remain unknown. We aimed to characterize mechanisms of resistance to lorlatinib occurring in patients with *ALK*-rearranged lung cancer and design new therapeutic strategies in this setting.

Experimental Design: Resistance mechanisms were investigated in five patients resistant to lorlatinib. Longitudinal tumor biopsies were studied using high-throughput next-generation sequencing. Patient-derived models were developed to characterize the acquired resistance mechanisms and Ba/F3 cell mutants were generated to study the effect of novel *ALK* compound mutations. Drug combinatory strategies were evaluated *in vitro* and *in vivo* to overcome lorlatinib resistance.

Results: Divers biological mechanism leading to lorlatinib resistance were identified. Epithelial-mesenchymal transition (EMT) mediated resistance in two patient-derived cell lines and was susceptible to dual SRC and ALK inhibition. We characterized three *ALK* kinase domain compound mutations occurring in patients, L1196M/D1203N, F1174L/G1202R and C1156Y/G1269A, with differential susceptibility to ALK inhibition by lorlatinib. We identified a novel by-pass mechanism of resistance caused by *NF2* loss of function mutations, conferring sensitivity to treatment with mTOR inhibitors.

Conclusion: This study shows that mechanisms of resistance to lorlatinib are diverse and complex, requiring new therapeutic strategies to tailor treatment upon disease progression.

Introduction

The anaplastic lymphoma kinase (ALK) is a member of the family of insulin-like tyrosine kinase receptors involved in the oncogenesis of several tumor types (1). *ALK* gene rearrangements occur in 3-6% of lung adenocarcinoma (2,3). Patients diagnosed with *ALK*-rearranged lung cancer benefit from treatment with ALK tyrosine kinase inhibitors (TKI) (4).

Lorlatinib is a potent third-generation ALK inhibitor able to overcome resistance to first and second generation ALK inhibitors, including those mediated by the G1202R mutation and has marked activity on brain metastasis (5). Clinical responses with lorlatinib were observed in 39% of patients previously treated with two or more ALK inhibitors and median PFS was 6.9 months (6,7). Nevertheless, as with first and second generation ALK inhibitors, resistance to lorlatinib treatment invariably occurs.

The spectrum of biological mechanisms driving lorlatinib resistance in patients remains to be elucidated. It has been recently reported that the sequential acquisition of two or more mutations in the *ALK* kinase domain (KD), also referred as compound mutations, is responsible for disease progression in about 35% of patients treated with lorlatinib, mainly by impairing its binding to the ALK kinase domain (8).

Herein we report the *in vitro* characterization of three resistance mechanisms detected in patients with *ALK*-rearranged lung cancer on lorlatinib, included in the prospective MATCH-R study (NCT02517892). These mechanisms include the occurrence of epithelial-mesenchymal transition (EMT) susceptible to combined ALK/SRC inhibition (patient MR57 and MR210), the acquisition of a novel compound mutation (G1202R/F1174L in MR144) and the pre-existing L1196M/D1203N (MR347) as well as NF2-loss of function mediated resistance overcome by mTOR inhibitors (MR135)

Materials and Methods

MATCH-R clinical trial

The MATCH-R study is a prospective single-institution trial running at Gustave Roussy Cancer Campus (Villejuif, France), aiming to identify mechanisms of resistance to targeted therapies in patients with advanced cancer (NCT02517892). Patients that achieved a partial or complete response, or stability of disease for at least six months with selected targeted agents were included in the study and underwent serial tumor biopsies. Extensive molecular tumor profiling was performed by panel targeted next-generation sequencing (NGS) (Ion torrent), whole exome sequencing (WES) and RNA sequencing (Illumina; Integragen) as previously described (9). For WES, mean coverage was 140X.

Development of patient-derived xenografts (PDX) in mice and in vivo pharmacological studies

All animal procedures and studies were performed in accordance with the approved guidelines for animal experimentation by the ethics committee at University Paris Sud (CEEA 26, Project 2014_055_2790) following EU regulation. Fresh tumor fragments from the patients MR57, MR135, MR144, MR210 and MR347 were implanted in the subrenal capsule of 6-week-old female NOD scid gamma (NSG) or nude mice obtained from Charles River Laboratories.

Cell lines

Patient-derived cell lines (MR57-S, MR57-R, MR135-R1, MR135-R2, MR210) were developed from PDX samples by enzymatic digestion with a tumor dissociation kit (Ref.130-095-929, Miltenyi Biotec) and mechanic degradation with the gentleMACs™ dissociator.

Cells were cultured with DMEM/F-12+GlutamMAX™ 10% FBS and 10% enriched with hydrocortisone 0.4 µg/ml, cholera toxin 8,4 ng/ml, adenine 24 µg/ml and ROCK inhibitor 5 µM (Y-27632, S1049 Selleckchem) until a stable proliferation of tumor cells was observed, as previously described (10). Culture media was then transitioned to DMEM and cultured in the presence of lorlatinib from 300 nM to 1 µM. The H3122 cell line harboring EML4-ALK rearrangement was cultured in RPMI 10% FBS. Parental Ba/F3 cells were purchased from DSMZ and cultured in DMEM 10% FBS in the presence of IL-3 (0.5 ng/ml). Ba/F3 cells were infected with lentiviral constructs as previously reported to express the EML4-ALK variant 3 fusion with or without ALK kinase domain mutations (11). Ba/F3 cells harboring the *EML4-ALK* fusion were selected in the presence of blasticidine (21 µg/ml) and IL-3 (0.5 ng/ml) until recovery, and a second selection by culturing the cells in the absence of IL-3. *EML4-ALK* rearrangement and *ALK* kinase domain mutations or *NF2* mutations were confirmed on the established cell lines by Sanger sequencing.

CRISPR-based NF2 knocking out

NF2 gene knock-out was performed with the CRISPR/Cas9 KO Plasmid (h) from Santa Cruz Biotechnology (sc-400504). CRISPR/Cas9 KO Plasmid (h) was transfected using Lipofectamine 3000 according to manufacturer's protocol. Green fluorescent protein-based cell sorting was performed for clonal selection. Single clones were screened for *NF2* gene disruption by RT-PCR followed by sequencing and Western Blot.

Site directed mutagenesis

Lentiviral vectors expressing the EML4-ALK variant 3 were created using the pLenti6/V5 directional TOPO cloning kit (#K495510, Thermofisher) according to manufacturer's

instructions. Point mutations were introduced using the QuickChange XL Site-Directed mutagenesis kit (#200516, Agilent) according to manufacturer's protocol using the following primers:

G1269A F- GAGTGGCCAAGATTGCAGACTTCGGGATGGCC

G1269A R- GGCCATCCCGAAGTCTGCAATCTTGGCCACTC,

C1156Y-F GACGCTGCCTGAAGTGTACTCTGAACAGGACGAAC,

C1156Y R- GTTCGTCCTGTTCAGAGTACACTTCAGGCAGCGTC,

E1154K F- CTGTGAAGACGCTGCCTAAAGTGTGCTCTGAACAG,

E1154K R- CTGTTCAGAGCACACTTTAGGCAGCGTCTTCACAG,

F1174L F- TGTTCTGGTGGTTTAATTTGCTGATGATCAGGGCTTCC,

F1174L R- GGAAGCCCTGATCATCAGCAAATTAAACCACCAGAACA,

G1202R F- GCTCATGGCGGGGAGAGACCTCAAGTCC,

G1202R R-GCTCATGGCGGGGAGAGACCTCAAGTCC.

D1203N F- ATGGCGGGGGGAAACCTCAAGTCCTTCC

D1203N R- GGAAGGACTTGAGGTTTCCCCCCGCCAT

L1196M F- GCCCCGGTTCATCCTGATGGAGCTCATGGCGGG

L1196M R- CCCGCCATGAGCTCCATCAGGATGAACCGGGGC

Reagents

Saracatinib (AZD0530) and vistusertib (AZD2014) were provided by AstraZeneca. Crizotinib (S1068), alectinib (S2762), brigatinib (S8229), dasatinib (S1021), erdafitinib (S8401), debio-1347 (S7665), lorlatinib (S7536) and entrectinib (S7998) were purchased from Selleck Chemicals.

For Western Blot assays the antibodies used were: pALK Y1282/1283 (9687S), pALK Y1604 (3341S), ALK (#3333S), pAKT (#4060S), AKT (#4961S), pERK (9101S), ERK (9102S), pS6 (4858S), S6 (2217S), cleaved Parp (9541S), BIM (2933S), Merlin (1288S), pPaxillin (2541S), Paxillin (2542S), Snail (3879S) and Vimentin (5741S) purchased from Cell Signaling Technology.

For IHC assays the antibodies used were ALK (#6679072001), E-Cadh (#790-4497) and CD31 (#760-4378) purchased from Ventana; N-Cadh (#M3613), Ki-67 (#M7240), beta catenin (#M3539), podoplanin (#M3619) and CD68 (#M0814) purchased from DAKO; Vimentin (#790-2917) purchased from Roche; pSRC (#6943S) and pMAPK (#4376) purchased from Cell Signaling Technology; Glut1 (#RP128-05) purchased from Clinisciences; CA-IX (#NB100-417SS) purchased from NovusBio, NF2/Merlin purchased from Sigma-aldrich (#HPA003097) and CD47 (#M5792) purchased from Spring.

Cell Viability and Apoptosis Assays

Cell viability assays were performed in 96-well plates using the Cell-Titer Glo Luminescent Cell Viability Assay (G7570, Promega). Apoptosis was measured using the caspase-Glo 3/7 Assay (G8091, Promega).

In vivo pharmacological studies

MR135-R2 PDX bearing athymic nude mice were treated with vistusertib (qdx1 then bidx1 then qdx1);4d off, Lorlatinib (qdx5/2d off) or their combination by oral gavage. Vistusertib was resuspended in 1% Tween80 in sterile deionized water and lorlatinib in sterile deionized water pH 3.0.

Circulating tumor DNA (ctDNA) analysis from patient's blood samples

A total of 20 ml of blood were collected in Streck BCT (Streck) or EDTA tubes and processed for DNA extraction. Molecular analysis from ctDNA was performed by Inivata (Cambridge, UK and Research Triangle Park, USA) using amplicon-based NGS (InVisionFirstTM-Lung) as previously reported (12) .

Actin microfilament staining with phalloidin

MR210, MR57-S and MR57-R cells were fixed in formaldehyde and permeabilized with PBS Triton X-100 (0.05%). Blocking solution with FBS 2% and BSA 1% was used. Alexa Fluor 488 Phalloidin (8878S, Cell Signaling) solution was diluted 1/200 in blocking buffer. Cells were incubated for one hour at room temperature, then washed with PBS and later incubated with DAPI 1/10.000 dilution for five minutes. Cells were imaged with an inverted IX73 microscope (Olympus).

Allelic distribution of ALK mutations

The *ALK* kinase domain was amplified by PCR and amplicons were subcloned into pCR2.1-TOPO vector (Invitrogen) according to manufacturer's protocol. Individual cDNA was sequenced by Sanger sequencing to determine the *cis/trans* status of mutations.

Modeling Tumor Clonal Evolution

Paired-end RNA sequencing (RNA-seq) data for MR144 sequential biopsies was mapped against the human genome version "hg19" through Burrows-Wheeler Aligner (BWA-MEM) (13). The resulting Sequence Alignment Map (SAM) was converted into binary version BAM files. PCR duplicates identified in BAM files were removed with "samtools fixmate". Realign Target Creator, and realigner of GATK were used to check and realign the sorted BAM files with predefined BED files for indels. The GATK-Base Recalibrator was used to generate tables for user-specified covariates and GATK-MuTect2 was used to calculate Variant Allelic Frequency (VAF). Computed VAFs of different time-points were adjusted according to tumor cell percentages and subjected to R-SciClone clustering analysis (14). The phylogeny of subclonal tumor evolution was determined using R-clonevol (15) and visualised with R-fishplot (16).

Computational modelling of ALK

All molecules for reconstruction and analysis of human ALK-kinases were taken from RCSB Protein Data Bank (PDB) and information obtained from UniProtKB database (17, 18). Full 3D-models of ALK-domains were built using I-TASSER server(19). Structure and assembling of polypeptide chains were analyzed using data of SCOP database (20). The secondary

structure of ALK-domain was verified based on self-optimized prediction method with alignment (SOPMA). Also, BioLuminate (Schrödinger) was used as a method for evaluating the role of amino acid mutations (21, 22). Geometry optimization and stability of reconstructed models were predicted based on results of molecular dynamics (MD) simulations. MD simulations were performed in an aqueous environment, using CHARMM force field and GROMACS 5.1.4 program package (23, 24). Each protein was solvated, optimized (10,000 steps steepest descent/conjugant gradient algorithms), equilibrated (30,000 steps) and relaxed during a free MD in water environment (50 ns). Lorlatinib topology was generated with online SwissParam tool(25). MD results were evaluated by RMSD, values of conformational energies and radius of gyration. Assessment of the amino acid composition, visualization and structure analysis were performed in PyMOL and BIOVIA DS Visualizer. CCDC GOLD 5.2.2 suite (www.ccdc.cam.ac.uk) was used for final exhaustive docking of hit compounds. The major part of docking options was turned on by default, however ChemScore function, which relies on the internal energy calculation, was altered to ASP algorithm(26). We kept GoldScore function as a primary function, as it provides best conformational search analysis. https://www.lifechemicals.com

Results

Resistance mechanisms to ALK TKI from MATCH-R clinical trial

From January 2015 to January 2019, 14 patients with *ALK*-rearranged tumors progressing on ALK TKI were included in the MATCH-R study. Four patients were excluded from the analysis due to inadequate biopsies for molecular profiling (Figure 1).

Among the eight patients with *ALK*-rearranged lung adenocarcinoma, tumor biopsies were obtained upon progression to crizotinib (n=1), ceritinib (n=3) and lorlatinib (n=4) (Table 1). NGS analysis of tumor biopsies from patients treated with crizotinib and ceritinib revealed the presence of secondary *ALK* kinase domain mutations in three cases (G1269A, L1196M/D1203N, and F1174L) and a *NOTCH1* variant of unknown significance in one additional case (Table 1). The ceritinib resistant patient with the compound mutation L1196M/D1203N (MR347) experienced primary resistance to lorlatinib and is therefore characterized here as an additional lorlatinib resistance mechanism. Among the four patients with *ALK*-rearranged lung cancer with acquired resistance to lorlatinib, *ALK* compound mutations were observed in two cases (C1156Y/G1269A for patient MR57 and G1202R/F1174L for patient MR144). Off-target mutations in *NF2* were encountered in two different temporo-spatial biopsies from patient MR135 obtained while on treatment with lorlatinib. The first biopsy was from an oligo-progressive lung lesion after 7 months of lorlatinib treatment that was treated with stereotactic radiation, and the second biopsy was obtained at the time of systemic progression from an adrenal metastasis after additional 8 months of treatment with lorlatinib. A single *ALK* C1156Y kinase domain mutation was found in one patient (MR210) after progression to lorlatinib, without evidence of additional genetic alterations. The *ALK* C1156Y mutation is known to confer resistance to crizotinib and

ceritinib, but remains sensitive to lorlatinib, as previously reported in preclinical studies (5). Thus, the C1156Y mutation is not likely to be responsible for lorlatinib resistance in this case. Patient-derived cell lines were developed from patients MR57, MR135 and MR210. Biological processes driving tumor resistance to lorlatinib were further explored using patient-derived cell lines.

Figure 1

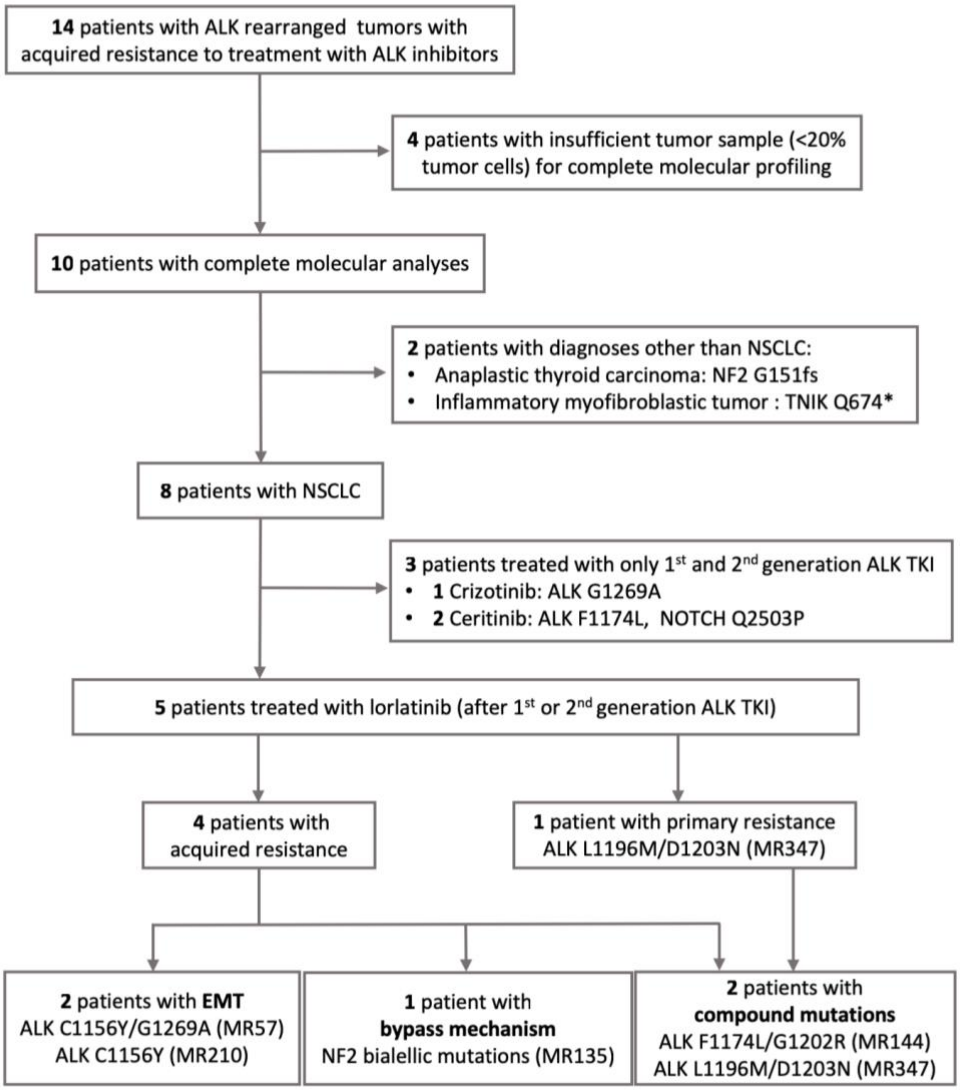

Figure 1. *Summary of ALK-rearranged patient included in the MATCH-R study. NSCLC: non-small cell lung cancer, EMT: epithelial mesenchymal transition*

ID	Diagnosis	Previous ALK TKI	NGS at progression to previous line of ALK TKI	Line of ALK TKI inclusion	ALK TKI MATCH-R inclusion	Response (RECIST) PFS	Targeted sequencing	Whole exome sequencing / RNA sequencing	Putative Resistance Mechanism
MR 39	LUAD	Crizotinib	No	2	**Ceritinib**	PR 14 months	No detectable alterations	*NOTCH1*:p.Q2503P	Unknown
MR 57	LUAD	Crizotinib	No	2	**Lorlatinib**	PR 7 months	*ALK*: p.C1156Y+p.G1269A	*ALK*: p.C1156Y+p.G1269A	EMT
MR 135	LUAD	Crizotinib	*NF2* c.8861G>A *NF2* S288X	2	**Lorlatinib**	PR 15 months	*PTPN11*: p.S502L TP53 p.R273P	*NF2*: p.K543N *NF2* c.886-1G>A	NF2 bypass
MR 143	ATC	No	NAP	1	**Crizotinib**	SD 5 months	TP53: p.E285*	TNIK: p.Q674*	Unknown
MR 144	LUAD	Crizotinib	ALK E1154K / G1202R	4	**Lorlatinib**	PR 4 months	*ALK*: p.G1202R+p.F1174L	*ALK*: p.G1202R+p.F1174L	*ALK*: p.G1202R+p.F1174L
		Ceritinib	N/A						
		Brigatinib	G1202R						
MR 154	MIT	Crizotinib	No	2	**Ceritinib**	SD 26 months	No detectable alterations	*NF2*: p.G151fs	NF2 bypass
MR 176	LUAD	No	N/A	1	**Crizotinib**	PR 30 months	No detectable alterations	*ALK*: p.G1269A	*ALK*: p.G1269A
MR 210	LUAD	Crizotinib Ceritinib	No	3	**Lorlatinib**	PR 16 months	*ALK*: p.C1156Y	*ALK*: p.C1156Y	EMT
MR 344	LUAD	Crizotinib	No	2	**Ceritinib**	PR 4 months	*ALK*: p.F1174L	*ALK*: p.F1174L; PIK3CB: p.E1051K	*ALK*: p.F1174L
MR 347	LUAD	Crizotinib	*ALK*: p.L1196M	2	**Ceritinib**	PR 5 months	ctDNA *ALK*: p.L1196M/D1203N	*ALK*: p.L1196M	*ALK*: p.L1196M/D1203N
				3	**Lorlatinib**	PD	N/A	N/A	N/A

Table 1. *Clinical and molecular features of patients with tumor molecular profiling on biopsies obtained upon resistance to ALK inhibitors in the MATCH-R study. TKI: tyrosine kinase inhibitor, PFS: progression-free survival, LUAD: lung adenocarcinoma, ATC: anaplastic thyroid carcinoma, MIT: myofibroblastic inflammatory tumor*

Epithelial-mesenchymal transition mediates lorlatinib resistance

A 59-year-old male was diagnosed with a metastatic *ALK* rearranged lung adenocarcinoma (Figure 2A). The patient received first-line treatment crizotinib achieving a partial response and a progression-free survival (PFS) of 4.2 months. At the time of disease progression to crizotinib, no tumor nor plasma was available. The patient received sequential second line treatment with lorlatinib at 75 mg daily achieving a partial response (-78% per RECIST criteria). After 6.9 months, disease progression was observed, the patient was

included in the MATCH-R trial (MR57) and a lung biopsy on the progressing primary site was performed.

Targeted NGS, WES and RNA sequencing showed the presence of both C1156Y and G1269A *ALK* mutations and the *EML4-ALK* variant 3 rearrangement (V3). cDNA Topo-TA cloning and sequencing of the *ALK* kinase domain, evidenced that both mutations were present in the same allele (compound mutation).

A PDX model was established directly from a biopsy and a cell line (MR57-S) was derived from the PDX, with a total elapsed time from the tumor biopsy to cell line establishment of 6 months. Cell survival assays showed that the patient derived cell line was sensitive to lorlatinib treatment (MR57-S), with an IC50 of 50 nM, suggesting that the C1156Y/G1269A compound mutation was not likely responsible for lorlatinib resistance (Supplementary Figure 1A). It remains to be elucidated if lorlatinib withdrawal during the time of PDX development and cell line establishment could have influenced the observed sensitivity of the MR57-S cell line. To further study the effect of this *ALK* compound mutation on ALK inhibitors sensitivity, we developed Ba/F3 engineered cells to express the EML4-ALK V3 with G1269A, C1156Y or compound C1156Y/G1269A mutations. Ba/F3 cells expressing EML4-ALK with the compound mutations were less sensitive to lorlatinib (IC50: 53 nM) than Ba/F3 cell expressing the C1156Y (IC50: 2.5 nM) or G1269A (IC50: 18 nM) single mutations (Supplementary Figure 1B). However, the doses required to induce cell death in these models were within the range of lorlatinib sensitivity, being lower than those required to target the G1202R mutation, known to be susceptible to lorlatinib inhibition in patients (5,6). The C1156Y/G1269A compound mutation conferred resistance to crizotinib, alectinib and entrectinib but not to brigatinib when tested *in vitro* (Supplementary Figure 1C).

The MR57-S cell line was exposed to incremental concentrations of lorlatinib until the tumor cells developed resistance, achieving stable growth at a dose of 300 nM. The MR57 resistant (MR57-R) cell line showed high levels of resistance to lorlatinib (IC50: 7.8 µM) (Supplementary Figure 1A). Sequencing of the ALK kinase domain in both MR57-S and MR57-R cells showed the presence of the C1156Y and G1269A mutations. MR57-R cells did not acquire any additional *ALK* kinase domain mutations during exposure to lorlatinib.

Immunoblot analysis of MR57 sensitive (MR57-S) and resistant (MR57-R) cells treated with incremental doses of lorlatinib showed that ALK inhibition resulted in inhibition of ERK, AKT and S6 phosphorylation and induction of apoptosis in MR57-S cells (Figure 2B). In contrast, MR57-R cells maintained high levels of ERK, AKT and S6 phosphorylation, with lower levels of apoptosis. This is in line with the occurrence of an off-target mechanism of resistance (*i.e.* the activation of a bypass track).

Because MR57-S and MR57-R cells had markedly different morphologies, we assessed the differential expression of EMT markers. Immunoblot analysis revealed that MR57-S cells expressed high levels of E-cadherin and lacked N-cadherin and vimentin, characteristic of an epithelial phenotype. In contrast, MR57-R cells lacked E-cadherin expression and had high levels of N-cadherin, Snail and vimentin expression, characteristic features of a mesenchymal phenotype (Figure 2B). RNA sequencing of the two cell lines confirmed the differential expression of EMT related genes at the mRNA level (Supplementary Figure 1D). Comparably, MR57-R cells had higher levels of vimentin, CDH-2 (N-cadherin), SNAIL, ZEB1, FGFR1 and TGFB1/2 mRNA expression and lower levels of EPCAM, CDH-1 (E-cadherin), and ICAM1 expression compared to MR57-S cells. In addition, we performed phalloidin staining of actin microfilaments on MR57-S and MR57-R cells. Lorlatinib sensitive cells manifested the formation of actin rings and proliferation in clusters,

distinctive of an epithelial phenotype (Supplementary Figure 1E). In contrast, MR57-R contained actin stress fibers, which is characteristic of a mesenchymal phenotype.

To assess whether EMT features were present in the patient's tumor upon progression to lorlatinib, we compared the expression of EMT markers by immunohistochemistry (IHC) on pre-crizotinib and at the time of disease progression with lorlatinib using FFPE specimens (Supplementary Figure 1F). EMT features were not observed in the patient´s tumor specimen upon lorlatinib progression, evidenced by the expression of E-cadherin and the absence of vimentin and N-cadherin expression. Cancer cells were spatially relocated in lymphatic vessels (CD31+, Podoplanin+), in a hypoxic (Carbonic Anhydrase 9 [CAIX+], Glucose Transporter 1 [Glut1+]) and immune evading microenvironment (CD47+ and CD68 low) with sustained MAPK phosphorylation. In the absence of EMT features in the tumor biopsy, these other factors could have contributed to disease progression by limiting drug availability. Nevertheless, the onset of an EMT program upon lorlatinib exposure in patient-derived cell line supports the role of EMT in lorlatinib resistance in this model *in vitro.*

A second patient became resistance to lorlatinib without evidence of any mutation causing TKI resistance (MR210). This 58-year-old never smoker female patient with metastatic ALK-rearranged NSCLC had a benefit over four years from crizotinib treatment (Figure 2C). The treatment was switched to ceritinib due to progressing bone metastasis, but ceritinib was suspended after one cycle due to toxicity. Treatment was switched to lorlatinib, achieving a response that lasted for 16 months, when oligoprogression in a bone lesion occurred. The patient was included in the MATCH-R trial (MR210) and a tumor biopsy was performed. The patient received treatment with cryoablation to the bone metastasis and currently continues to benefit from treatment with lorlatinib, ongoing for 35 months. The MR210 cell line was directly resistant to lorlatinib and similarly to MR57 displayed EMT

features. Phalloidin staining confirmed the presence of actin stress fibers and the mesenchymal phenotype (Figure 2D).

We evaluated the expression of EMT markers by IHC on pre-crizotinib and post-lorlatinib FFPE specimens. While E-cadherin and N-cadherin expression were of similar intensity and percent positive cells among both samples, we observed an increase in vimentin expression in the post-lorlatinib specimen. This would suggest a partial EMT in the tumor at the time of resistance consistent with the observed EMT in the patient derived cell line (Supplementary Figure 1G).

Combined SRC and ALK inhibition overcome EMT mediated lorlatinib resistance

To overcome the resistance in these models, we tested 66 pharmacological compounds on MR57-R and MR210 cell lines in the presence or absence of lorlatinib. The SRC inhibitor saracatinib in combination with lorlatinib showed a potent synergistic effect on both mesenchymal cell lines (Figure 2E and F). No cytotoxic effect was observed with saracatinib on MR57-S cells with epithelial features (Supplementary Figure 1H). In concordance, a synergistic cytotoxic effect was observed in mesenchymal cells treated with dasatinib (another SRC inhibitor) and lorlatinib (Supplementary Figure 1I) and not in the epithelial cells (Supplementary Figure 1J). Interestingly, FGFR inhibitors also sensitized MR210 cells to lorlatinib treatment (and to a lower extent in MR57 - data not shown) as it has recently been shown for EGFR mutant NSCLC (Figure 2F) (17).

Immunoblot analysis showed that MR57-R mesenchymal cells had higher levels of paxillin phosphorylation (a surrogate for SRC activation), compared to the epithelial MR57-S cells, suggesting that SRC was driving EMT in this model, as previously reported (18) (Figure

2B and G). Consistently, treatment with saracatinib and lorlatinib inhibited ERK, AKT and S6 phosphorylation in MR57-R cells which translated in a mild increase in the expression of apoptosis markers such as cleaved PARP and BIM (Figure 2G).

To study if the cytotoxic effect of combining SRC and ALK inhibition could be due to a reversion of the mesenchymal state to an epithelial phenotype, we exposed MR57-R cells to 30 days of treatment with lorlatinib, saracatinib or their combination. We observed a partial reversion in E-cadherin expression in MR57-R cells treated with saracatinib (Supplementary Figure 1K). This effect was not observed when saracatinib was combined with lorlatinib. This suggests that continued exposure of MR57-R cells to lorlatinib can induce death in cells undergoing partial EMT reversal. Accordingly, we performed actin microfilament staining and observed that cells treated with saracatinib alone exhibited lower levels of actin stress fibers and increased formation of actin rings (Figure 2H), suggesting that SRC inhibition can promote a partial EMT reversal in the long-term.

Figure 2

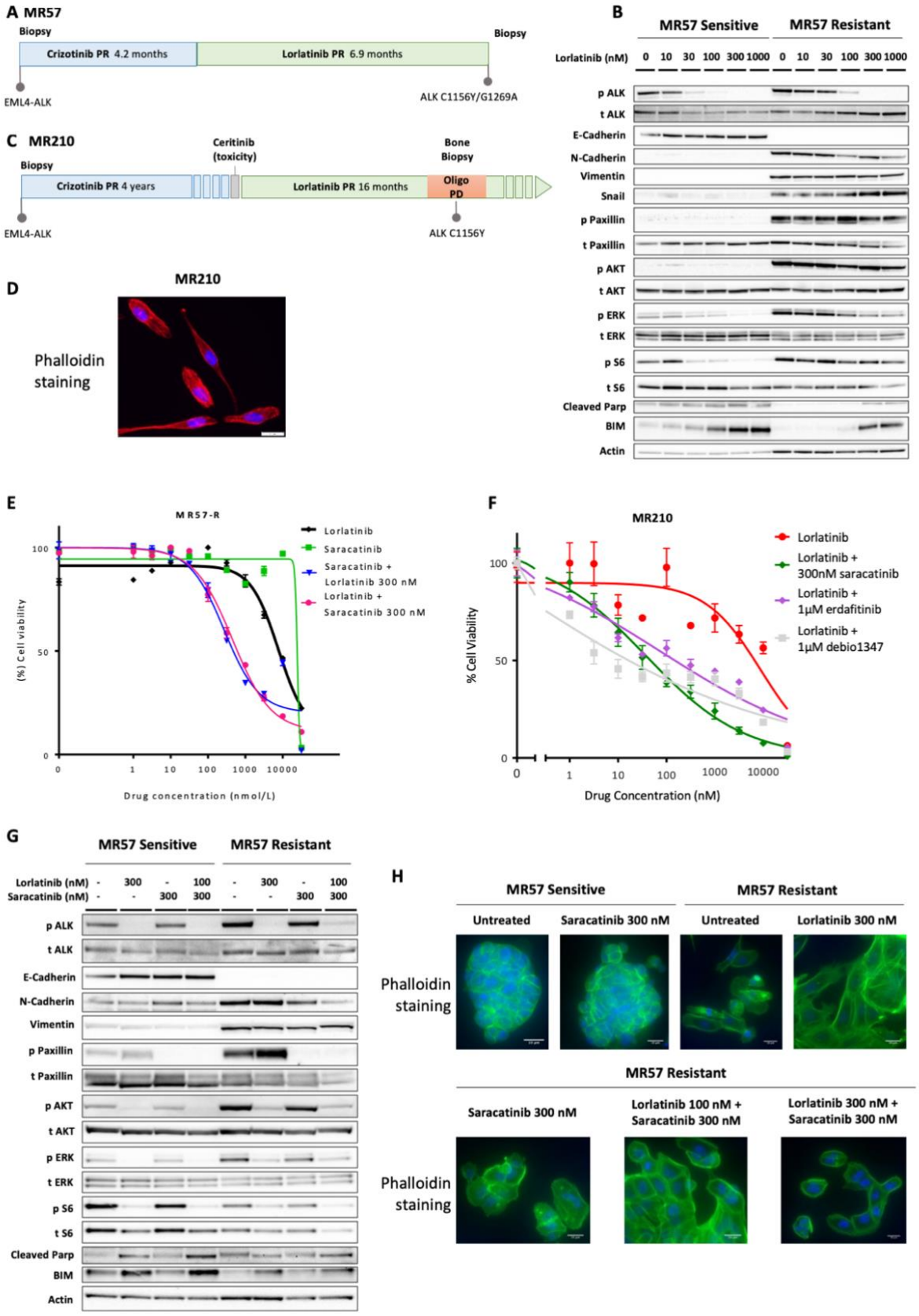
A MR57
Biopsy
Crizotinib PR 4.2 months
Lorlatinib PR 6.9 months
Biopsy
EML4-ALK
ALK C1156Y/G1269A
C MR210
Biopsy
Crizotinib PR 4 years
Ceritinib (toxicity)
Lorlatinib PR 16 months
Bone Biopsy
Oligo PD
EML4-ALK
ALK C1156Y
B
MR57 Sensitive
MR57 Resistant
Lorlatinib (nM) 0 10 30 100 300 1000 0 10 30 100 300 1000
p ALK
t ALK
E-Cadherin
N-Cadherin
Vimentin
Snail
p Paxillin
t Paxillin
p AKT
t AKT
p ERK
t ERK
p S6
t S6
Cleaved Parp
BIM
Actin
D
MR210
Phalloidin staining
E
MR57-R
Lorlatinib
Saracatinib
Saracatinib + Lorlatinib 300 nM
Lorlatinib + Saracatinib 300 nM
(%) Cell viability
Drug concentration (nmol/L)
F
MR210
Lorlatinib
Lorlatinib + 300nM saracatinib
Lorlatinib + 1µM erdafitinib
Lorlatinib + 1µM debio1347
% Cell Viability
Drug Concentration (nM)
G
MR57 Sensitive
MR57 Resistant
Lorlatinib (nM) - 300 - 100 - 300 - 100
Saracatinib (nM) - - 300 300 - - 300 300
p ALK
t ALK
E-Cadherin
N-Cadherin
Vimentin
p Paxillin
t Paxillin
p AKT
t AKT
p ERK
t ERK
p S6
t S6
Cleaved Parp
BIM
Actin
H
MR57 Sensitive
MR57 Resistant
Untreated
Saracatinib 300 nM
Untreated
Lorlatinib 300 nM
Phalloidin staining
MR57 Resistant
Saracatinib 300 nM
Lorlatinib 100 nM + Saracatinib 300 nM
Lorlatinib 300 nM + Saracatinib 300 nM
Phalloidin staining

Figure 2. *SRC and ALK inhibition overcomes lorlatinib resistance mediated by EMT.* ***A,*** *Treatment course of patient MR57 (PR, partial response).* ***B,*** *MR57-S and MR57-R cells were treated with increasing concentrations of lorlatinib for 24hs. Cell lysates were immunoblotted to detect the selected proteins.* ***C,*** *Treatment course of patient MR210 (PD, progressive disease).* ***D,*** *Phenotype of MR210 epithelial and mesenchymal cells labelled with Cy3 Phalloidin and DAPI.* ***E,*** *MR57-R cells were treated with the indicated doses of lorlatinib and saracatinib alone or in combination, for 7 days. Cell viability was assessed with Cell Titer Glo.* ***F,*** *MR210 cells were treated with single agents lorlatinib, saracatinib, erdafitinib and debio-1347 or in combination for 7 days. Cell viability was assessed with Cell Titer Glo.* ***G,*** *MR57 lorlatinib sensitive (epithelial) and resistant (mesenchymal) cells were treated with the specified concentrations of lorlatinib and saracatinib for 24hs. Cell lysates were probed with antibodies against the indicated proteins.* ***H,*** *Phenotypes of MR57 epithelial and mesenchymal cells labelled with Alexa Fluor 488 Phalloidin and DAPI after treatment with lorlatinib and saracatinib for 30 days.*

Novel lorlatinib resistant ALK compound mutations

A 58-year-old non-smoker female was diagnosed with a metastatic *ALK* rearranged lung adenocarcinoma. The patient achieved a partial response with a 9.2 months PFS on first line treatment with crizotinib (Figure 3A). At disease progression, the patient was enrolled in the MATCH-R study (MR144). RNA sequencing confirmed the EML4-ALK V3 fusion and showed the presence of the *ALK* kinase domain resistant mutation G1202R (VAF: 7%) and an unreported E1154K variant (VAF: 29%) on different alleles (Supplementary Figure 2A). Amplicon-based NGS analysis of ctDNA also detected the G1202R and a I1268V mutation,

but not the E1154K variant (Supplementary Figure 2B). Because lorlatinib was not available at that time, the patient received a short course of ceritinib treatment with rapid disease progression, and treatment was switched to brigatinib. A mixed response was observed with the occurrence of new lesions after 2.5 months of treatment. A second biopsy was performed and only the G1202R mutation was detected at a higher allelic frequency (VAF: 67%). The patient started lorlatinib treatment but the benefit lasted only 3.7 months. A third biopsy was performed, and RNA sequencing showed the presence of both, a G1202R mutation (VAF: 100%) and a F1174L mutation (VAF: 56%) confirmed to be in *cis* by TOPO-TA cloning and sequencing of ALK kinase domain (Supplementary Figure 2C). This was consistent with ctDNA sequencing which showed a rise in G1202R detection and the appearance of the F1174L mutation. Interestingly, ctDNA analysis detected four additional co-occurring *ALK* kinase mutations, not detected in the biopsy: C1156Y, G1269A, S1206F and T1151M (Supplementary Figure 2B). Solely, the G1202R/S1206F mutations were confirmed to be in the same read (*cis)* with amplicon-based NGS. C1156Y and T1151M were confirmed to be in *trans,* but due to the size of the amplicons covering the ALK kinase domain, the allelic distribution of the other mutations could not be assessed by this method. These other ALK KD mutations detected with ctDNA were not found in the sequencing analysis of the tumor biopsy, reflecting that these mutations could arise from polyclonal tumor cell sub-populations absent in the tumor biopsy.

To further characterize the clonal evolution on sequential ALK inhibitors, a FishPlot model was generated from WES compiling the three sequential patient biopsies (Figure 3B). While no *ALK* resistant mutation was detected prior to ALK TKI, multiple clones emerged at crizotinib resistance including a G1202R carrying cell population and an E1154K mutated population. Subsequent treatments with second generation ALK TKIs led to the disappearance of the E1154K population and the persistence of the G1202R carrying cells.

Finally, at disease progression on lorlatinib, we observed an enrichment of the G1202R mutated tumor cell population and the appearance of the F1174L mutation within this population. This case illustrates the tumor cell population dynamics when exposed to different generations of ALK TKI, in accordance with the previously described sequential acquisition of *ALK* kinase domain mutations *in cis* (8).

A 40-year-old male patient with metastatic *ALK*-rearranged lung cancer received crizotinib for four months (Figure 3C). The patient was included in the MATCH-R trial (MR347), and tissue and ctDNA NGS detected the *ALK* gatekeeper L1196M mutation, previously known to confer resistance to crizotinib(19). The patient received ceritinib for 5 months and a second tumor biopsy was obtained from a progressive lung lesion. Targeted NGS, WES and RNA sequencing from the tissue detected only the *ALK* L1196M mutation. ctDNA NGS further detected the presence of a solvent front D1203N mutation, present in *cis* with the L1196M, revealing a sequential development of L1196M/D1203N compound mutation. The treatment was then switched to lorlatinib but disease progression was immediately documented, proving primary resistance to lorlatinib.

Lorlatinib activity against ALK compound mutations

We generated Ba/F3 cells expressing the EML4-ALK fusion with single mutations E1154K, F1174L, G1202R, L1196M, D1203N and the G1202R/F1174L, L1196M/D1203N compound mutations. Ba/F3 cells were treated with crizotinib, alectinib, brigatinib, entrectinib and lorlatinib to test the differential effect of these mutations on the sensitivity to ALK inhibitors. The E1154K mutation did not confer resistance to any ALK TKI (Supplementary Figure 2D). Its selection on crizotinib treatment remains, therefore, to be elucidated. While F1174L mutation did not confer resistance to lorlatinib, high concentrations of lorlatinib were required to induce a cytotoxic effect on EML4-ALK$_{G1202R}$ and EML4-ALK$_{G1202R/F1174L}$

expressing cells (5). Slightly higher concentrations of lorlatinib were required to induce cell death in Ba/F3 cells expressing EML4-ALK$_{G1202R/F1174L}$ (IC50: 123 nM) compared to cells expressing EML4-ALK$_{G1202R}$ (IC50: 83 nM) (Figure 3D) which could be sufficient to confer resistance in the patient. L1196M and D1203N single mutations conferred a 10-fold shift in IC50 compared to non-mutated cells but the L1196M/D1203N compound mutation induced a more than 300-fold higher IC50 confirming the highly lorlatinib resistant feature of this novel compound mutation (Figure 3E and Supplementary Figure 2E).

To better characterize the direct impact of those compound mutations on lorlatinib efficacy, we assessed ALK phosphorylation across these models exposed to incremental concentrations of lorlatinib. In concordance with the cell viability assay, ALK phosphorylation with the compound mutation L1196M/D1203N was maintained at high doses of lorlatinib (1 µM) (Figure 3F). Interestingly, Ba/F3 cells expressing the other compound mutation G1202R/F1174L displayed higher basal levels of ALK phosphorylation compared with Ba/F3 cells expressing the single mutations or no secondary mutation (Figure 3G and Supplementary Figure 2F). Computational modelling of ALK further supports our finding. The F1174L mutation does not affect lorlatinib binding. However, in the context of the G1202R/F1174L compound mutation, a greater kinase stability is achieved, which could explain higher basal levels of ALK phosphorylation, and possibly contribute to resistance in this case (Figure 3H).

Figure 3

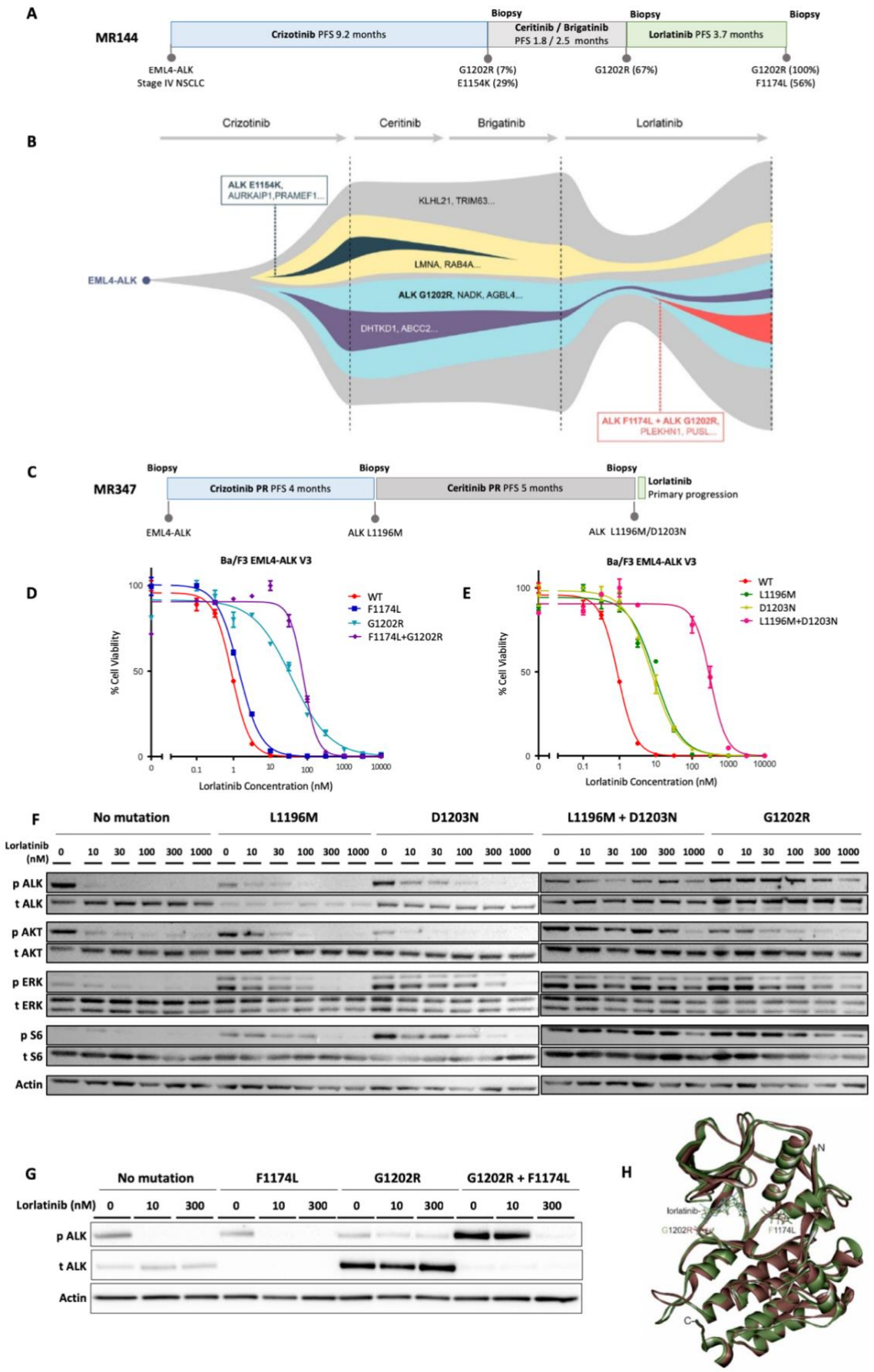
A
MR144
Crizotinib PFS 9.2 months
Biopsy
Ceritinib / Brigatinib
PFS 1.8 / 2.5 months
Biopsy
Lorlatinib PFS 3.7 months
Biopsy
EML4-ALK
Stage IV NSCLC
G1202R (7%)
E1154K (29%)
G1202R (67%)
G1202R (100%)
F1174L (56%)
B
Crizotinib
Ceritinib
Brigatinib
Lorlatinib
ALK E1154K, AURKAIP1,PRAMEF1...
KLHL21, TRIM63...
LMNA, RAB4A...
EML4-ALK
ALK G1202R, NADK, AGBL4...
DHTKD1, ABCC2...
ALK F1174L + ALK G1202R, PLEKHN1, PUSL...
C
MR347
Biopsy
Crizotinib PR PFS 4 months
Biopsy
Ceritinib PR PFS 5 months
Biopsy
Lorlatinib
Primary progression
EML4-ALK
ALK L1196M
ALK L1196M/D1203N
D
Ba/F3 EML4-ALK V3
% Cell Viability
Lorlatinib Concentration (nM)
WT
F1174L
G1202R
F1174L+G1202R
E
Ba/F3 EML4-ALK V3
% Cell Viability
Lorlatinib Concentration (nM)
WT
L1196M
D1203N
L1196M+D1203N
F
No mutation
L1196M
D1203N
L1196M + D1203N
G1202R
Lorlatinib (nM)
0 10 30 100 300 1000
p ALK
t ALK
p AKT
t AKT
p ERK
t ERK
p S6
t S6
Actin
G
No mutation
F1174L
G1202R
G1202R + F1174L
Lorlatinib (nM)
0 10 300
p ALK
t ALK
Actin
H
N
C
F1174L

Figure 3. *Resistance to lorlatinib mediated by ALK kinase domain compound mutations.* ***A****, Clinical course of patient MR144 and allelic frequencies of ALK resistant mutations (from RNA sequencing) with sequential treatments.* ***B,*** *Fish Plot illustrating the tumor clonal evolution obtained by WES analysis during treatment with ALK inhibitors. The ALK E1154K and G1202R subclones emerged independently upon resistance to crizotinib. After disease progression with brigatinib, the ALK G1202R clone predominated and the E1154K clone became undetectable. At lorlatinib resistance, a subclone emerged from the ALK G1202R clone acquiring an additional F1174L mutation.* ***C,*** *Clinical course of patient MR347.* ***D,*** *Cell survival assay of Ba/F3 models with the indicated ALK single and the F1174L/G1202R compound mutations treated with lorlatinib for 48hs.* ***E,*** *Cell survival assay of Ba/F3 models with the indicated ALK single and the L1196M/D1203N compound mutations treated with lorlatinib for 48hs.* ***F,*** *ALK and downstream kinases phosphorylation in Ba/F3 mutated cells treated with the indicated concentrations of lorlatinib for 3hs.* ***G,*** *Direct comparison of ALK phosphorylation in the same Ba/F3 models by immunoblotting of cell lysates after 3hs treatment with lorlatinib showing higher levels of ALK phosphorylation with the F1174L/G1202R compound mutation.* ***H,*** *Visual representation of aligned wild-type (green) and F1174L/G1202R mutated (brown) ALK structures in complex with lorlatinib.*

NF2 loss of function mediates resistance to lorlatinib

A 44-year-old male was diagnosed with *ALK*-rearranged metastatic lung adenocarcinoma (Figure 4A). The patient experienced disease progression after 11 months on crizotinib. The treatment was switched to lorlatinib achieving a rapid partial response. Oligo-progressive disease occurred after 7 months of treatment with a new single lesion in the left lower lobe. The patient was included in the MATCH-R study (MR135), a biopsy of the lesion was performed and stereotactic radiotherapy (50 Gy) treatment was applied. Targeted NGS and WES of the biopsy revealed both, a *NF2* S288X non-sense mutation and a *NF2* splicing site mutation (NM_000268.3:c.886-1G>A). A PDX model was developed from this first site of progression (R1) and a patient derived cell line was established (MR135-R1).

After 8 months of lorlatinib treatment, multiple new lesions appeared, achieving a total benefit of lorlatinib treatment for 15 months. A biopsy of the right adrenal gland was performed confirming the presence of *ALK*-rearranged lung adenocarcinoma. Interestingly, WES and RNA sequencing of this biopsy showed the same splicing site mutation (NM_000268.3:c.886-1G>A), coexisting with a new *NF2* K543N mutation. A second PDX model was developed and a second lorlatinib resistant patient derived cell line was established (MR135-R2). Sequencing of NF2 mRNA from both cell lines revealed a 9-base pair (bp) skipping in exon 10 as a consequence of the splicing site mutation (Supplementary Figure 3A) but the absence of the S288 non-sense mutation and no secondary ALK KD mutations. The K543N *NF2* mutation was only present in MR135-R2 in concordance with tumor biopsy sequencing results. Both the 9 bp skipping (20) and the K543N mutation were predicted to be pathogenic (cancergenomeinterpreter.org). Merlin expression was detected by WB in the MR135-R1 cell line as well as in the pre- and post-biopsies by IHC staining, suggesting a loss of function but not a loss of expression mechanism of resistance (Supplementary Figure 3B). NF2 mutations

are rare events (1.5%) in lung adenocarcinoma, and do not seem to overlap with ALK rearrangements (according to cBioportal) (21).

NF2 mutations K543N and S288* were not detected in the tumor biopsy prior to lorlatinib treatment. Importantly, the *NF2* splicing site mutation was present prior to lorlatinib treatment. The acquisition of two different second *NF2* events attests for the temporo-spatial convergence between metastatic sites. This preexisting *NF2* splicing site mutation predisposed cancer cells to resist to lorlatinib by an NF2 loss of function mechanism.

Targeting lorlatinib resistance mediated by NF2 loss with mTOR inhibitors

NF2 encodes the merlin protein, a key tumor suppressor implied in the regulation of the PI3K-AKT-mTOR pathway through mTOR inhibition (22). We performed a drug screen in the MR135-R1 identifying the selective dual mTOR1-2 inhibitor, vistusertib (AZD2014, AstraZeneca), and the multi-kinase inhibitor, ponatinib, as hits in this cell line.

Both MR135-R1 and MR135-R2 cell lines were highly sensitive to vistusertib and the combination of vistusertib and lorlatinib (Figure 4B, MR135-R1) (Supplementary Figure 3C, MR135-R2). The activity of an mTOR inhibitor was confirmed by using the clinically available rapamycin analogue everolimus (Supplementary Figure 3D). Ponatinib, a multikinase inhibitor targeting ABL, VEGR, FGFR3, PDGFRA and RET, showed an important synergistic effect with lorlatinib in this cell line with a 57- to 80-fold IC50 reduction with the combination compared to lorlatinib single agent (Supplementary Figure 3E). However, we did not identify a by-pass mechanism related to the activation of tyrosine kinase receptors (RTK) targeted by ponatinib by phospho-receptor tyrosine kinase (p-RTK) arrays (data not shown).

Western blot analysis in MR135-R1 showed that ALK inhibition with lorlatinib alone had no inhibitory effect on the phosphorylation of the downstream signaling pathways (Figure 4C). Treatment of this cell line with vistusertib alone or in combination with lorlatinib inhibited S6 phosphorylation and increased the level of the pro-apoptotic BH3-only protein BIM and the proteolytic cleavage of PARP. This effect was more potent with the combination of vistusertib and lorlatinib. Similarly, the combination of lorlatinib and ponatinib reduced AKT, ERK and S6 phosphorylation, and increased apoptosis as compared to either treatment alone (Figure 4C).

To further assess the activity of the combined treatment against lorlatinib resistant ALK-positive tumors *in vivo*, we examined the efficacy of lorlatinib and vistusertib against the corresponding MR135-R2 PDX. As shown in Figure 4D, treatment of MR135-R2 PDX tumor-bearing mice with the combination was significantly more effective than with single agents in controlling tumor growth.

Independent validation of NF2 loss-mediated lorlatinib resistance

We performed *NF2* knock-out (KO) by CRISPR-CAS9 gene editing in ALK-rearranged H3122 cell line to further validate the implication of NF2 loss of function in lorlatinib resistance. The resulting H3122-NF2KO cell line harbored a genomic 22,803 bp deletion causing a 434 bp frameshift deletion at the mRNA level (Exon 4-12). Immunoblot analysis confirmed the lack of merlin expression in H3122-NF2KO cells (Figure 4E).

Consistent with the MR135 cell lines, H3122-NF2KO cells were less sensitive to lorlatinib treatment than the parental cell line with an IC50 of 41.8 nM compared to 1.3 nM, respectively (Figure 4F). The shift in the IC50 value was also observed for other ALK TKI

(Supplementary Figure 3F). We next assessed the magnitude of this effect in a time-course cell proliferation assay simultaneously with a caspase activity assay. H3122-NF2KO cells continued to proliferate in the presence of high doses of lorlatinib and exhibited low caspase activity compared to the parental cell line at each time point (Figure 4G-4H). Western blot analysis revealed that merlin deficient cells maintained higher levels of S6 phosphorylation compared to merlin proficient cells (Figure 4I). Consistently with the caspase-3/7 activity assay, H3122-NF2KO cells had decreased levels of cleaved PARP after 48 hours of treatment with lorlatinib. Importantly, vistusertib alone or in combination with lorlatinib potently inhibited S6 phosphorylation and induced PARP cleavage in H3122-NF2KO cells (Supplementary Figure 3G). This further supports the importance of merlin integrity in the regulation of mTOR signaling, evidenced by the overactivation of mTOR secondary to *NF2* knock out in this model (Supplementary Figure 4.).

Figure 4

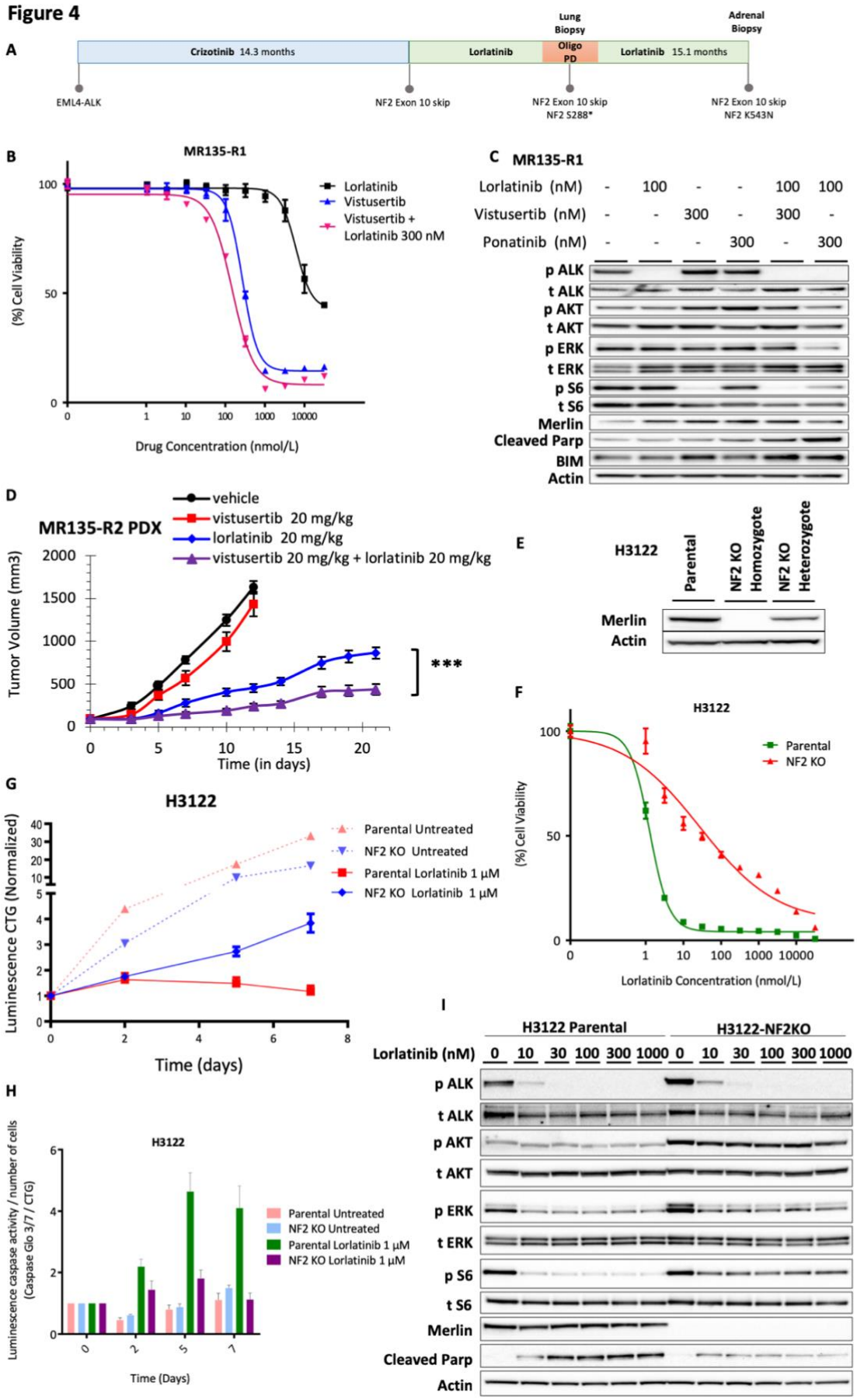
A
Crizotinib 14.3 months
Lorlatinib
Lung Biopsy
Oligo PD
Lorlatinib 15.1 months
Adrenal Biopsy
EML4-ALK
NF2 Exon 10 skip
NF2 Exon 10 skip
NF2 S288*
NF2 Exon 10 skip
NF2 K543N
B
MR135-R1
(%) Cell Viability
Drug Concentration (nmol/L)
Lorlatinib
Vistusertib
Vistusertib + Lorlatinib 300 nM
C
MR135-R1
Lorlatinib (nM) - 100 - - 100 100
Vistusertib (nM) - - 300 - 300 -
Ponatinib (nM) - - - 300 - 300
p ALK
t ALK
p AKT
t AKT
p ERK
t ERK
p S6
t S6
Merlin
Cleaved Parp
BIM
Actin
D
MR135-R2 PDX
vehicle
vistusertib 20 mg/kg
lorlatinib 20 mg/kg
vistusertib 20 mg/kg + lorlatinib 20 mg/kg
Tumor Volume (mm3)
Time (in days)
***
E
H3122
Parental
NF2 KO Homozygote
NF2 KO Heterozygote
Merlin
Actin
F
H3122
Parental
NF2 KO
(%) Cell Viability
Lorlatinib Concentration (nmol/L)
G
H3122
Parental Untreated
NF2 KO Untreated
Parental Lorlatinib 1 µM
NF2 KO Lorlatinib 1 µM
Luminescence CTG (Normalized)
Time (days)
H
H3122
Parental Untreated
NF2 KO Untreated
Parental Lorlatinib 1 µM
NF2 KO Lorlatinib 1 µM
Luminescence caspase activity / number of cells (Caspase Glo 3/7 / CTG)
Time (Days)
I
H3122 Parental
H3122-NF2KO
Lorlatinib (nM) 0 10 30 100 300 1000 0 10 30 100 300 1000
p ALK
t ALK
p AKT
t AKT
p ERK
t ERK
p S6
t S6
Merlin
Cleaved Parp
Actin

Figure 4. *NF2 loss of function mediates resistance to lorlatinib.* ***A,*** *Clinical course of patient MR135 and mutational profile of samples obtained on lorlatinib progression (PD, progressive Disease).* ***B,*** *Cell survival assay assessed with Cell Titer Glo of MR135 lorlatinib resistant cells from biopsy 1 (MR135-R1) treated for 7 days with the indicated concentrations of lorlatinib and vistusertib (AZD2014) alone or in combination.* ***C,*** *Immunoblot analysis from cell lysates of MR135-R1 treated for 24hs with the specified doses of lorlatinib, vistusertib (AZD2014) and ponatinib alone or in combination using indicated antibodies.* ***D,*** *Athymic nude mice bearing MR135-R2 PDX were administered lorlatinib or vistusertib 20 mg/kg orally. Tumor volumes, mean ±SD (n =8); (*** $p < 0.001$). **E,** Cell lysates from H3122 parental and H3122 cells with NF2 heterozygous deletions or homozygous deletions, generated by CRISPR-CAS9 gene editing, were immunoblotted to detect merlin expression. H3122 cells with bi-allelic NF2 knock-out lacked merlin expression.* ***F,*** *Cell survival assay of H3122 parental and H3122 NF2 knock-out (NF2 KO) cells treated with lorlatinib for 7 days. Cell survival was assessed by Cell Titer Glo.* ***G****, Cell proliferation assay of H3122 parental and H3122 NF2 KO cells untreated and treated with lorlatinib measured at baseline, day 2, day 5 and day 7. Cell viability was assessed with Cell Titer Glo.* ***H,*** *Caspase 3/7 activation (Caspase 3/7-Glo assay) relative to the number of live cells simultaneously assessed in the cell proliferation assay previously described.* ***I,*** *H3122 parental and NF2 KO cells were treated with the indicated doses of lorlatinib for 24hs. Cell lysates were immunoblotted to detect the selected proteins.*

Discussion

Lorlatinib, which has been recently granted FDA approval, is the new standard treatment for patients progressing after crizotinib and a second generation ALK inhibitor or after upfront treatment with ceritinib or alectinib, and the last remaining available line of ALK-targeted therapy (6,7,23). With this study, we contributed to understand the adaptive mechanisms driving resistance to this targeted agent trough the longitudinal assessment of tumor biopsies and ctDNA by deep molecular profiling and the development of PDX and cell lines.

The sequential accumulation of mutations on a single allele of the *ALK* kinase domain has been recently described by Yoda and colleagues to mediate resistance in about 35% of patients previously exposed to first- and second-generation TKI (8). In addition to these pivotal findings, we identified and characterized three novel compound mutations from patient tumor biopsies (F1174L/G1202R, L1196M/D1203N and C1156Y/G1269A). The C1156Y/G1269A compound mutation retained sensitivity to lorlatinib both in Ba/F3 cells and the patient-derived cell line suggesting that co-occurring off-target mechanisms of resistance can drive disease progression even in the presence of compound mutations. Similarly to the previously described L1196M/G1202R mutation, the L1196M/D1203N mutation conferred high level of lorlatinib resistance. On the other hand, the G1202R/F1174L compound mutation resulted in a mild increase in resistance to lorlatinib compared to the single G1202R mutation, and is potentially targetable by increasing lorlatinib doses *in vitro*. However, this approach would not be feasible in patients, limited by the risk of increased toxicities. This is further supported by a recent study reporting the acquisition *in vitro* of the F1174L mutation arising from G1202R mutant Ba/F3 cells, exposed to low doses of lorlatinib using ENU mutagenesis screening, conveying low levels of resistance to this drug (24). In this patient,

the detection in ctDNA of multiple secondary ALK mutations, of which G1202R and S1206F were confirmed to be in *cis*, shows that compound mutations can be polyclonal events.

Our studies on patient derived cell lines allowed to further explore off-target mechanisms of resistance to lorlatinib, contributing to past efforts in the design of novel therapeutic strategies (25). We developed two patient-derived cell lines that underwent EMT *in vitro* on treatment with lorlatinib involving SRC activation. EMT had previously been implied in resistance to ALK inhibitors and other targeted therapies in lung cancer (26–29). In addition, it is also known that SRC activation plays a key role in the development of EMT throughout different cancer types (30). Crystal and colleagues had previously reported that several ALK resistant patient-derived cell lines were susceptible to combined ALK and SRC inhibition. In the present study, we further demonstrated that this association is highly effective in lorlatinib resistant patient derived cell lines undergoing EMT, and showed that SRC inhibition could partially restore E-cadherin expression in mesenchymal cells without completely reverting them to an epithelial phenotype. Interestingly, as recently shown for EGFR mutant NSCLC, FGFR inhibitors sensitized ALK-rearranged EMT cell lines to lorlatinib *in vitro* (17). There are no effective therapies against lung cancer undergoing EMT, our work further supports the exploration of combination strategies in clinical trials for patients with off-target resistant mechanisms.

Finally, we identified NF2 loss of function as a novel bypass mechanism of resistance to lorlatinib (MR-135) and subsequently confirmed these findings *in vitro* by *NF2* knock-out in the H3122 cell line. In this case, the NF2 splicing site mutation was present at the time of progression to crizotinib, and in this context, the patient experienced initial response to lorlatinib treatment. At the time of resistance, additional deleterious events in *NF2* occurred and led to a potent bypass mechanism. We hypothesize that NF2 loss of function was a functional convergence among multiple metastatic sites where sequential genomic events

led to biallelic *NF2* deleterious mutations. The patient-derived cell lines were resistant to lorlatinib and sensitized by mTOR inhibition *in vitro* and *in vivo*, constituting a novel potential treatment approach in this context.

This study has several limitations, the first being the number of patients evaluable for resistance mechanisms and reported in this study. Among the four patients who achieved a partial response with lorlatinib, the PFS ranged from 3.7 (MR144) to 16 months (MR210) which seems shorter than reported in the phase II study of lorlatinib (7). Further studies are needed to disclose the full spectrum of resistance mechanisms to lorlatinib including from patients with prolonged benefit. Secondly, pre-lorlatinib tumor biopsies and plasma samples were not available in all cases, limiting the analysis of the impact of baseline genomic alterations in lorlatinib resistance. Thirdly, during the development of patient-derived cell lines, the selective pressure introduced by passages *in vitro* and treatment exposure, may result in the outgrowth of more aggressive tumor cells and force the acquisition of EMT features.

In summary, the mechanisms of resistance to lorlatinib in patients with *ALK*-rearranged lung cancer can be diverse and complex. We have shown here that longitudinal tumor samplings combined with patient derived models can provide new insights on tumor dynamics and biological processes underlying disease progression, thereby, contributing to the design of novel therapeutic strategies.

Authors contributions:

JCS and LF conceived and designed the study. GR, LB, JPT, LL, FA, CM, BB, JCS and LF developed the methodology. GR, LM, DP, FF, LB, AZR, KH, OD, LM, CN, MNC, SM, LL, PT, FB, CR and LF acquired data. GR, FF, LB, RF, JG, TS, KH, PT, FB, OD and LF conducted experiments. GR, LM, DS, JYS, AZR KO, BB and LF analyzed and interpreted data (including computational analysis). All authors wrote, reviewed, and/or revised the manuscript. GR, LM, DP, AG, LB, AZR, JYS, RLF, TS, JH, OD, LM, JG, AAL, CN, MNC, SM, LL, CR, TDB, LT, EA, FA, CM, CS, BB and LF provided technical, or material support (e.g., reporting or organizing data, constructing databases). CM, FA, JCS, BB and LF supervised the study.

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Supplementary Files

Supplementary Figure 1: EMT mediated lorlatinib resistance (corresponding to main Figure 2). **A,** Cell survival assay of MR57 lorlatinib sensitive (MR57-S) and resistant (MR57-R) cell lines treated with the indicated doses of lorlatinib for 7 days. Cell survival was assessed with Cell Titer Glo. **B,** Cell survival assay of Ba/F3 models of single and compound ALK mutations treated with the indicated doses of lorlatinib for 48hs. Cell survival was assessed with Cell Titer Glo. **C,** Cell survival assay assessed with Cell Titer Glo of Ba/F3 models harboring the compound C1156Y/G1269A mutation treated with the indicated ALK inhibitors for 48hs. **D,** Log2 fold change in the expression of key genes implied in EMT from RNA sequencing of MR57 lorlatinib resistant (MR57-R) and sensitive (MR57-S). The differential expression of E-Cadherin, N-cadherin, vimentin and SNAI1 supports the characterization of the mesenchymal phenotype in MR57-R cells. **E,**

MR57 lorlatinib sensitive and resistant cells labelled with Alexa Fluor 488 Phalloidin. MR57 sensitive cells exhibit epithelial features by forming compact clusters with extensive intercellular contacts associated with subcortical actin microfilaments. MR57 resistant cells display significant formation of actin stress fibers, characteristic of a mesenchymal phenotype. **F,** Pre-crizotinib and post-lorlatinib lung biopsies from MR57 underwent hematoxylin and eosin staining (HES) and immunohistochemical staining for ALK, E-cadherin (E-Cad), N-Cadherin (N-Cadh), Vimentin, pSRC, pMAPK, Ki-67, Podoplanin, Glucose transporter 1 (Glut1), Carbonic anhydrase 9 (CA-IX), beta catenin (B catenin), CD47 and CD68. There was no evidence of EMT in the tumor tissue upon lorlatinib progression as evidenced by high levels of E-cadherin expression and the absence of N-cadherin or vimentin expression. Beta-catenin retained its membrane localization and no nuclear staining was detected. Interestingly, hematoxylin and eosin (H&E) staining revealed spatial tumor relocation to lymphatic vessels (expressing CD31 and podoplanin). This carcinomatous lymphangitis allowed a sustained tumor cell growth (with high Ki-67 index) and MAPK pathway phosphorylation while on lorlatinib. This relocation was associated with a hypoxic environment [expression of carbonic anhydrase 9 (CA-IX) and Glut1] and innate immunity escape [expression of CD47 and low content in CD68 macrophages]. Overall, the study of patient tumor histology did not confirm EMT-related resistance, but a tumor relocation that could have impacted the drug accessibility to tumor cells. **G,** Pre-crizotinib and post-lorlatinib FFPE biopsies from MR210 underwent HES and immunohistochemical staining for E-cadherin (E-Cad), N-Cadherin (N-Cadh) and Vimentin. The increase in vimentin expression in the post-lorlatinib specimen would suggest a partial EMT in the tumor at the time of resistance. **H**, Cell survival assay assessed with Cell Titer Glo of MR57-S cell line treated with the indicated doses of saracatinib or lorlatinib or their combination for 7 days. **I**, Cell survival assay assessed with Cell Titer Glo of MR57 lorlatinib resistant (MR57-R) cell line treated with the indicated doses of dasatinib or lorlatinib or their combination for 7 days. **J**, Cell survival assay assessed with Cell Titer Glo of MR57-S cell line treated with the indicated doses of dasatinib or lorlatinib or their combination for 7 days. **K**, MR57-S and MR57-R cells were treated for 30 days with the indicated concentrations of lorlatinib, saracatinib or the combination. Immunoblotting of the cell lysates for the selected

EMT markers showed partial restoration in E-Cadherin expression in MR57-R cells exposed to saracatinib.

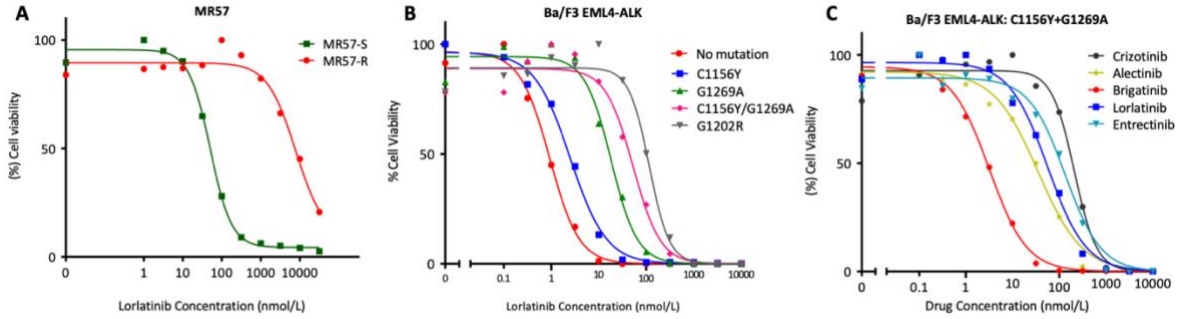
Supplementary Figure 2: ALK secondary mutations (corresponding to main Figure 3). **A,** Proportion of sequenced colonies with the G1202R and E154K mutations in different alleles from TOPO-TA cloning of the ALK kinase domain from RNA (cDNA) extracted from the tumor biopsy of MR144 obtained after progression to crizotinib. **B,** Allelic frequencies of ALK mutations detected in circulating tumor DNA sequencing from MR144 during treatment with ALK inhibitors (PD, progressive disease; PR, partial response). **C,** Proportion of sequenced colonies with the G1202R and F1174L mutations *in cis* from TOPO-TA cloning of the ALK kinase domain from DNA extracted from the tumor biopsy of MR144 obtained after progression to lorlatinib. **D,** Cell survival assay of Ba/F3 models of EML4-ALK, non-mutant and with the novel E1154K variant, treated with the indicated doses of different ALK inhibitors for 48hs. **E,** Mean IC50 values from three replicates for Ba/F3 cells harboring EML4-ALK rearrangements with single and compound mutations treated with ALK inhibitors. **F,** ALK phosphorylation of Ba/F3 cells treated with the indicated concentrations of lorlatinib for 3hs.

Supplementary Figure 3: *NF2* deleterious mutations and sensitivity to lorlatinib (corresponding to main Figure 4). **A**, Sanger sequencing of NF2 (cDNA) from MR135-R1 cells showing NF2 exon 10 skipping of 9bp secondary to the indicated intron 9 splicing acceptor site mutation, also present in MR135-R2 cell line. **B**, Pre- and post-lorlatinib FFPE biopsies from MR135 underwent Merlin immunohistochemical staining. Merlin protein expression was detected in the pre- and post-biopsies. **C,** Cell survival assay of MR135 lorlatinib resistant cells from biopsy 2 (MR135-R2) treated with the indicated doses of vistusertib (AZD2014) and lorlatinib single agents and in combination for 7 days. **D**, Cell survival assay of MR135-R1 cells treated with everolimus and lorlatinib as indicated for 7 days. **E**, Cell survival assay of MR135-R1 cells treated with the indicated doses of ponatinib and lorlatinib as monotherapy or in combination for 7 days. **F**, Cell

survival assay of H3122 parental and H3122 NF2 KO cells treated with the indicated concentrations of crizotinib, alectinib, brigatinib and entrectinib for 7 days. H3122 NF2 KO cells were less sensitive across different ALK inhibitors compared to parental H3122 cells. Cell survival was assayed with Cell Titer Glo in all experiments. **G**, H3122 parental and NF2 KO cells were treated with the specified doses of lorlatinib and vistusertib (AZD2014) for 24hs. Cell lysates were immunoblotted to detect the specific proteins. The combination of vistusertib and lorlatinib enhanced apoptosis induction in H3122 NF2 KO cells.

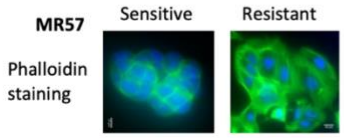
Supplementary Figure 4: NF2 inhibition of mTORC1 and its canonical pathway. **A,** NF2 functions as a tumor suppressor gene. By its canonical pathway NF2 indirectly inhibits the Rho GTPase Rac1 and Cdc42. Therefore RAC1 does not activate the serine/threonine p21-activating kinases (PAKS) and the MAPK effectors JNK and c-Jun. Merlin also inhibits mTORC1, and therefore the phosphorylation of p70 S6 Kinase 1 (S6K1) and eIF4E Binding Protein (4EBP). In this context, S6K1 does not phosphorylate S6. 4EBP, is a repressor protein and inhibits the eukaryotic translation initiation factor 4E (eIF4E) (98). In the context of mTOR inhibition by merlin, 4EBP inhibits eIF4E, and by the canonical pathway and mTORC1 inhibition, merlin negatively regulates cell proliferation. **B,** Lorlatinib inhibits the EML4-ALK fusion protein and thus, inhibits the phosphorylation of ALK downstream signaling pathways including the PI3K/AKT/mTOR pathway. In cells with proficient merlin function, in addition merlin mediates mTORC1 inhibition. In merlin deficient cells, mTORC1 even in the setting of adequate ALK inhibition, loses the negative regulation of mTOR and in turns is activated, phosphorylating S6K1 and S6 promoting ALK independent downstream oncogenic signaling. Figure A was adapted from *Beltrami et al. Anticancer Cancer 2013*.

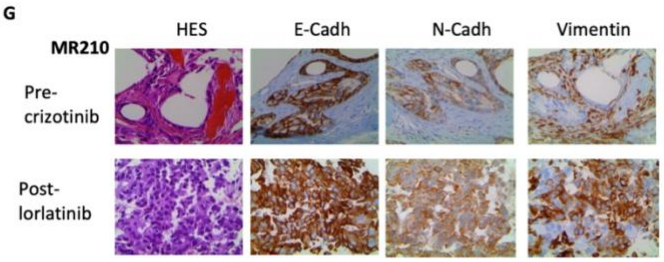
Supplementary Figure 1

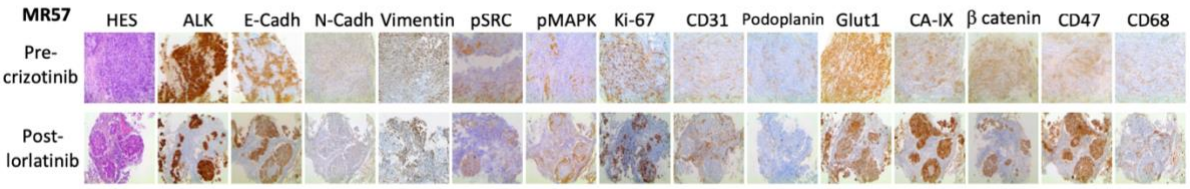


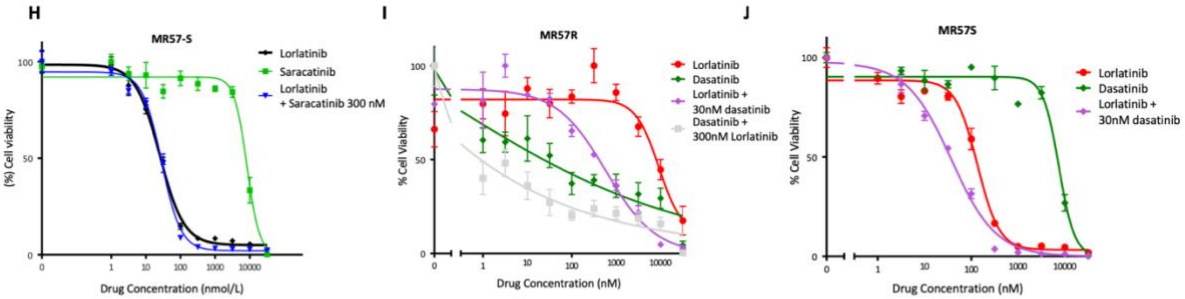
D

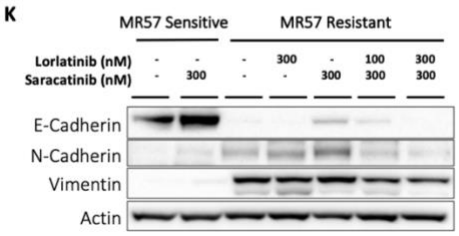
Gene	Log2 Fold change (MR57 R/S)
VIM	6,74
FGFR1	4,38
ZEB1	4,24
TGFB1I1	4,09
CDH2 (N-Cadherin)	3,93
TGFB2	3,92
SNAI1	3,75
TGFA	-4,19
EPCAM	-5,75
CDH1 (E-Cadherin)	-5,78
ICAM1	-6,53



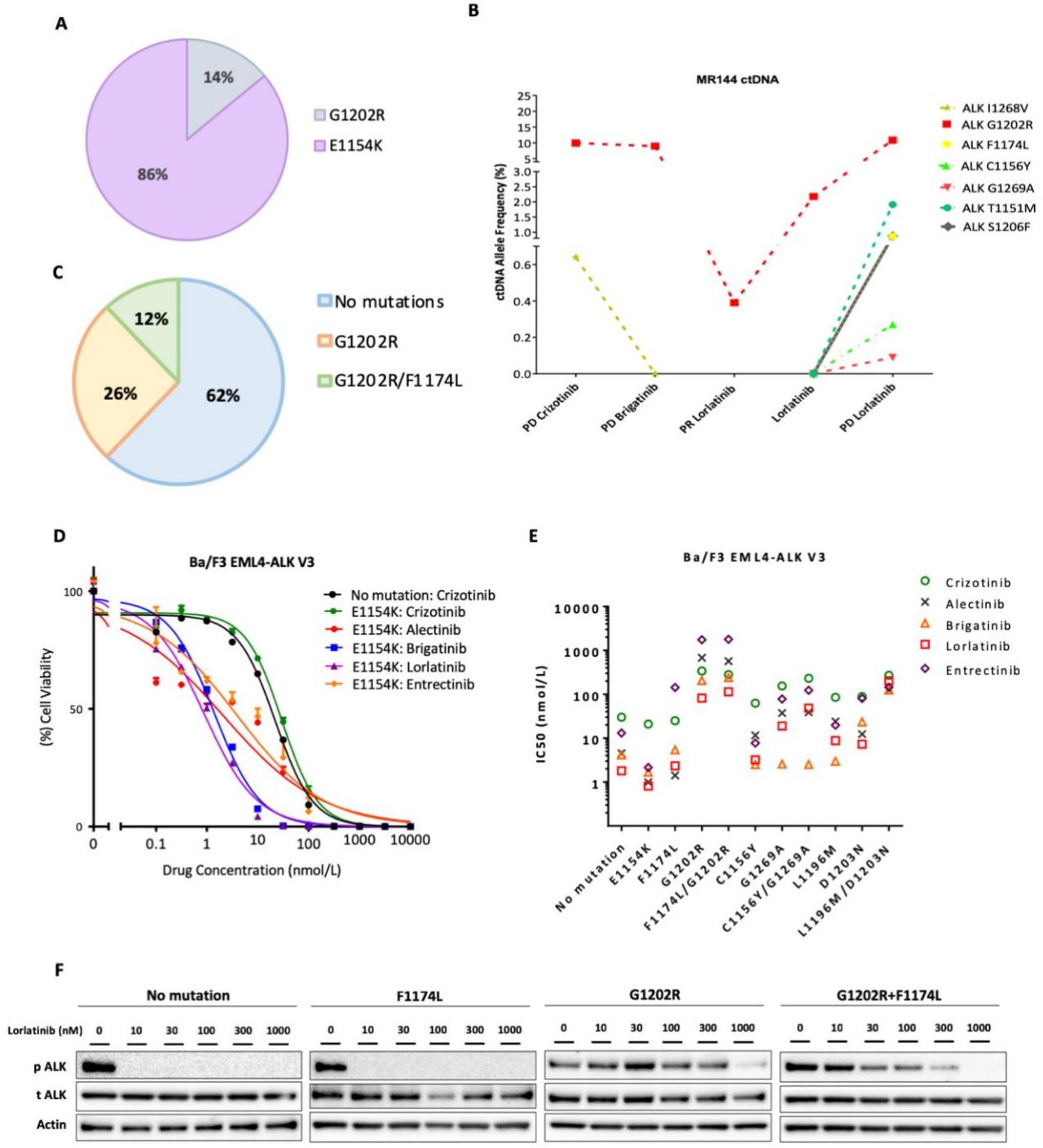




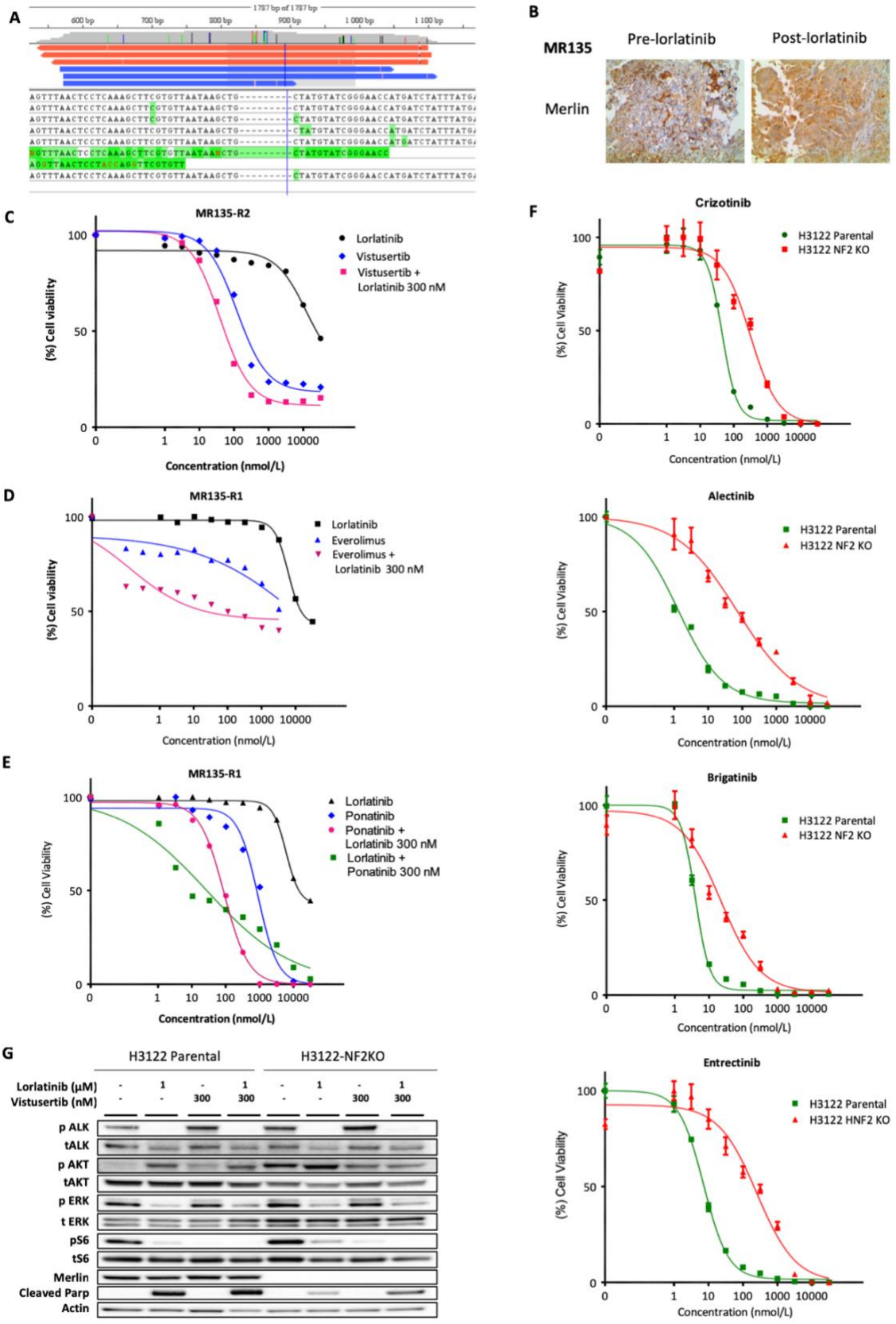




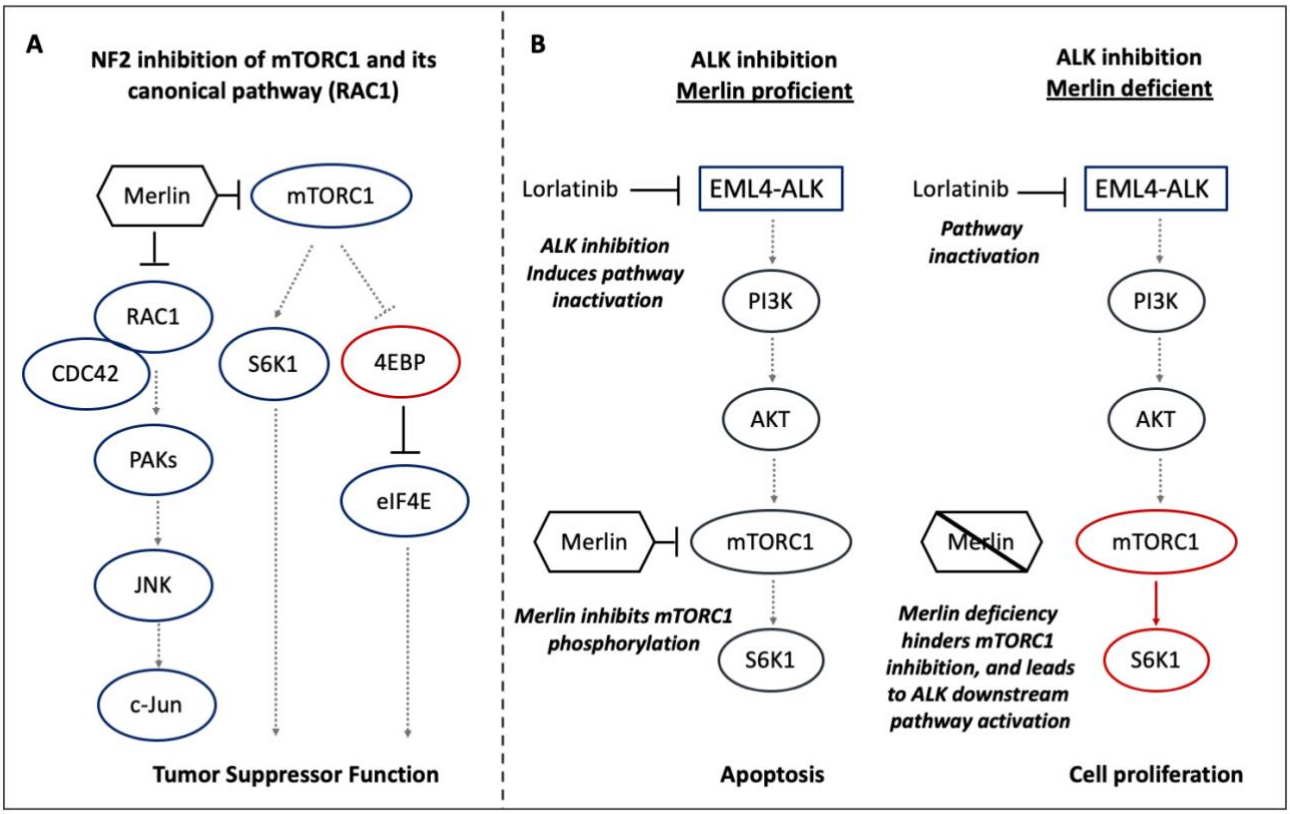
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Part III. Discussion and perspectives

1. Implementing strategies to study resistance to lorlatinib in patients with lung cancer

The evolution of science, technology and drug development has propelled the oncology field into the era of targeted therapies, improving outcomes for patients with advanced-stage lung cancer. In parallel with the development of kinase inhibitors, the study of resistance to kinase inhibitors has contributed thoroughly to the comprehension of cancer adaptation and evolution, as well as the development of new generation kinase inhibitors (212). This has been possible by optimizing the development of patient-derived cell lines and xenografts together with the recent advances in molecular diagnosis (223). However, in few academic institutions around the world, the study of resistance to kinase inhibitors has been implemented in a systematic and prospective fashion.

This study is the first to report on mechanisms of resistance to targeted therapies within the scope of the institutional MATCH-R trial held at Gustave Roussy Campus. In our study, we have shown that the prospective inclusion of patients in the MATCH-R trial allowed to interrogate the tumor biology at different time-points during treatment and at the time of progression to the third generation ALK inhibitor lorlatinib. By this mean, we were able to fully characterize novel mechanisms of resistance to this compound by generating patient-derived tumors and integrating genomic and RNA sequencing in the process.

As part of the strategy implemented to study resistance mechanisms occurring in patients treated with lorlatinib, we developed a research workflow based on the development of patient derived cell lines and xenograft models. Establishing representative patient-derived cell lines constituted one of the major

challenges we had to sort throughout the course of this project. Tumor processing and logistic variables together with the intrinsic properties of cancer cells determines the success rate of cell line development. The success rate is influenced by the size of tumor biopsies and the proportion of cancer cells, time from biopsy to tumor processing and engraftment in mice, careful selection and identification of cancer cells growing in culture, adapting culture media requirements, selecting lorlatinib resistant cells and validating the genomic features of the cell line with those observed in tissue NGS.

From the 13 biopsies with adequate tumor sampling obtained from 10 patients experiencing resistance to ALK TKIs, we successfully developed 4 patient derived cell lines (MR57-S, MR135-R1, MR135-R2 and MR210), with a success rate of 31%. In addition, we derived a fifth cell line, the MR57 resistant cell line by exposing MR57-S cells to lorlatinib in vitro. This was the cornerstone to pursue the characterization of novel mechanisms of resistance to lorlatinib, by allowing to test multiple compounds to identify potential hits through drug screen assays and activated phosphokinases by immunoblotting assays.

Validating the role of compound mutations emerging at the time of lorlatinib resistance required the development of reliable models of on-target resistance. After confirmation of the allelic distribution of ALK mutations, we cloned the full EML4-ALK variant 3 cDNA into lentiviral vectors, introduced multiple single and compound ALK mutations and developed Ba/F3 models by successfully infecting and selecting ALK-dependent cells. This allowed to test the effect of these mutations against most available ALK TKIs with a high degree of reproducibility and certainty.

Finally, to confirm that NF2 deleterious mutations can induce resistance to lorlatinib, we developed a second ALK cell line model to reproduce this effect by inducing complete NF2 knock out on H3122 cells using CRISPR-Cas9 gene editing. It was challenging to obtain an NF2 knock out cell line by this method, as most cell clones did not survive the simultaneous biallelic loss of this tumor suppressor gene. However, in the established NF2 knockout H3122 clone, we could reproduce the findings observed in the lorlatinib resistant patient-derived

cell line, and further validate the role of mTOR overactivation in a clean model of resistance.

2. Novel mechanisms of resistance to lorlatinib

The discovery of compound ALK mutations and the negative effect of most mutation combinations on lorlatinib binding was the sole described mechanism of resistance to lorlatinib reported to date. In our study, we contributed to the existing evidence by characterizing novel off-target mechanisms of resistance to lorlatinib: the role of SRC- mediated resistance by the induction of EMT and the downstream overactivation of mTOR, induced by NF2 loss of function mutations. We also studied the effect on lorlatinib of new compound mutations found in tumors, the C1156Y + G1269A and G1202R + F1174L mutations.

EMT induced by SRC activation prompts lorlatinib resistance and can be targeted with SRC inhibitors

In our study, we found that SRC activation mediates resistance to lorlatinib by promoting EMT in vitro and showed that dual combination of SRC and ALK inhibition induces cell death in SRC-dependent cell undergoing EMT. The role of SRC activation in resistance to first and second-generation ALK inhibitors was previously reported by Crystal and colleagues (223). In hand with their findings, we further linked the role of SRC-dependent resistance to the induction of EMT in ALK positive lung cancer cells in vitro. A recent study by Fukuda and colleagues show that, in the context of ALK resistance mediated by EMT, reversing the EMT state in vitro by using HDAC inhibitors was necessary to resensitized cancer cells to ALK inhibition (251). Differently, in our study, we prove that direct targeting of SRC and ALK, results in high levels of apoptosis in overtly mesenchymal cells, but SRC inhibition alone does not provoke this effect, even when partially reverting the EMT state. This proves that cancer cells undergoing SRC mediated EMT can still be co-dependent on ALK and SRC signaling for survival *in vitro* (297).

One of the pitfalls of our study, is the lack of evidence of EMT features in the biopsy specimen from the patient, and the fact that the original cell line developed (MR57-S) was sensitive to lorlatinib. We don´t know if lorlatinib withdrawal during the time of PDX development and cell line establishment could have influenced the observed sensitivity of the MR57-S cell line, and if epigenetic modifications could have mediated this phenomenon. In this cell line, the induction of a mesenchymal resistant phenotype by lorlatinib treatment *in vitro*, led us to hypothesis that a pre-programmed EMT state may have been present in lorlatinib tolerant persister cells, and this is currently being studied in our team.

Compound mutations can have differential impact on lorlatinib resistance

Previous reports on compound mutations have shown to confer high levels of resistance to lorlatinib treatment, mainly by partnering G1202R mutations with a second ALK kinase domain mutation (216,279,298). Previous studies from Shaw and colleagues, showed that compound mutation C1156Y+L1198F, even in the absence of a G1202R mutation, conferred resistance to lorlatinib but resensitized cells to crizotinib treatment (298). In our study we showed that the C1156Y+G1269A did not cause resistance to lorlatinib and could also be targeted with brigatinib. Because of this, the differential effect of compound mutations should be incorporated in the treatment decision process. In case of detecting this compound mutations after progression to first- or second- generation ALK TKI, our findings support pursuing treatment with brigatinib or lorlatinib.

Furthermore, we also characterized a novel compound mutation that confers resistance to lorlatinib, the G1202R + F1174L. In contrast with the high levels of resistance reported with G1202R compound mutations, this compound mutation does not seem to fully abrogate lorlatinib binding, as full ALK phosphorylation can be suppressed with higher lorlatinib doses. Interestingly, we found that baseline ALK phosphorylation was significantly higher in ALK G1202R + F1174L mutant Ba/F3 cells, compared to single mutant cells. This suggests that the kinase affinity for ATP is enhance in this context, potentially contributing to the lower ALK inhibitory potency observed. However, we did not validate this hypothesis in the current study, as we did not perform kinase affinity assays for

this compound mutation. Okada and colleagues also reported this compound mutation while performing an ENU mutagenesis screen do identify resistance mechanisms to lorlatinib, further confirming our findings (216).

In addition, we characterized a highly resistant L1196M/D1203N compound mutation emerging after treatment with crizotinib and ceritinib and conferring primary resistance to lorlatinib. This also suggests that compound conferring primary resistance to lorlatinib can emerge during treatment with earlier generation ALK TKIs and that assessment of ALK kinase domain mutations prior to lorlatinib treatment can help in the treatment selection of these patients.

NF2 loss induces resistance to lorlatinib by mTOR overactivation and can be reversed with mTOR inhibitors

The identification and characterization of *NF2* loss of function mutations resulting in mTOR overactivation is the most relevant contribution of our study to the existing evidence on resistance to lorlatinib. Based on our results, ALK resistance driven by NF2 loss of function alterations can be overcome by combined ALK and mTOR inhibition *in vitro* and *in vivo*

The role of NF2/merlin in mTOR regulation has been extensively studied in NF2 mutant schwannomas, meningiomas and mesotheliomas in the context of type II neurofibromatosis disease (106,108). This has encouraged the development of clinical trials assessing the efficacy of mTOR inhibitors in this setting (NCT02831257, NCT03433183). However, in lung cancer, *de novo* NF2 mutations are a rare event, and are mutually exclusive with ALK rearrangements (299,300). Redaelli and colleagues have previously reported that combined ALK and mTOR inhibition had a synergistic effect in NPM-ALK lymphoma cells (301). In our study, we did not observe this in other cell line models of lorlatinib resistance or other models of TKI resistance, suggesting that this combination was selectively potent in the setting of NF2 loss. An ongoing phase I trial is studying the safety and efficacy of ceritinib in combination with everolimus in the first line treatment for patients with ALK-driven lung cancers. This study will provide some clinical perspective on the feasibility of this combination.

One of the limitations of our study is that we did not deepen into the basic processes involved in mTOR overactivation by merlin loss, in the context of ALK resistance, and whether it resembles those observed in neurofibromatosis-associated cancers needs to be further explored.

3. Challenges and future perspectives in the treatment of patients with ALK-rearranged lung cancer

The findings of the present work support the notion that mechanisms of resistance to lorlatinib are diverse and complex and, even when challenging, pursuing new ways of optimizing and developing effective ALK targeted treatments is crucial

One of the most important challenges moving forward, is to find novel ways to target compound mutations, reported in about 35% of patients experiencing resistance to lorlatinib (279). As the list of defined compound mutations continues to grow, it is less likely that an ATP-competitive ALK TKI will be able to inhibit ALK in the context of all the published compound mutation combinations (216,279). In my opinion, future strategies should aim to target ALK without depending on binding properties of kinase inhibitors to the kinase domain. By sparing the need to bind to the catalytic pocket, developing allosteric ALK inhibitors could be an innovative treatment strategy in the setting of intricate compound mutations.

Another novel way to target ALK in this context is being explored with the development of protein degraders, a group of drugs that induce protein ubiquitination and proteasomal degradation by the cereblon E3 ligase complex. Protein degraders are called bifunctional proteolysis targeting chimeras (PROTACs). Nathanael Gray's group has recently published the chemical structure and development of ALK degraders. These degraders are composed of an ALK inhibitor (ceritinib or TAE684) bound by a linker to the cereblon ligand

pomalidomide (302). Pomalidomide recruits the E3 ubiquitin ligase complex, ultimately resulting in ALK selective ubiquitination and proteasomal degradation. ALK degradation results in lower levels of total ALK in cancer cells and, in consequence, decreased ALK phosphorylation and enhanced apoptosis.

Today, in the clinical setting of resistance to lorlatinib, there are few available treatment options for patients experiencing disease progression with lorlatinib. Thus, the development of clinical trials for patients progressing on this treatment is urgently needed. In the presence of well characterized resistant compound mutations, treatment with chemotherapy is the sole clinically available option. Only two mutations have been reported to resensitize ALK-rearranged cancer cells to earlier generation ALK TKIs, the L1198F mutation to crizotinib and the ALK L1256F mutation to alectinib and, if detected at progression, treatment with the earlier generation ALK TKIs should be considered (216,298).

Combination strategies targeting ALK and off-target mechanisms of resistance like we showed for SRC and mTOR activation *in vitro* should be explored. Given that this is the first study to date reporting on bypass mechanisms of resistance to lorlatinib, it remains to be elucidated if SRC activation and NF2 mutations or other alterations in the PI3K/AKT/mTOR axis will occur frequently. Our findings *in vitro* and *in vivo* support the development of SRC/ALK and mTOR/ALK combinations in the setting of resistance. However, this is limited by the lack of clinical biomarkers to detect SRC or mTOR overactivation in tumor samples. Case reports and small studies have shown that combining MET or RET inhibitors with the third generation EGFR TKI osimertinib is effective when MET amplification or RET-fusions emerge as bypass resistance (71,254,303). Hopefully, these combinatorial strategies with osimertinib can be adopted to overcome lorlatinib resistance.

Other future strategy to tackle of-target resistance to lorlatinib is to modulate effectors that regulate common oncogenic signaling pathways. Dardaei and colleagues identified SHP2 as a potent activator of MAPK signaling in the setting of resistance to different ALK TKI (143). In the absence of detectable on-target resistance to lorlatinib, combining lorlatinib with SHP2 inhibition could be a rational strategy to pursue in clinical trials. Early trials assessing the safety of

SHP2 inhibitors are currently ongoing for patients with tumors harboring molecular alterations in the EGFR and MAPK pathway (NCT03114319).

With the current evidence, showing that compound mutations are developed by the sequential acquisition of single ALK mutations to different ALK TKI, developing strategies to prevent the onset of resistance is necessary. Lorlatinib is the most potent ALK inhibitor developed, and preclinical data in PDX models supports the efficacy of upfront treatment with this drug (272). This was also observed in the clinical setting, where first line treatment with lorlatinib in the phase I/II study yielded a 90% response rate among 30 patients with prolonged progression-free survival durations. The efficacy of lorlatinib compare to crizotinib I the first line setting is currently being studied in a phase III randomized trial (NCT03052608).

In the current scenario, the development of robust biomarkers to tailor the treatment with ALK TKIs is necessary. In EML4-ALK rearranged cancers, the rearrangement variant might play a role in this setting. Lin and colleagues have reported that on target resistance mutations are more commonly detected in tumor with EML4-ALK variant 3 rearrangements compared to variant 1 rearrangement (57% vs 30%), and this difference is more striking with the G1202R mutation (32% vs 0%). Previously reported preclinical studies showed that in non-mutant EML4-ALK cells, treatment with lorlatinib upfront did not induce resistance by single ALK mutations (272,279). Based on this, treating patients harboring variant 3 EML4-ALK rearranged cancers with lorlatinib in the first line setting could prevent the emergence of single ALK resistance mutations, including the G1202R mutation, and thus, block the future acquisition of compound mutations. This might not be as relevant for EML4-ALK variant 1 tumors, in which the incidence of acquired secondary mutations is lower and following a sequential treatment strategy with crizotinib, ceritinib or alectinib in the first line could be a suitable option (162). Though the variant type doesn’t seem to influence progression free survival outcomes with alectinib or lorlatinib, it could be considered in future trial designs due to the differential predisposition in the type of resistance mechanisms observed.

Moving forward with this research field, it's important to continue to study resistance mechanisms to lorlatinib occurring in patients. In addition to characterizing individual cases, like in our study, our research group will continue to develop a biobank of patient derived models that will allow us to explore more common and shared biological mechanisms of resistance across patient's tumors. For this, we need to study beyond the genomic alterations in ALK, or the modulation of well characterized signaling pathways. Exploring other hallmarks of cancer survival like epigenetic modulation of gene expression, cell cycle effectors and antiapoptotic mechanisms together with the unique features of drug tolerant (or persister) cells may derive in new ways of understanding resistance to ALK TKIs. This could be coupled to study the effect of new epigenetic modifiers, like next generation HDAC inhibitors, or cyclin-dependent kinases in this setting.

Furthermore, the influence of the tumor microenvironment in resistance to ALK TKIs is not well understood and needs to be studied. Paracrine signaling by multiple cell populations like immune cells, fibroblasts and endothelial cells have been previously reported to induce EMT and bypass mechanisms in EGFR and ALK rearranged lung cancer cells in vitro (234). In this line, the study of circulating exosomes may provide novel insights in the influence signaling from distant metastatic sites on systemic progression. Exosomes contain growth factors, EMT inducers, miRNAs and long-non-coding RNAs, amongst many other molecules that may influence the tumor microenvironment and also directly impact lung cancer cells. (304).

Finally, we hope that with our study we have proven that conjoint research efforts of basic, translational and clinical investigators, in partnership with drug development units can shed a light on mechanisms of resistance to novel compounds, like lorlatinib in ALK-dependent lung cancer patients. Most importantly, we hope that our findings could contribute to the development of novel treatment strategies to improve patients care.

Conclusions

In the translational science field, our research provides novel insights on biological mechanisms of resistance to lorlatinib in patients with ALK-rearranged non-small cell lung cancer. We revealed that these mechanisms can be diverse and complex constituting the first report integrating on- and off-target mechanisms of resistance to lorlatinib. We showed that SRC activation can mediate resistance to lorlatinib in vitro by inducing epithelial mesenchymal transformation. We also demonstrated that SRC and ALK inhibition can induce cell death in highly mesenchymal cells. In addition, we characterized the biological effect of novel compound mutations occurring at lorlatinib progression in patients, proving that compound mutations found in patients can confer differential sensitivity/resistance to lorlatinib, and can also emerge as polyclonal effects. Lastly, we demonstrated that *NF2* loss of function mutations result in lorlatinib resistance by mTOR overactivation and can be reverted by combining ALK and mTOR inhibition.

In the clinical field, our findings support the development of combination treatment strategies to tackle off-target resistance mechanisms in patients progressing on lorlatinib. In addition, with our work we show that the prospective and systematic assessment of tumor biology through molecular profiling and the development of cell line models from patients treated with targeted therapies is feasible and useful to study and develop new strategies to improve patient's outcomes.

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1. Article II: "Harnessing resistance to targeted and immunotherapy at Gustave Roussy: design and feasibility of the MATCH-R clinical trial".

Harnessing resistance to targeted and immunotherapy at Gustave Roussy: design and feasibility of the MATCH-R clinical trial

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ABSTRACT

The recent advances in the development of molecular targeted agents and immunotherapy provide substantial benefits in patients with advanced cancer, allowing improvements in disease control, survival outcomes and quality of life. Due to their malignant nature, some tumor cells invariably acquire the capacity to adapt and evade the lethal effect of these novel agents. Unraveling the biological processes driving tumor resistance is necessary to support the development of innovative treatment strategies. The MATCH-R trial is a single institution study aiming to characterize the molecular mechanisms of resistance to a wide-range of novel anticancer agents in patients with advanced cancer, regardless of tumor type. For this purpose, deep molecular profiling of tumors biopsies from patients progressing on treatment with selected therapies is performed. In parallel, patient-derived xenografts and cell line models are developed for translational research purposes. Herein, we present the study design and feasibility of the ongoing MATCH-R study at Gustave Roussy Cancer Campus. Amongst 333 included patients, adequate tumor biopsies were performed in 303 cases (91%). From these biopsies, 278 (83%) were contributive for NGS analyses and 54 patient-derived xenograft (PDX) models were established.

Keywords: Resistance; biopsies; models; targeted therapy; immunotherapy, personalized medicine.

INTRODUCTION

Cancer research has led to significant advances in the understanding of tumor biology and immunology, providing rational for the development of novel treatment strategies (305). In part, this has been possible due to the accessibility of high throughput molecular biology techniques, the improvements in developing patient-derived models and the collaborative efforts of the research community to deeply study cancer biology (29,223).

In recent years, the breakthrough of highly effective treatments such as immune checkpoint inhibitors and molecular targeted therapies has improved outcomes for patients affected by different types of cancer and radically changed their management (306,307). Many innovative approaches, using antibody-drug conjugates, monoclonal antibodies, cell-cycle inhibitors, endocrine therapies, DNA repair and epigenetic modulators have become standard therapeutic options for selected cancer patients (308). This vast landscape of drugs in development, used either as monotherapy or in combination, will continue to improve cancer care in the near future (309–313).

In this context, the development of reliable biomarkers is key to predict patients benefit from therapies and avoid unnecessary toxicities. Targetable molecular alterations in *EGFR*, *BRAF*, *MET*, *RET, ROS1, ALK, NTRK, KIT* predict responses to selective kinase inhibitors (53,59,174,314–318). PD-L1 staining, tumor mutational burden, T-effector signatures and mutational signatures are currently being studied as predictive biomarkers of treatment with immune checkpoint inhibitors (319).

However, when prolonged disease control can be achieved, disease progression, secondary to acquired resistance to antineoplastic treatments, eventually occur. Multiple resistance mechanisms to targeted therapies have been characterized, shedding a light on the evolution of cancer cells under treatment pressure (320). This has subsequently guided the development of novel compounds capable of overcoming these barriers to provide patients with new therapeutic alternatives (173,321).

As new treatments are developed, cancer cells will consequently adapt to sustain tumor proliferation and dissemination (322). Hence, it is necessary to design research strategies intended to systematically study resistance mechanisms to cancer therapies.

Herein, we report the study design and feasibility of the MATCH-R study, a prospective single institution trial, designed to identify novel mechanisms of acquired resistance in patients with advanced cancer treated with molecular targeted agents and immunotherapy.

METHODS

Study Design and eligibility criteria.

The MATCH-R trial (NCT02517892) is a prospective, single institution study held at Gustave Roussy Cancer Campus. The primary objective of this study is to characterize molecular mechanisms of acquired resistance to targeted therapies and immunotherapy in patients with advanced cancer by high-throughput next generation sequencing (NGS) and the development of patient derived xenografts (PDX) and cell lines. Patients must have achieved either an initial response, defined as partial response (PR) or complete response (CR) by RECIST 1.1, or stable disease (SD) of at least 24 weeks, and develop disease progression while actively receiving molecular targeted therapy or immunotherapy. Key eligibility criteria for study inclusion are summarized in Table 1.

All patients participating in the study are fully informed and sign an informed consent. The study was approved by an institutional review committee and was conducted in accordance with the Declaration of Helsinki. Demographic and clinical data are prospectively collected together with pathology records and integrated with molecular analysis and translational research studies.

Inclusion Criteria
Unresectable or metastatic cancer diagnosis
Treatment with selected targeted agents or immunotherapy.
Disease progression while actively on treatment after achieving an initial response to treatment (defined as a partial or complete response by RECIST 1.1 or stable disease lasting longer than 24 weeks).
Progressing tumor lesion accessible to core biopsies, including malignant pleural effusion and ascites.
The interval of time between the last dose of the selected therapy and the tumor biopsy should be less or equal to one month
Available tumor tissue, acquired before the initiation of the selected therapy.
Exclusion Criteria
Clinical contraindications to biopsy procedure (coagulation abnormalities).

Table 1. Key inclusion and exclusion criteria

Baseline or pre-treatment samples are obtained either from diagnostic formalin fixed paraffin embedded (FFPE) pathology blocks or from fresh biopsies if available. Post-progression tumor samples are obtained by core biopsies stored as frozen samples and embedded in paraffin (Figure 1), as well as from serosal effusions. If considered safe, concomitant target lesions with stable disease are biopsied and analyzed to compare genetic alterations driving disease progression in subclonal populations. The target lesions undergo several biopsies to provide adequate material for pathological diagnosis, complete molecular profiling and to develop patient-derived models. Importantly, blood sampled are collected longitudinally throughout the treatment and at progression in selected patients for circulating tumor DNA (ctDNA) sequencing.

The expected events for the primary objective are the apparition of new molecular alterations, the disappearance of existing alterations, the change in the proportion of cells with the alteration or significant change in the allele frequency.

The molecular events are grouped by gene at the patient level. The objective is to identify genes that are altered in more than 10% of the patients who develop resistance. Genes for which an event is found in at least 2 patients will be selected. We plan to study 52 patients per molecular targeted agent or molecular family of agents.

The study was amended from its original design that required only a post-treatment biopsy (cohort 1) to include specific cohorts of patients with paired pre- and post-treatment biopsies (cohorts 2-4). This aimed to increase the precision of this study in the assessment of truly acquired mechanisms of resistance of anti-cancer drugs. These cohorts include: patients treated with EGFR/ALK inhibitors in oncogene driven non-small cell lung cancer (NSCLC EGFR+/ALK+) (cohort 2), patients treated with immunotherapy for lung cancer and bladder cancer (cohort 3) and patients with prostate cancer resistant to androgen deprivation therapy (ADT) (cohort 4).

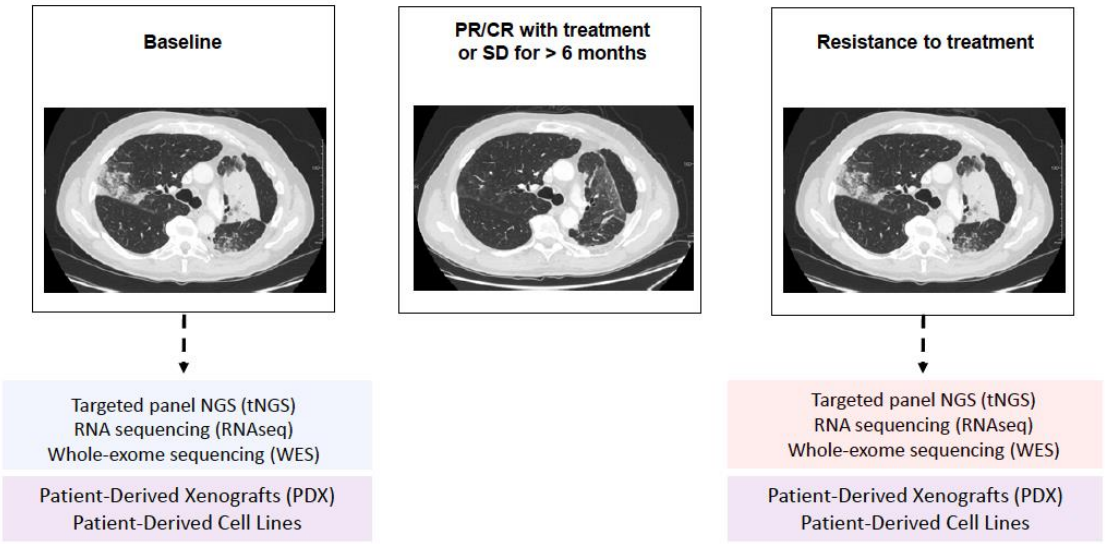

Figure 1. MATCH-R study design. Tumor biopsies are obtained at treatment resistance and at baseline. Tumor samples undergo deep molecular analysis, and some are selected for the development of patient derived xenografts.

Molecular analyses

Tumor biopsies are evaluated by senior pathologists to estimate the percentage of tumor cells, using a threshold of 10% tumor cells to perform molecular analysis. Targeted NGS is performed with the Ion Torrent PGM (ThermoFisher Scientific) sequencer using a customized panel (Mosc4) covering 82 cancer genes developed with Ion AmpliSeq custom design, as previously reported (283). The bioinformatic analysis is performed using TorrentSuite software, variantCaller (ThermoFisher Scientific). Filtering and annotations of variants are completed, and pathogenicity is defined by molecular geneticists and biologists. If the proportion of tumor cells is higher than 30%, whole exome sequencing (WES), and RNA sequencing (RNAseq) are also performed as previously reported (283,323). Of notice, the amount of molecular data from the MATCH-R study is subsequently integrated with results provided by further translational research efforts using MATCH-R patient derived models.

Establishment of patient derived models

All animal procedures and studies are performed in accordance with the approved guidelines for animal experimentation by the ethics committee at University Paris Sud (CEEA 26, Project 2014_055_2790). Fresh tumor fragments are implanted in the subrenal capsule of NOD scid gamma (NSG) or nude mice obtained from Charles River Laboratories. Xenografts are then serially propagated subcutaneously from mice to mice. From passage 3, selective pressure with the inhibitor for which the patient acquired resistance is applied, to avoid expansion of sensitive tumor cell populations. This is performed through a collaboration with the PDX-dedicated CRO (XenTech).

Patient-derived cell lines are developed from (a) patient biopsies or (b) PDX samples. (a) Patient biopsies are cut in petri dishes and incubated with Liberase™ DH Research Grade (Ref 5401054001, Sigma Aldrich) at 37°c for 1h; (b) PDX samples are processed by enzymatic digestion with the tumor dissociation kit (Ref.130-095-929, Miltenyi Biotec) and mechanic degradation

with the gentleMACs™ dissociator. Cells are cultured with DMEM/F-12+GlutamMAX™ 10% FBS and 10% enriched with hydrocortisone 0.4 µg/ml, cholera toxin 8,4 ng/ml, adenine 24 µg/ml and ROCK inhibitor 5 µM (Y-27632, S1049 Selleckchem) until a stable proliferation of tumor cells is observed, as previously described (284).

RESULTS

Study population

From January 1st 2015 and as of June 15th 2018, a total of 333 patients were included in the study (Figure 2). Thirty patients (9%) were later excluded from the analysis due to screen failure (n=5), withdrawal of consent (n=2), absence of tumor biopsy (n=12) and inadequate tumor content in the biopsy for molecular analysis (n=11) (Figure 2). From the 303 patients with adequate tumor biopsies (tumor cellularity ≥ 10%), 159 (52.5%) were included in cohort 1 (Global Match-R), 12 (4%) in cohort 2 (NSCLC EGFR+/ALK+), 57 (18.8%) in cohort 3 (Immunotherapy) and 75 (24.8%) in cohort 4 (Prostate cancer). The study is currently open to enrolment.

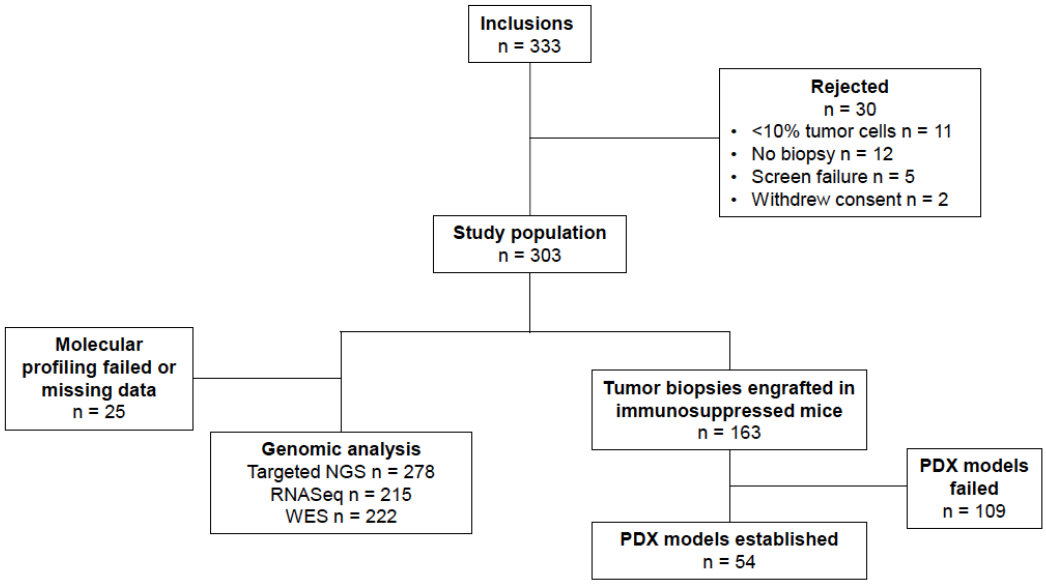

Figure 2. Study flowchart.

At the interim cut-off for feasibility assessment, median age (interquartile range) for the study population was 65 years (55-71), with a higher proportion of men (60.1%). The most common cancer types were non-small cell lung cancer (NSCLC) (n=142) followed by prostate (n=75), urothelial (n=30), gastrointestinal (n=17), gynecological (n=13) and breast cancer (n=8). Patients with less frequent tumor types were also included (Figure 3A).

Regarding the last cancer therapy received at the time of inclusion, 127 patients (42%) experienced disease progression with targeted therapies, 101 (33%) with immunotherapy and 75 (25%) with anti-androgen therapy (Figure 3B).

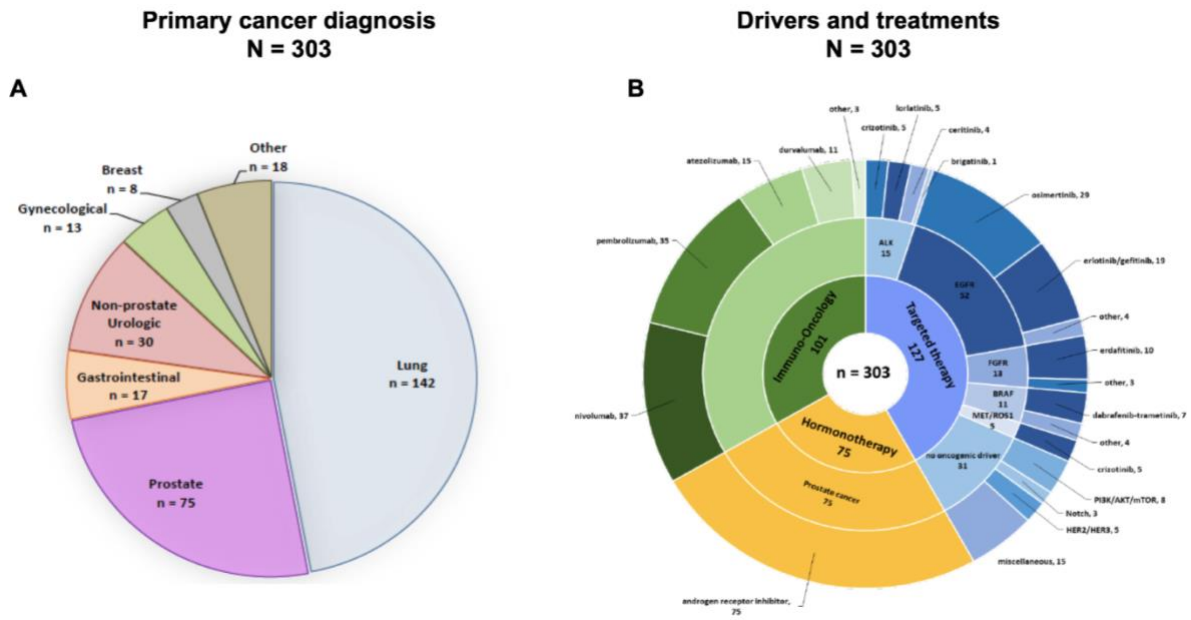

Figure 3. A, Proportion of histological sub-types and **B,** Distribution of molecular drivers and anticancer treatments of patients included in MATCH-R.

Feasibility of tumor biopsies

Among the 314 biopsies performed at the time of resistance, only 11 (3.6%) contained less than 10% tumor cells and were not inadequate for molecular profiling. Overall, the mean tumor content of all 303 biopsies that underwent NGS was 49%. In most cases, the procedure was safe and well

tolerated, and procedure-related adverse events were reported in 24 patients (7.6%), of which the most common was the development of pneumothorax (Table 2). In 12 patients that did not undergo tumor biopsy, this was due to technical or clinical factors including lack of accessible tumor sites, renal insufficiency, previous pneumothorax and anxiety, among others.

Adverse events		**n**
Total		24
Bleeding		2
Pneumothorax	All grades	14
	Grade 1	5
	Grade 2	1
	Grade 3	7
Other		10

Table 2. Adverse events related to biopsy procedure

Feasibility of molecular analysis

From the 303 biopsies with ≥ 10% tumor cell that underwent next generation sequencing, 278 (92%) were evaluable for analysis (Figure 2). Of these, all underwent successful targeted NGS, 222 samples (73%) were analyzed with whole exome and 215 (71%) with RNA sequencing. Importantly, 197 samples (65%) were fully characterized by targeted NGS, WES and RNA sequencing. These preliminary feasibility results show that systematic and complete molecular profiling of tumors that acquire resistance to different anti-cancer therapies is achievable.

Establishment of patient-derived models of resistance

Up to this interim cut-off, 163 patient tumor biopsies have been grafted in immune-deficient mice (Table 3). The success rate for the development of PDX models reached 33%, being the highest for bladder urothelial carcinomas (72.7%). The most frequently engrafted tumors were from patients with NSCLC (n=59) and castration-resistant prostate cancer (n=60) with success rates of 30% and 27%, respectively. Among the 54 established PDX models, 12 were developed from FGFR-driven tumors resistant to erdafitinib, 9 from osimertinib resistant EGFR mutant lung cancers, and 4 ALK-rearranged lung cancer after progression to lorlatinib treatment (Figure 4). In prostate PDX models, 10 grafted biopsies were obtained before hormone therapy and 6 were from anti-androgen resistant tumors, and in one case, paired pre and post-treatment PDX models were developed. The remaining established PDX models derived from patients treated with ATR, NOTCH, MEK or BRAF inhibitors.

Importantly, upon treatment with the same drugs that the patient had experience disease progression, 11/12 (91%) PDX models tested recapitulated the pharmacological response observed in patients, both from progression and stable sites (data not shown). This suggests that a timely application of selective pressure of treatment *in vivo* allows to reproduce, in preclinical models, the resistance mechanisms that were acquired in patients.

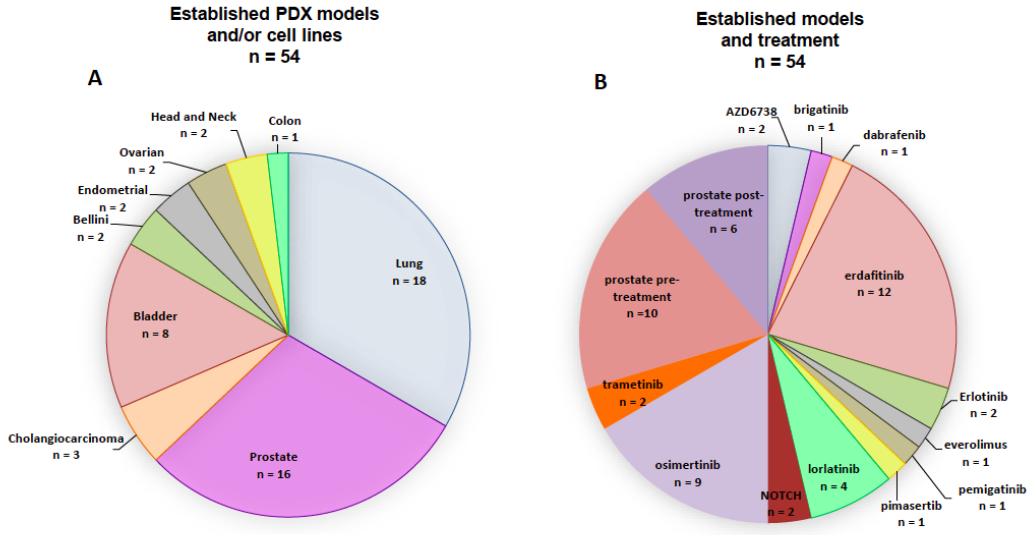

Figure 4. A, PDX and/or cell lines models developed according to histological sub-types and **B,** initial driver from patients included in MATCH-R.

Cancer Type	Tumor grafted (n)	PDX models developed (n)	Success rate (%)
Lung	59	18	30.5
Prostate	60	16	26.7
Cholangiocarcinoma	15	3	20
Bladder	11	8	72.7
Bellini Tumor	3	2	66.7
Endometrial	4	2	50
Ovarian	3	2	66.7
Head and Neck	3	2	66.7
Colon	3	1	33.3
Adenoid Cystic Carcinoma	1	0	0
Total	**163**	**54**	**33.1**

Table 3. Feasibility of the development of Patient-derived xenograft models per cancer type.

DISCUSSION

Systematic molecular profiling of tumors has been proposed as a diagnostic tool to tailor treatment according to the patient's cancer genotype and phenotype. Multiple clinical trials have been conducted to assess the clinical benefit of this approach (26,283,324,325). In the MOSCATO-01 trial, led by Gustave Roussy Cancer Campus, 33% of heavily pre-treated patients, allocated to a specific therapy based on molecular findings, achieved clinical benefit (283). The MATCH-R trial will provide new insights on acquired resistance mechanisms to a variety of antineoplastic treatments, in a wide range of cancer types. The preliminary feasibility results show that, in our platform, 92% of tumor samples with ≥ 10% tumor cells are suitable for molecular analysis, with complete molecular profiling achievable in 65% of cases. The information obtained from this study is integrated in the clinical context of the patient and discussed in molecular tumor boards to design tailored therapeutic options in the setting of resistance. When feasible, patient-derived *in vivo* and *in vitro* models of resistance are developed to further characterize mechanisms of resistance. This study uses a systematic approach to tackle this issue by optimizing logistics and standard operative procedures to provide high-throughput molecular profiling in the context of the clinical evolution of the patient. This collection of PDX/cell line models will be a useful preclinical tool to identify pivotal mechanisms underlying acquired resistance to current therapies and develop novel treatment strategies.

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2. Article III: “Making the first move in EGFR-driven or ALK-driven NSCLC: first-generation or next-generation TKI?” Nature Reviews Clinical Oncology; Volume 15, pages 694–708 (2018)

Making the first move in *EGFR*-driven or *ALK*-driven NSCLC: first-generation or next-generation TKI?

*Gonzalo Recondo[1], Francesco Facchinetti[2], Ken A. Olaussen[1], Benjamin Besse[1,3] and Luc Friboulet[1]**

Abstract | The traditional approach to the treatment of patients with advanced-stage non-small-cell lung carcinoma (NSCLC) harbouring *ALK* rearrangements or *EGFR* mutations has been the sequential administration of therapies (sequential treatment approach), in which patients first receive first-generation tyrosine-kinase inhibitors (TKIs), which are eventually replaced by next-generation TKIs and/or chemotherapy upon disease progression, in a decision optionally guided by tumour molecular profiling. In the past few years, this strategy has been challenged by clinical evidence showing improved progression-free survival, improved intracranial disease control and a generally favourable toxicity profile when next-generation EGFR and ALK TKIs are used in the first-line setting. In this Review, we describe the existing preclinical and clinical evidence supporting both treatment strategies — the 'historical' sequential treatment strategy and the use of next-generation TKIs — as frontline therapies and discuss the suitability of both strategies for patients with *EGFR*-driven or *ALK*-driven NSCLC.

The treatment of patients with lung cancer is rapidly evolving. In the past 20 years, the clinical management of these patients has shifted from a histology-based approach towards a molecularly driven approach, owing to the development of targeted therapies against the driver mutations of this disease, which affect a number of kinases[1–3]; this strategy has improved the outcomes for patients, which is important considering the high incidence and mortality of this disease[4].

Approximately 50% of Asian patients with non-small-cell lung carcinoma (NSCLC) and 11–16% of patients in Western countries harbour mutations in *EGFR*, which affect the kinase domain of EGFR[5–7]. The majority of these alterations (>90%) are deletions within exon 19 or L858R point mutation[8]. Genomic rearrangements involving the *ALK* gene occur in 3–6% of patients with NSCLC[9,10]. Other genomic alterations (in *MET*, *ROS1*, *HER2*, *BRAF*, or *RET*) are less frequent.

In the past decade, the first-generation EGFR tyrosine-kinase inhibitors (TKIs) gefitinib, erlotinib, and icotinib, and the second-generation TKI afatinib were established as standard-of-care first-line therapies for patients with NSCLC harbouring activating mutations in *EGFR*[11]. Despite high initial response and disease control rates, virtually all the patients receiving these TKIs eventually experience tumour progression owing to the emergence of therapeutic resistance[12]. Resistance to TKIs is most commonly acquired de novo during treatment, but can also occur owing to the outgrowth of pre-existing resistant subclones[13]. In approximately 50% of patients, resistance was mediated by the acquisition of the 'gatekeeper' mutation T790M, which results in sterical blockade of first-generation or second-generation TKI binding and also increases the kinase affinity for ATP[14–17]. Osimertinib is an irreversible third-generation EGFR TKI that is active against exon 19 deletions and L858R mutation, regardless of the presence of T790M mutation[18]. This TKI forms a covalent bond to the cysteine residue at position 797 and has lower activity than the aforementioned TKIs against wild-type EGFR protein. Osimertinib was initially approved by the FDA and European Medicines Agency (EMA) as the standard-of-care treatment for patients with tumours harbouring the *EGFR*^{T790M} mutation after progression upon treatment with a first-line EGFR TKI[19–21].

Since 2011, the first-generation TKI crizotinib has been the frontline treatment for NSCLC harbouring translocations involving *ALK*[22]. As with EGFR TKIs, all patients ultimately develop resistance to this agent, and secondary point mutations in the kinase domain are responsible for drug resistance in approximately 20% of patients[23]. Unlike mutations causing *EGFR* resistance, a diverse range of mutations in *ALK* affect the kinase domain, and their incidence increases to 56% with

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Key points

- Patients with *EGFR*-driven or *ALK*-driven non-small-cell lung carcinoma (NSCLC) benefit from therapies targeting those alterations, but relapse occurs systematically.
- Several generations of tyrosine kinase inhibitors (TKIs) have been developed to address the acquisition of therapeutic resistance.
- The historical treatment approach involving sequential administration of TKIs is associated with long overall survival durations.
- Clinical evidence from the past few years indicates that the use of next-generation TKIs in the frontline setting is associated with major improvements in progression-free survival, control of intracranial disease and tolerability.
- For most patients with *EGFR*-driven or *ALK*-driven NSCLC, the choice of first-line of treatment should favour next-generation TKIs.

sequential exposure to ALK TKIs[23]. Ceritinib, alectinib, and brigatinib are second-generation ALK inhibitors with activity against a wide spectrum of secondary resistance mutations affecting the ALK kinase domain[24–26]. These TKIs were first developed in the setting of crizotinib resistance, in which they had shown potent activity in preclinical studies[24–26]. Similarly, lorlatinib, a third-generation ALK inhibitor, has been developed to be administered after progression following treatment with first-generation and/or second-generation TKIs[27]. In this Review, 'next-generation TKI' refers to the third-generation *EGFR* TKI osimertinib, the second-generation *ALK* TKIs ceritinib, alectinib, and brigatinib, and the third-generation *ALK* TKI lorlatinib.

In the 'historical' sequential treatment approach, patients with NSCLC receive frontline therapy with a first-generation TKI and 'switch' to next-generation TKIs and/or chemotherapy upon disease progression. In 2017, however, next-generation inhibitors have emerged as treatment options in the first-line setting, on the basis of the increased efficacy observed when directly compared with historical first-line TKIs[28–30]. The lack of comparative survival outcomes has hampered the elucidation of the most beneficial strategy for patients in the long term. Herein, we present the evidence currently available on the antitumour activity of EGFR and ALK TKIs, reported in both preclinical and clinical studies, and discuss the advantages and drawbacks of both strategies for patients with *EGFR*-driven or *ALK*-driven NSCLC.

Historical approach: sequential treatment

EGFR TKIs. The publication of two studies in 2004 (REFS[31,32]) describing the predictive value of sensitizing mutations in *EGFR* on the activity of EGFR inhibitors is a key landmark in the development of potent drugs to treat molecularly selected patients with NSCLC[31,32]. Multiple phase III trials comparing the first-generation EGFR TKIs erlotinib, gefitinib, or icotinib, as well as the second-generation TKI afatinib, with platinum-based chemotherapy as frontline therapies for patients with advanced-stage disease have been reported[33–50] (TABLE 1). A consistent benefit in favour of EGFR TKIs is observed across studies in terms of progression-free survival (PFS), response rates, and disease control rates. The median PFS with these compounds ranged from 8.0–13.1 months, compared with 4.6–6.9 months with chemotherapy (range of HRs 0.16–0.48). Given this impressive PFS benefit, an important overall survival benefit was expected[51]. Nevertheless, median overall survival durations were equivalent for both trial arms across studies (19.3–34.8 months), predominantly owing to the high rates of treatment crossover (54–95%). The findings of these studies also provided the first demonstration that, in the context of oncogene addiction, the clinical benefit derived from treatment with TKIs is independent of whether the patients were treated upfront or after first-line chemotherapy.

For the treatment of patients with the most frequent *EGFR* mutations (L858R and exon 19 deletions), the choice of a first-generation or second-generation EGFR inhibitor depends on the physician's preference, the toxicity profile, and the local availability of each agent. No differences in the efficacy of erlotinib, gefitinib, or afatinib in terms of PFS and overall survival have been detected in comparative studies (CTONG 0901 (REF.[52]) and LUX-Lung 7 (REFS[53,54])). Icotinib has been demonstrated to be non-inferior to gefitinib, leading to its approval in 2014 in China as a frontline treatment for patients with advanced-stage *EGFR*-mutant NSCLC but its development in Western countries was not pursued[45]. In the ARCHER 1050 trial, dacomitinib, another second-generation irreversible EGFR TKI, was associated with longer PFS and overall survival durations than gefitinib (34.1 months versus 26.8 months, HR 0.76; $P = 0.044$)[55,56] (TABLE 1). This improvement was achieved at the cost of higher toxicity (frequency of grade 3 adverse events 63% versus 41%) and a detrimental effect on quality of life (QOL)[55]. Similarly, the addition of erlotinib to bevacizumab extended PFS duration for an average of 6 months compared with erlotinib monotherapy[47], although again at the expense of increased toxicity (frequency of grade 3 adverse events 91% versus 53%); the combination regimen was approved by the EMA in 2016 as a first-line treatment option[46].

For patients treated with first-line EGFR TKIs, blood-based and/or tumour sampling analysis upon disease progression is mandatory to study the T790M mutational status, owing to the clinical benefits shown for patients in this subgroup who received sequential treatment with osimertinib in multiple studies[18,21,57–59] (TABLE 1). In the AURA 3 randomized phase III trial[21], for example, osimertinib was associated with better median PFS durations and overall response rates (ORRs) than cisplatin plus pemetrexed in the second-line setting (TABLE 1). In comparison with the chemotherapy regimen, patients receiving osimertinib also had an improved QOL, with better scores for lung cancer symptoms and a lower incidence of grade ≥3 adverse events (23% versus 47%). At a median follow-up duration of 8.3 months, 71% of patients receiving chemotherapy had crossed over to receive osimertinib after disease progression, and the median overall survival had not been reached in either treatment arm. The extended benefit of the sequential administration of a first-generation EGFR TKI followed by osimertinib observed in this study[21] drove the approval of this compound for patients with NSCLC harbouring the T790M mutation and disease progression after treatment with first-generation or second-generation EGFR TKIs.

Table 1 | **Clinical trials testing EGFR TKIs in sequential strategy**

Trial	Trial design (phase, primary end point and treatment arms, including number of patients harbouring *EGFR* mutations)[a]	Median follow-up duration (months)	Outcomes (ORR, median PFS and median OS)	Refs
First generation				
IPASS	• III • PFS • Gefitinib (n = 132) versus carboplatin + paclitaxel (n = 129)	17	• 71.2% versus 47.3% • 9.5 mo versus 6.3 mo (HR 0.48; P < 0.001) • 21.6 mo versus 21.9 mo (HR 1.00; P = 0.99)	33,34
First-SIGNAL	• III • OS • Gefitinib (n = 26) versus cisplatin + gemcitabine (n = 16)	35	• 84.6% versus 37.5% • 8 mo versus 6.3 mo (HR 0.54; P = 0.086) • 27.2 mo versus 25.6 mo (HR 1.04)	35
WJTOG3405	• III • PFS • Gefitinib (n = 86) versus cisplatin + docetaxel (n = 86)	34 (59.1 for OS analysis)	• 62.1% versus 32.2% • 9.2 mo versus 6.3 mo (HR 0.49; P < 0.0001) • 34.8 mo versus 37.3 mo (HR 1.25)	36,37
NEJ002	• III • PFS • Gefitinib (n = 114) versus carboplatin + paclitaxel (n = 114)	23.4	• 73.7% versus 30.7% • 10.8 mo versus 5.4 mo (HR 0.30; P < 0.001) • 27.7 mo versus 26.6 mo (HR 0.89; P = 0.48)	38,39
OPTIMAL (CTONG-0802)	• III • PFS • Erlotinib (n = 82) versus carboplatin + gemcitabine (n = 72)	25.9	• 83% versus 36% • 13.1 mo versus 4.6 mo (HR 0.16; P < 0.0001) • 22.8 mo versus 27.2 mo (HR 1.19; P = 0.27)	40,41
ENSURE	• III • PFS • Erlotinib (n = 110) versus cisplatin + gemcitabine (n = 107)	28.9 (erlotinib arm) and 27.1 (chemotherapy arm)	• 62.7% versus 33.6% • 11 mo versus 5.5 mo (HR 0.34; P < 0.0001) • 26.3 mo versus 25.5 mo (HR 0.91; P = 0.61)	42
EURTAC	• III • PFS • Erlotinib (n = 86) versus platinum + gemcitabine or paclitaxel (n = 87)	18.9 (erlotinib arm) and 14.4 (chemotherapy arm)	• 63.6% versus 17.8% • 9.7 mo versus 5.2 mo (HR 0.37; P < 0.0001) • 19.3 mo versus 19.5 mo (HR 1.04; P = 0.87)	43
BELIEF	• II • PFS • Erlotinib + bevacizumab (n = 109)	21.4	• 77% • 13.2 mo whole cohort; 16.0 mo T790M$^+$ • 28.2 months	46
JO25567	• II • PFS • Erlotinib + bevacizumab (n = 75) versus erlotinib (n = 77)	20.4	• 69% versus 64% • 16 mo versus 9.7 mo (HR 0.54; P = 0.0015) • NA	47
CTONG 0901	• III • PFS • Erlotinib (n = 128) versus gefitinib (n = 128)	22.1	• 56.3% versus 53.3% • 13.0 mo versus 10.4 mo (HR 0.81, P = 0.11) • 22.9 mo versus 20.1 mo (HR 0.84; P = 0.25)	52
CONVINCE	• III • PFS • Icotinib (n = 148) versus cisplatin + pemetrexed (up to four cycles) eventually followed by pemetrexed maintenance (n = 137)	18 (icotinib arm) and 15.7 (chemotherapy arm)	• NR • 11.2 mo versus 7.9 mo (HR 0.61; P = 0.006) • 30.5 mo versus 32.1 mo (P = 0.89)	44
ICOGEN	• III • PFS (non-inferiority in full data set) • Icotinib (n = 29) versus gefitinib (n = 39)	NA	• 62.1% versus 53.8% • 7.8 mo versus 5.3 mo (HR 0.78; P = 0.32) • 20.9 mo versus 20.2 mo (HR 1.1; P = 0.76)	45
Second generation				
LUX-Lung 3	• III • PFS • Afatinib (n = 230) versus cisplatin + pemetrexed (n = 115)	41	• 56% versus 23% • 11.1 mo versus 6.9 mo (HR 0.58: P = 0.001) • Whole cohort: 28.2 mo versus 28.2 mo (HR 0.88; P = 0.39) • Exon 19 deletion: 33.3 mo versus 21.1 mo (HR 0.54; P = 0.002)	48,50
LUX-Lung 6	• III • PFS • Afatinib (n = 242) versus cisplatin + gemcitabine (n = 122)	33	• 66.9% versus 23% • 11.0 mo versus 5.6 mo (HR 0.28; P < 0.0001) • 23.1 mo versus 23.5 mo (HR 0.93; P = 0.61)	49,50
LUX-Lung 7	• IIB • PFS, TTF and OS • Afatinib (n = 160) versus gefitinib (n = 159)	42.6	• 70% versus 56% • 11.0 mo versus 10.9 mo (HR 0.73; P = 0.017) • 27.9 mo versus 24.5 mo (HR 0.86; P = 0.26)	53,54
ARCHER-1050	• III • IRC-assessed PFS • Dacomitinib (n = 227) versus gefitinib (n = 225)	31.3	• 75% versus 70% • 14.7 mo versus 9.2 mo (HR 0.59; P < 0.0001) • 34.1 mo versus 26.8 mo (HR 0.76; P = 0.0438)	55,56

Table 1 (cont.) | **Clinical trials testing EGFR TKIs in sequential strategy**

Trial	Trial design (phase, primary end point and treatment arms, including number of patients harbouring *EGFR* mutations)[a]	Median follow-up duration (months)	Outcomes (ORR, median PFS and median OS)	Refs
Third generation				
AURA (dose-escalation and expansion cohorts)	• I • Safety and efficacy • Osimertinib • First line (n = 60 patients), second line or beyond (n = 193)	19.1 and NA	• 77% and 61% • 20.5 mo and 9.6 mo • NA	18,120
AURA (extension cohort)	• Phase II • ORR • Osimertinib (n = 201) • Second line or beyond, prior treatment with erlotinib (58%), gefitinib (58%) and/or second-generation EGFR TKI (24%)	13.2	• 62% • 12.3 mo • Pooled analysis OS: 26.8 mo • Median treatment exposure: 16.4 mo	57,59
AURA 2	• Phase II • ORR • Osimertinib (n = 210) • Second line or beyond, prior treatment with erlotinib (57%), gefitinib (58%) and/or second-generation EGFR TKI (20%)	13.0	• 70% • 9.9 mo • Pooled analysis OS: 26.8 mo • Median treatment exposure: 16.4 mo	58,59
AURA 3	• Phase III • ORR • Osimertinib (n = 279) versus platinum + pemetrexed (n = 140) • Second line, prior treatment with gefitinib (59%), erlotinib (34%) or afatinib (7%)	8.3	• 71% versus 31% • 10.1 mo versus 4.4 mo (HR 0.30; P < 0.001) • NA	21

IRC, independent review committee; mo, months; NA, not available; NR, not reported; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; T790M+, patients with NSCLC harbouring T790M mutation in *EGFR*; TKI, tyrosine-kinase inhibitor; TTF, time to treatment failure. [a]Line of treatment only stated for third-generation inhibitors; all the first-generation and second-generation inhibitors were tested in the first-line setting.

ALK TKIs. Crizotinib is a first-generation TKI of ALK, MET, and ROS1, and was the first agent to be approved for the treatment of patients with NSCLC harbouring ALK translocations[60–63]. Two randomized phase III trials established the superiority of crizotinib over chemotherapy in patients with advanced-stage NSCLC, either as a first-line therapy[22] or in patients with disease progression after receiving a platinum-based regimen[64]. In the PROFILE 1014 study[22], greater response rates and median PFS durations were achieved with crizotinib than with platinum-based therapy (TABLE 2). Again, no significant differences in overall survival were observed, with a 4-year survival of 56.6% with crizotinib and 49.1% with chemotherapy[65]. This effect was mostly due to the high crossover rates (84.2%) from crizotinib to the experimental arm. In an exploratory analysis, after adjusting for crossover, the median overall survival was 59.8 months with crizotinib and 19.2 months with chemotherapy.

Sequential treatment strategies with ALK inhibitors have been developed with the aim of extending overall survival durations[61–63,66–81] (TABLE 2). Unlike EGFR inhibitors, a wide repertoire of ALK TKIs is available for patients with disease progression after treatment with crizotinib; the second-generation ALK TKIs ceritinib, alectinib, and brigatinib have been developed to overcome most resistance mechanisms[24–26]. Treatment with ceritinib was associated with improved outcomes compared with second-line chemotherapy (ORR 39.1% versus 6.9%, and a median PFS gain of ~4 months) in patients with disease relapse after receiving crizotinib and platinum-based chemotherapy[72] (TABLE 2). In the same disease setting, the results of the phase III ALUR trial[77] and the phase II ALTA trial[76] demonstrated beneficial outcomes with alectinib and brigatinib, respectively (TABLE 2). The third-generation ALK TKI lorlatinib has activity against resistance mutations arising after treatment with first-generation and/or second-generation TKIs, including the G1202R mutation[27]. Lorlatinib has been tested in a dose-escalation phase I study[66] and in a phase II trial[81] (TABLE 2).

One of the major concerns in the management of patients with *ALK*-translocated tumours is the high risk of developing brain metastases; 22–33% of patients present with central nervous system (CNS) involvement at diagnosis, and the prevalence of brain metastases increases to 45–70% upon progression on crizotinib treatment[68,69,73,75,82]. The improved CNS activity of second-generation and third-generation ALK TKIs results from both their higher CNS penetration and increased potency compared with crizotinib[27]. Intracranial responses have been observed in 45% of patients receiving ceritinib[69], 64% of those receiving alectinib[83], and 67% treated with brigatinib[84]. Brigatinib was associated with an intracranial PFS of 18.4 months with the standard dose[84]. Importantly, even 42% of patients in a heavily pretreated cohort (≥2 lines of ALK TKIs) had intracranial disease control with lorlatinib, and the cerebrospinal fluid concentration documented for lorlatinib was 75% of the plasma concentration[66].

Translational studies of resistance

A number of 'back-to-benchside' studies have been conducted with the aim of characterizing the mechanisms underlying clinical resistance to EGFR or ALK TKIs. The results from these studies can provide a

Table 2 | **Clinical trials testing ALK TKIs in sequential strategy**

Trial	Trial design (phase, primary end point and treatment arms, including number of patients and dosing schedule when relevant)[a]	Median follow-up duration	Outcomes (ORR, median PFS and OS)	Refs
First generation				
PROFILE 1001	• I • ORR, DOR, TTR, PFS, 6–12 mo OS, and safety profile • Crizotinib (n = 149)	16.3	• 60.8% • 9.7 mo • 1-year OS 74.8%	61,62
PROFILE 1005	• II • ORR • Crizotinib (n = 1069)	NA	• 54% • 8.4 mo • 21.8 mo	63
PROFILE 1007	• III • PFS • Crizotinib (n = 173) versus pemetrexed or docetaxel (n = 174)	12.2 mo (crizotinib) and 12.1 mo (chemotherapy)	• 65% versus 20% • 7.7 mo versus 3.0 mo (HR 0.49; P < 0.001) • 20.3 mo versus 22.8 mo (HR 1.02; P = 0.54)	64
PROFILE 1014	• III • PFS • Crizotinib (n = 172) versus platinum + pemetrexed (n = 171)	46 mo	• 74% versus 45% • 10.9 mo versus 7.0 mo (HR 0.45; P < 0.001) • NR (45.8 mo–NR) versus 47.5 mo (32.2 mo–NR; HR 0.76; P = 0.048)	22,65
Second generation				
ASCEND-1	• I • MTD • Ceritinib (n = 246) • First line (33%) or second line after crizotinib (66%)	11.1 mo	• 72% or 56% • 18.4 mo or 6.9 mo • NR or 16.7 mo	67,68
ASCEND-2	• II • ORR • Ceritinib (n = 140) • Second line after crizotinib	11.3 mo	• 38.6% • 5.7 mo • 14.9 mo	69
ASCEND-3	• II • ORR • Ceritinib (n = 124) • First line	8.3 mo	• 63.7% • 11.1 mo • NA	70
ASCEND-4	• III • PFS • Ceritinib (n = 189) versus platinum + pemetrexed (n = 187) • First line	19.7 mo	• 72.5% versus 26.7% • 16.6 mo versus 8.1 mo (HR 0.55; P < 0.00001) • NE (29.3 mo–NE) versus 26.2 mo (22.8 mo–NR; HR 0.73; P = 0.056)	71
ASCEND-5	• III • PFS • Ceritinib (n = 115) versus pemetrexed or docetaxel (n = 116) • Second line after crizotinib	16.5 mo	• 39.1% versus 6.9% • 5.4 mo versus 1.6 mo (HR 0.49; P < 0.0001) • 18.1 mo versus 20.1 mo (HR 1.00; P = 0.5)	72
AF-001JP	• I/II • DLT and MTD (phase I) or ORR (phase II) • Alectinib (n = 46) • First line	36 mo[b]	• 93.5% • NR; 3-year PFS: 62% • NE; 3-year OS: 78%	73,74
AF-002JG	• I/II • Recommended phase II dose • Alectinib (n = 47) • Second line after crizotinib	4.2 mo	• 55% • NA • NA	75
NP28761/ NP28673	• II • ORR • Alectinib (n = 225; n = 189 evaluable for response) • Second line after crizotinib	92.3 weeks	• 51.3% • 8.3 mo • 29.1 mo	76,146
ALUR	• III • PFS • Alectinib (n = 72) versus docetaxel or pemetrexed (n = 35) • Second line after crizotinib	6.5 mo	• 37.5% versus 2.9% • 9.6 mo versus 1.4 mo (HR 0.15; P < 0.001) • 12.6 mo (9.7 mo–NR) versus NR (NR–NR; HR 0.89)	77
NCT01449461	• Recommended phase II dose (phase I) or ORR (phase II) • Brigatinib (n = 79) • First line (10%, n = 8), second line after crizotinib (85%, n = 68) or third line after crizotinib and ceritinib (5%, n = 3)	>31 mo[b]	First-line brigatinib (n = 8): • 100% • 34.2 mo • NR (2-year OS 100%) Brigatinib after crizotinib (n = 71): • 73% • 13.2 mo • 30.1 mo (2-year OS 61%)	78,79

Table 2 (cont.) | **Clinical trials testing ALK TKIs in sequential strategy**

Trial	Trial design (phase, primary end point and treatment arms, including number of patients and dosing schedule when relevant)[a]	Median follow-up duration	Outcomes (ORR, median PFS and OS)	Refs
Second generation (cont.)				
ALTA	• II • ORR • Brigatinib 90 mg daily (n = 112) versus brigatinib standard dose[c] (n = 110) • Second line after crizotinib	19.6 mo (90 mg daily) or 24.3 mo (standard dose)	• 46% versus 56% • 9.2 mo versus 15.6 mo • 29.5 mo (18.2 mo–NR) versus 34.1 mo (27.7 mo–NR)	80,147
Third generation				
NCT01970865	• I • MTD • Lorlatinib (n = 41) • First line (2.4%), second line (34.2%), third line (56.1%) or fourth line (7.3%)	17.4 mo	• 46% • 9.6 mo (whole cohort), 13.5 mo (second line), and 9.2 mo (third line and beyond) • NA	66,81
	• II • ORR • Lorlatinib (n = 228) • First line (13.1%), second line or beyond, prior treatment with crizotinib only (11.8%), crizotinib + chemotherapy (14.1%), non-crizotinib ALK TKI (12.3%), any two ALK TKIs (28.5%), or any three ALK TKIs (20.2%)	NA	• 90%, 69%, 33% or 39% • NR, NR, 5.5 mo after treatment with ALK inhibitor other than crizotinib, and 6.9 mo after ≥2 lines of ALK TKIs • NA	66,81

DLT, dose-limiting toxicity; DOR, duration of response; mo, months; MTD, maximum tolerated dose; NA, not available; NE, not estimable; NR, not reported; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; TKI, tyrosine-kinase inhibitor; TTR, time to treatment recurrence. [a]Line of treatment stated for second-generation and third-generation inhibitors; all the first-generation inhibitors were tested in the first-line setting. [b]Updated presented data from the original publication. [c]90 mg daily for 7 days and then 180 mg daily.

rationale for optimizing sequential treatment strategies, because a better understanding of the biological implications of therapeutic resistance can guide clinicians to provide the most adequate treatment upon disease progression (FIG. 1).

As discussed, the acquisition of the gatekeeper T790M mutation in *EGFR* is the most common mechanism of resistance to first-generation EGFR TKIs (detected in 50–60% of patients)[12,14,85,86]. The activation of 'bypass' signalling mechanisms is also relevant in this scenario, and involves potential therapeutic targets, such as MET, AXL, IGF1R, and other members of the EGFR family[87–90]. Resistance to third-generation EGFR TKIs has also been described[91]: the most common tertiary mutation in *EGFR* is C797S in 24–40% of patients, which affects the covalent binding site of osimertinib[92–94]. This tertiary mutation can be present in *cis* or *trans* with the T790M mutation[95]. The results of preclinical studies suggest that combinations of brigatinib or other novel EGFR inhibitors with anti-EGFR monoclonal antibodies are an effective treatment option when C797S is present in *cis*[96,97]. Resistance dependent on the presence of the tertiary mutation in *trans* can be overcome by combining first-generation and third-generation EGFR TKIs[98,99].

A range of secondary mutations affecting the kinase domain of ALK confer resistance to different ALK TKIs. The following mutations have been implicated in resistance to crizotinib: G1269A, C1156Y, E1210K, I1171T, L1152R, S1206C/Y, I1151T/N/S, F1174C/L/V, V1180L, and L1196M[23,100–104]. F1174C/L/V, 1151Tins, L1152P, and C1156Y mutations are associated with resistance to ceritinib[24]. Both V1180L and I1171T/N/S alterations confer resistance to alectinib, and double mutations in E1210K and S1206C or D1203N have been reported in patients with resistance to brigatinib[23,105]. G1202R is the most common resistance mutation emerging on treatment with second-generation ALK inhibitors and is only targetable with lorlatinib[23,27,106,107]. Interestingly, the acquisition of both the C1156Y and L1198F mutations upon lorlatinib treatment has been reported to resensitize the tumour to crizotinib[108]. After the description of this initial case report, the results of the first extensive preclinical and clinical study of mutations causing resistance to lorlatinib were published in 2018 by Yoda and colleagues[109]. Using *N*-ethyl-*N*-nitrosourea-generated mutagenesis screening to determine the secondary mutations in *ALK* that can arise upon lorlatinib treatment, these investigators found that single mutations in *ALK* cannot cause resistance to lorlatinib. Indeed, only double *ALK* mutations in *cis* were detected upon resistance to lorlatinib, both in preclinical experiments and in patient-derived samples. Thus, observations of the stepwise accumulation of resistance mutations in *ALK* suggest that upfront treatment with lorlatinib could markedly delay the onset of on-target resistance, leading to a more durable clinical benefit than the current sequential treatment approach.

Off-target resistance mechanisms, such as bypass pathway activation, have also been reported in patients with resistance to first-generation and second-generation ALK TKIs[23,102,110]. The results of preclinical studies revealed that treatment with second-generation ALK TKIs could overcome resistance to crizotinib that develops without the acquisition of secondary mutations in *ALK*[24]. This observation mainly suggests that crizotinib has lower inhibitory potency against ALK than do second-generation ALK TKIs, facilitating tumour growth upon modest activation of bypass signalling mechanisms. By contrast, treatment with lorlatinib does not overcome resistance to second-generation

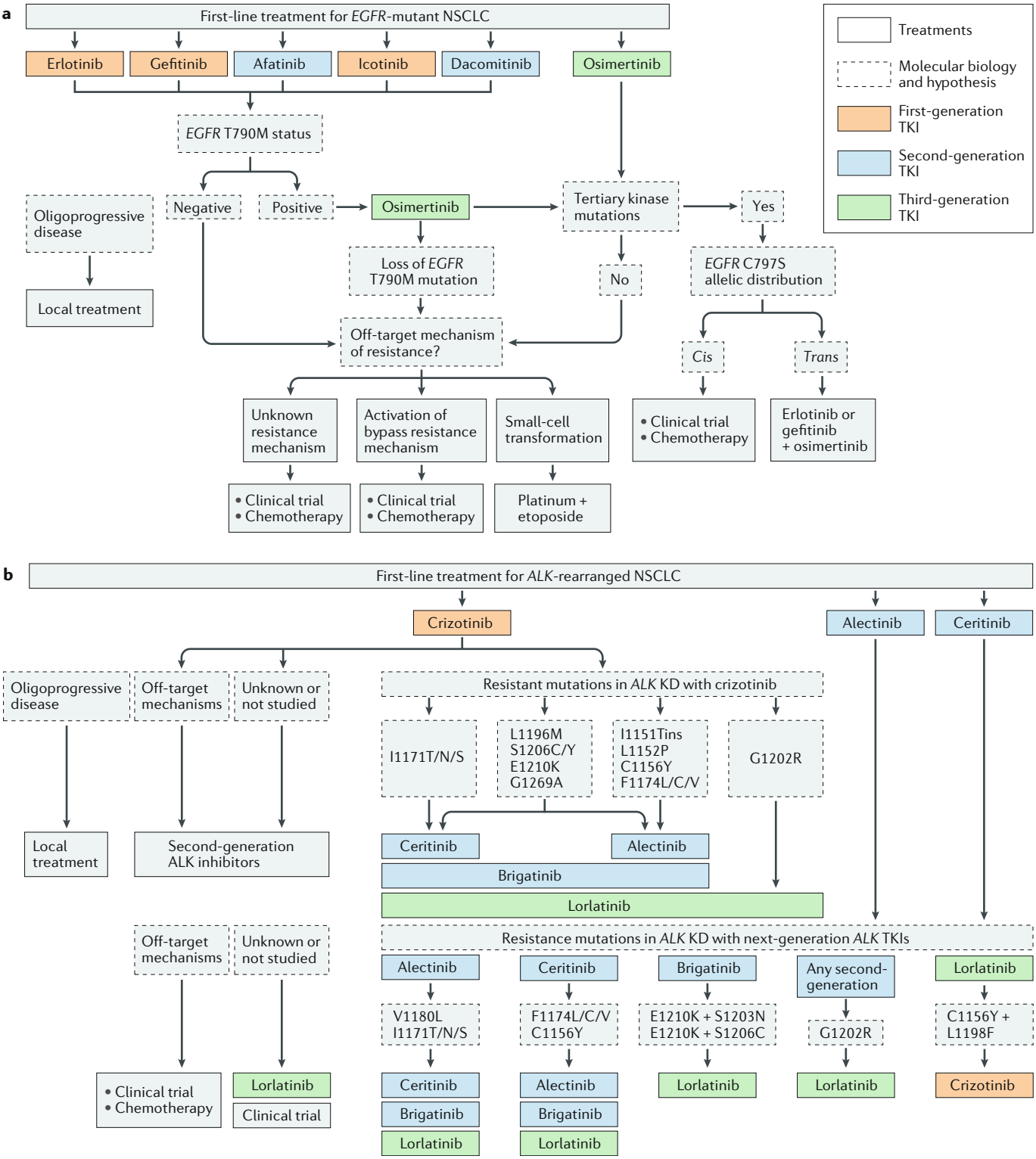

Fig. 1 | **Biomarker integration in the management of patients with NSCLC.** This chart depicts the optimal sequencing strategies for the selection of frontline tyrosine-kinase inhibitors (TKIs; either first generation or next generation), adapted to the occurrence of secondary mechanisms of resistance in patients with non-small-cell lung carcinoma (NSCLC) harbouring *EGFR* mutations (part **a**) or *ALK* rearrangements (part **b**). KD, kinase domain.

TKIs mediated by off-target mechanisms[23,27]. On the basis of these observations, bypass mechanisms involving robust oncogenic pathways, such as MAP2K1, SRC, EGFR, or PI3K, that are activated upon treatment with second-generation ALK TKIs have been proposed to also drive resistance to third-generation ALK TKIs[23].

A series of laboratory studies have focused on the brain penetration of both EGFR and ALK TKIs.

In studies using mouse models, alectinib was superior to crizotinib in controlling metastatic disease in the CNS[111]; moreover, responses to lorlatinib were observed even in mice with disease progression after alectinib treatment[27]. Importantly, evidence from several of these preclinical studies suggests that next-generation TKIs provide optimal long-term outcomes when used as frontline treatments[19,26,27].

Other preclinical studies were aimed at providing a biological rationale to explain systematic relapse in patients treated with TKIs despite major initial responses. Several studies have shown that a small subpopulation of tumour cells (<5%) cultured in the presence of a TKI remain alive and are reprogrammed into a drug-tolerant state[112–117]. These cells, with limited or no growth during months of TKI treatment, are referred to as 'persister' cells and provide a reservoir of cells from which drug-resistance mechanisms could emerge. Initial studies have suggested that epigenetic reprogramming of TKI-persister cells involves the histone demethylase KDM5A and thus could be selectively targeted by histone deacetylase inhibitors[112]. The results of preclinical studies indicate that persister cells can later cause tumour regrowth through the de novo acquisition of diverse genetically driven resistance mechanisms, such as secondary mutations or activation of bypass signalling[113,115]. Eradicating persister cancer cells early during the course of treatment might therefore block or drastically postpone the onset of resistance. Persister cells display an impaired apoptotic response to TKI (as assessed by annexin V staining)[115], and, thus, treatment with inhibitors of the BCL-2 family anti-apoptotic proteins has been proposed to be a potentially effective therapeutic strategy; the combination of osimertinib and navitoclax is currently being tested in patients with NSCLC harbouring the *EGFR* T790M mutation (NCT02520778)[115]. Two studies with results published in 2017 revealed a common persister-cell-specific dependency on the lipid hydroperoxidase GPX4, targeting of which prevented tumour relapse in mice[116,117].

Finally, tumour heterogeneity occurs early in the course of cancer progression: in patients with resectable NSCLC, a median of 30% of the somatic mutations detected are subclonal[118]. Tumour heterogeneity is an important factor contributing to the development of therapeutic resistance because it contributes to both the selective expansion of pre-existing resistant clones and the adaptive resistance of persister tumour cells[115]. In patients with NSCLC harbouring *EGFR* mutations and with disease progression after a first-generation or second-generation TKI, the allelic fraction of T790M mutations can, for instance, affect the therapeutic response to third-generation EGFR TKIs[119]. Observations in patients treated with osimertinib[95] or lorlatinib[109] indicate that clones resistant to third-generation TKIs can emerge upon sequential treatment with first-generation and second-generation EGFR or ALK TKIs, affecting the choice of the next optimal treatment strategy. In line with these observations, preclinical and clinical studies performed during first-line treatment with third-generation ALK and EGFR TKIs revealed that the emergence of resistance driven by on-target mutations can be delayed[19,27,120]. Mice bearing EGFR[19] and ALK[27] TKI-sensitive tumours treated with first-generation and third-generation inhibitors showed prolonged tumour responses and delay of resistance with third-generation TKIs. In two cohorts of patients with *EGFR*-mutated NSCLC treated with upfront osimertinib in the phase I AURA study, none of the evaluable patients had disease progression owing to T790M mutation[120]. Overall, in addition to enabling the interpretation of the outcomes of clinical studies, the studies discussed herein highlight the importance of characterizing the molecular mechanisms of resistance to TKIs during or after each line of treatment using blood or tissue sampling to inform clinical decision-making.

Paradigm shift for first-line therapy

The historical trend in the management of patients with cancer has been to move more-potent, more-specific, and possibly less-toxic drugs to the first-line treatment setting. Similarly to chemotherapy, the magnitude of efficacy of next-generation TKIs generally increases in accordance with an earlier administration during the course of treatment with targeted therapies[21,28,29,71,72,77]. Indeed, several single-arm early phase trials in patients with NSCLC who had not received any previous TKI showed prolonged disease control upon first-line treatment with osimertinib[120], ceritinib[70], or alectinib[74] (in comparison with data available for first-generation and second-generation TKIs). In 2017, additional evidence of major PFS benefits emerged from three phase III trials, supporting the upfront use of next-generation TKIs over the standard first-line EGFR TKIs and crizotinib (TABLE 3).

EGFR TKIs. In the randomized phase III FLAURA study[28], osimertinib was compared as a frontline therapy with the standard choice of gefitinib or erlotinib in patients with NSCLC harbouring *EGFR* exon 19 deletions or L858R point mutation[28]. As expected, the median PFS was significantly prolonged by almost 9 months with osimertinib compared with first-generation TKIs (HR 0.46; $P < 0.001$), although the ORRs were similar between trial arms (TABLE 3). The median time to second-line treatment or death was 23.5 months with osimertinib and 13.8 months with first-line EGFR TKI, and the median time to third-line treatment was not reached and 25.9 months, respectively. Brain imaging was mandatory at study entry, as well as during the course of the study for patients with brain metastases; at study entry, 19% of patients in the osimertinib arm and 23% in the control arm had brain metastases. Fewer patients treated with osimertinib had disease progression in the CNS (6% versus 15%) or extracranial disease progression (38% versus 54%), compared with the control arm[28]. The benefit in PFS was maintained for patients with brain metastases (15.2 months with osimertinib versus 9.6 months with first-generation TKIs; HR 0.47; $P < 0.001$). Osimertinib was better tolerated than first-line TKIs (34% versus 45% of patients had grade 3 adverse events). Accordingly, the rate of treatment discontinuation was 13% in the osimertinib arm compared with

Table 3 | **Clinical trials comparing first-generation and next-generation TKIs in the frontline setting**

Trial	Trial design (phase, primary end point and treatment arms, including number of patients and dosing schedule when relevant)	Median follow-up duration	Outcomes (ORR, median investigator-assessed PFS, median IRC-assessed PFS, OS and grade ≥3 AEs)	Refs
ALK TKIs				
ALEX	• III • Investigator-assessed PFS • Alectinib (n = 152; 600 mg b.i.d.) versus crizotinib (n = 151)	22.8 mo (alectinib arm) and 27.8 mo (crizotinib arm)[a]	• 82.9%[a] versus 75.5% • 25.7 mo (95% CI 19.9 mo–NE) versus 10.4 mo (95% CI 7.7–14.6 mo; HR 0.50; P < 0.001); 34.8 mo[a] versus 10.9 mo (HR 0.43; 95% CI 0.32–0.58) • 1-year OS 84.3% versus 82.5% (HR 0.76; P = 0.24) • 44.7%[a] versus 51%	29,121
J-ALEX	• III • IRC-assessed PFS • Alectinib (n = 103; 300 mg b.i.d.) versus crizotinib (n = 104)	12 mo (alectinib arm) and 12.2 mo (crizotinib arm)	• 92% versus 79% • NA; HR 0.34 (95% CI 0.21–0.55) • Not reached (95% CI 20.3 mo–NE) versus 10.2 mo (95% CI 8.2–12.0 mo; HR 0.34; P < 0.0001) • NA (immature data) • 26% versus 52%	30
EGFR TKIs				
FLAURA	• III • Investigator-assessed PFS • Osimertinib (n = 279) versus gefitinib or erlotinib (n = 277)	15 mo (osimertinib arm) and 9.7 mo (first-generation TKI arm)	• 80% versus 76% • 18.9 mo versus 10.2 mo (HR 0.46; P < 0.001) • 17.7 mo versus 9.7 mo (HR 0.45; P < 0.001) • 18 mo OS 83% versus 71% (HR 0.63; P = 0.007, nonsignificant owing to immature data) • 34% versus 45%	28

AE, adverse event; b.i.d., twice daily; mo, months; IRC, independent review committee; NA, not available; NE, not estimable; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; TKI, tyrosine-kinase inhibitor.[a] Updated data.

18% in the control arm. Of note, QT interval prolongations were more frequent with osimertinib than with first-line TKIs (10% versus 4%). In this trial, crossover to subsequent treatment with osimertinib was permitted in patients in whom the T790M mutation was detected after progression upon treatment with first-generation EGFR TKIs. Among the 129 patients who received treatment after disease progression in the control arm, 48 patients (37%) crossed over to receive treatment with osimertinib; data on the overall survival of patients who received treatment after disease progression are eagerly awaited.

ALK TKIs. Ceritinib was the first second-generation TKI approved as a first-line treatment option for patients with *ALK*-rearranged NSCLC on the basis of the superior efficacy over platinum-based chemotherapy observed in the ASCEND-4 study[71] (TABLE 2). In this study, the incidence of grade 3–4 adverse events was higher with ceritinib than with chemotherapy (65% versus 40%), but treatment discontinuations owing to toxicity occurred in 5% of patients treated with ceritinib versus 11% in the control arm. This study was designed before crizotinib was established as standard first-line therapy in this disease setting; taking toxicities into consideration, ceritinib remains a valid option for first-line treatment. Encouraging results from a phase I/II trial of brigatinib (NCT01970865) include a median PFS of 34.2 months in 8 patients treated upfront with this agent[78]. In another phase II trial, the ORR was 90% in a cohort of 30 patients receiving frontline lorlatinib and the median PFS had not been reached at the time of reporting; mature results of this ongoing study will provide further insight into the clinical outcomes derived from lorlatinib treatment[81].

Alectinib is the first ALK inhibitor that was compared against crizotinib in the first-line setting in two randomized studies: the phase III trials J-ALEX[30], conducted in Japan, and the international ALEX trial[29] (TABLE 3). None of the patients enrolled in J-ALEX had been previously treated with an ALK TKI, but 36% of them had received chemotherapy. Alectinib was associated with a significant PFS benefit (TABLE 3), as well as a more favourable toxicity profile than crizotinib: grade 3 adverse events were reported in 26% of patients receiving alectinib versus 52% of those receiving crizotinib, and fewer patients required dose interruptions (29% versus 74%) or toxicity-related treatment suspensions (9% versus 20%).

All the patients enrolled in the ALEX trial[29] received alectinib in the frontline setting. The median PFS duration and ORR were higher with alectinib than with crizotinib; according to the last update[121], median PFS was 34.8 months with alectinib and 10.9 months with crizotinib (HR 0.43; 95% CI 0.32–0.58 months). Crossover was not permitted in the study protocol, hampering the direct comparison of outcomes obtained by administering alectinib using sequential or upfront strategies. One strength of this study[29], however, was the evaluation of CNS activity through mandatory brain MRI at study entry and every 8 weeks during treatment. Baseline brain metastases were detected in 42% of patients allocated to receive alectinib and in 38% of patients in the crizotinib group. Patients with measurable CNS metastases had an intracranial response rate of 81% (45% of them being complete responses) with alectinib and 50% (9% complete responses) with crizotinib. The median duration of CNS responses was 17.3 months with alectinib and 5.5 months with crizotinib, and the 12-month cumulative incidence of brain metastases was significantly

Box 1 | **Arguments supporting different frontline treatment strategies**

Arguments in favour of using first-generation tyrosine-kinase inhibitors (TKIs) upfront

- Mature follow-up data available supporting long survival for patients treated with sequential TKIs
- Multiple subsequent treatment options available in the event of resistance

Arguments in favour of using next-generation TKIs upfront derived from studies comparing with first-generation TKIs

- In preclinical studies: longer disease control in mice
- Reduced toxicity in most cases
- Enhanced therapeutic activity in the central nervous system
- Prolonged progression-free survival
- Reduced need for subsequent molecular diagnostic

lower with alectinib than with crizotinib (9.4% versus 41.4%), showing that alectinib provides superior control against the development of brain metastases compared with crizotinib. Interestingly, the difference in PFS between arms can be mainly attributed to the higher rates of CNS-related disease progression with crizotinib, because no significant differences in extra-CNS progression rates were observed between arms (24% and 22% with alectinib and crizotinib, respectively). Comparative trials of crizotinib with brigatinib (NCT02737501), lorlatinib (NCT03052608), or ensartinib (NCT02767804) will provide further information on the efficacy of all next-generation ALK TKIs in the first-line setting.

Choice of upfront treatment strategy

With the management of patients with advanced-stage *EGFR*-driven and *ALK*-driven NSCLC on the verge of a paradigm change, the risk–benefit balance of choosing between sequential treatment or next-generation upfront strategies needs to be taken into consideration when optimizing treatment strategies. Several arguments favour each strategy, and, thus, the choice remains complex (BOX 1).

Traditional sequential approach. This approach has been in place for a longer time than the next-generation upfront strategy, and, thus, sufficient data support an impressive long-term survival with therapies involving sequencing TKIs. The long-term benefit of providing sequential therapies is based on the response rates and the duration of PFS that can be achieved with next-generation inhibitors upon resistance to first-generation TKIs. In patients with NSCLC harbouring mutations in *EGFR*^{T790M}, a pooled analysis update of the AURA 2 and AURA extension studies[59] revealed a median global overall survival of 26.8 months. The 2-year overall survival was 56% for the entire cohort. The mature survival outcomes of the AURA 3 study[21] and data on treatment outcomes from the ASTRIS study[122] have not yet been published; these results should provide insight into the clinical benefits derived from osimertinib treatment in patients with *EGFR*^{T790M}-mutated NSCLC.

In patients with *ALK*-rearranged NSCLC, results from the PROFILE 1014 trial showed, at a median follow-up duration of 46 months, that median survival was not reached (95% CI 45.8 months–not reached) and that 4-year overall survival was 56.6% in patients treated with crizotinib, of whom 33% received subsequent next-generation TKIs[65]. The French national IFCT-1302 retrospective study[123] analysed the survival outcomes of 318 patients with *ALK*-rearranged NSCLC involved in an expanded crizotinib access programme[123]. In this study, 31.9% of patients received the second-generation *ALK* inhibitors ceritinib or alectinib after disease progression on frontline crizotinib. The median overall survival duration from the first dose of crizotinib was not reached for patients who received sequential treatment, and 3-year survival was 59.2% (both ceritinib and alectinib analysed together). Impressively, the median overall survival from the time of diagnosis of metastatic NSCLC was 89.6 months. This duration is highly superior to that observed in patients with NSCLC not driven by alterations in *EGFR* or *ALK* and treated with chemotherapy in 'real-world' settings (~10 months)[124].

The studies discussed support the notion that effective sequential strategies with upfront first-generation inhibitors can lead to impressive overall survival in some patients with NSCLC in which the driver alterations have been characterized; whether upfront next-generation inhibitors could provide a similar long-term benefit remains to be established. The available preclinical and clinical evidence suggests that no clinical benefit is derived from treatment with first-generation TKIs after disease progression on next-generation TKI treatment, with the exception of *ALK* L1198F[108], MET amplification[125], and *EGFR* C797S mutation in *trans*[95], thus limiting the availability of targeted therapeutic options when next-generation inhibitors are used upfront.

Next-generation ALK and EGFR TKIs upfront. This therapeutic option is associated with prolonged PFS durations, improved disease control in the CNS, and a more favourable toxicity profile than treatment with first-generation TKIs — providing a major argument in favour of upfront treatment with next-generation TKIs. In the ALEX[29] and FLAURA[28] studies, the difference in the incidence of grade 3 adverse events with first-generation versus next-generation TKIs was ~10%, favouring the latter. With the upfront administration of next-generation TKIs, T790M or secondary *ALK* mutational screening does not need to be performed on a continuous basis, an approach that is convenient in centres where repeated molecular diagnosis is not available. Indeed, the medical practice environment needs to be considered in decisions of the best therapeutic strategy for patients. Close monitoring and timely access to molecular diagnostics and treatment options are essential to providing optimal care.

In the ALEX[29] and FLAURA[28] studies, alectinib and osimertinib showed greater efficacy in the treatment of brain metastases than first-generation TKIs; thus, these agents should be considered for patients in this setting[126–128]. The prevention or delay of the onset of brain metastases is key to controlling morbidity and reducing the needs and costs for localized CNS therapies[129]. In this context, the results of the ongoing evaluation of

responses to frontline lorlatinib are awaited[81]. Indeed, results from studies in mouse models suggest that frontline lorlatinib could dramatically delay the emergence of resistance, including those with brain metastases[27]. Despite having superior potency and the widest spectrum of activity against secondary mutations, lorlatinib might not replace alectinib as the standard-of-care ALK TKI in the first-line setting because of its association with an increased incidence of neurological adverse effects; lorlatinib, however, might represent the ideal second-line treatment option after disease progression on alectinib.

An important argument in favour of using next-generation upfront originates from the emerging evidence from studies of persister cells. An intuitive hypothesis is that a 'hitting hard first' strategy would help to limit the number of drug-tolerant cells that would later lead to disease progression; however, to our knowledge, direct comparisons of the persistence capacities of cancer cells treated with first-generation or next-generation TKIs have not been performed. Understanding the molecular mechanisms supporting the viability of these cells and how they can be targeted therapeutically are key questions that have not yet been solved.

Another key aspect that remains to be elucidated is whether frontline treatment with next-generation TKIs can decrease the emergence of subclonal heterogeneity involving TKI resistance mechanisms, either with a mutational or non-mutational component. Importantly, the existence of intratumour heterogeneity is evidenced by simultaneous oncogenic alterations that can mediate resistance to EGFR or ALK TKIs, including the co-occurrence of *EGFR* with *ALK* alterations or *ALK* with *KRAS* alterations, which present a challenge for treatment selection[23,130–134]. To address this issue, multiple combinations of ALK or EGFR TKIs with other kinase inhibitors targeting MET (NCT02143466), MEK (NCT03392246, NCT03087448, NCT03202940, and NCT02143466), JAK (NCT02917993 and NCT03450330), mTOR (NCT02503722 and NCT02321501), SRC (NCT02954523), AXL (NCT03255083) or CDK4/6 inhibitors (NCT03455829 and NCT02292550), or apoptotic modulators, such as navitoclax (NCT02520778), are ongoing. The aim of these strategies is to revert, delay or prevent the onset of off-target resistance. In addition, several studies have intended to modulate the antitumour immune response by combining an EGFR or ALK TKI with anti-programmed cell death 1 (PD-1) and/or anti-programmed cell death 1 ligand 1 (PD-L1) monoclonal antibodies, which generally lack efficacy as single agents in patients with oncogene-addicted NSCLC[135]. Nevertheless, toxicity issues have already hampered the development of combinations of osimertinib with durvalumab and of crizotinib with nivolumab. In the phase Ib TATTON study, recruitment into the combination arm (osimertinib plus durvalumab) was closed owing to the occurrence of interstitial lung disease in 38% of patients[136]. In the multicohort phase I/II CheckMate 370 trial, the combination of nivolumab and crizotinib was associated with severe hepatic toxicity in 38% of patients, with two adverse-event-related deaths[137]. By contrast, preliminary data of the combination of crizotinib or lorlatinib with avelumab and of alectinib with atezolizumab have shown an acceptable safety profile[138,139].

Integrative strategy. In the absence of survival data after disease progression from head-to-head comparative trials, investigators rely on the sum of PFS from studies held in different therapy lines to establish comparisons. This provocative approach is not supported statistically[140] but can provide an estimation, in the absence of valid surrogates, of the theoretical benefit of sequential targeted therapies in patients with advanced-stage NSCLC (FIG. 2).

Relying on the results from clinical trials[22,69,72,77,80], patients with *ALK*-translocated NSCLC would derive a median PFS of 16–25 months from frontline crizotinib followed by a next-generation ALK TKI, compared with 34.8 months with alectinib[121]. Likewise, patients with *EGFR*-mutated NSCLC would derive a PFS benefit ranging from 21–27 months[21,36,41,47,53] with sequential treatment, a value close to the 18.9 months reported for frontline osimertinib in the FLAURA study[28]. Of note, chemotherapy is the standard treatment for patients with T790M-negative NSCLC with disease progression after receiving first-generation EGFR TKIs. For these patients, the median PFS with cisplatin-based chemotherapy after progression upon treatment with first-line EGFR TKIs was reported to be 5.4 months[141]; thus, frontline treatment with a first-generation TKI would provide a slightly inferior sum of PFS than frontline osimertinib.

In addition, a subset of patients treated with TKIs can develop oligoprogressive disease. In this scenario, and especially in the setting of brain metastasis, patients can benefit from a 6-month gain in PFS when local ablative treatments (such as surgery or radiotherapy) are applied[142]. These local ablative treatments are crucial because they enable the continuation of previously administered systemic therapies, delaying the switch to the next treatment line and prolonging systemic disease control.

The economic burden of novel drugs can also influence the choice of upfront TKIs — for example, osimertinib is more expensive than afatinib[143]. In the absence of definitive evidence of meaningful overall survival benefits, the prolonged administration of costly therapeutic agents might not be easily accepted by regulatory authorities.

In this new era, a growing need exists for the development of clinical trials to enable further understanding of the best sequential therapeutic strategy in the setting of advanced-stage NSCLC. Monitoring resistance onset using sequencing of circulating cell-free DNA can provide new insights into the effect of early treatment of subclinical resistance[144]. In the setting of *EGFR*-mutated NSCLC, the ongoing phase II APPLE trial[145] will shed light on this matter, evaluating the overall survival outcomes of patients treated sequentially with a first-line EGFR TKI and switching to osimertinib upon progression, compared with treatment with osimertinib upfront.

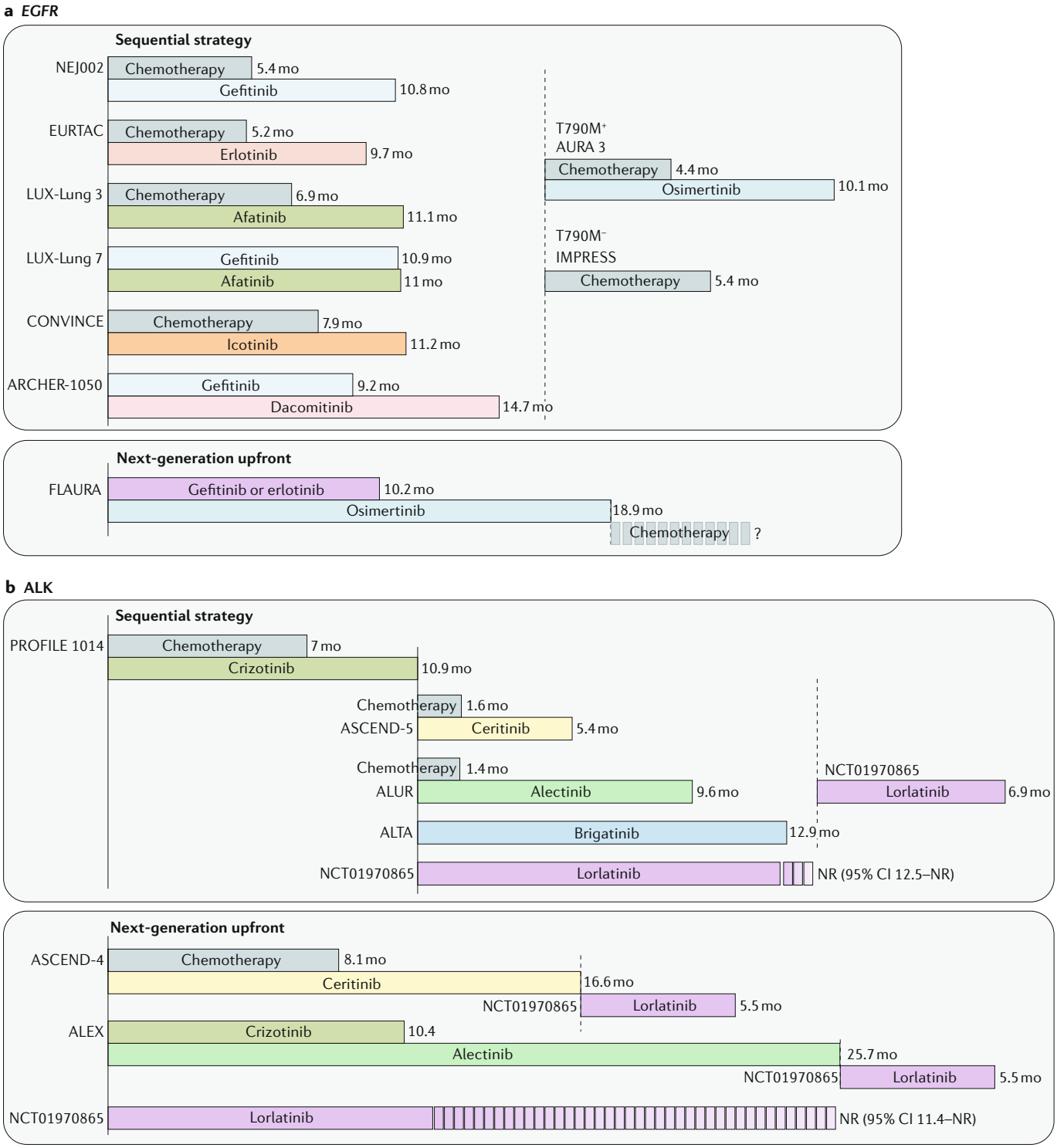

Fig. 2 | **Comparison of PFS results in selected clinical trials testing TKI sequencing in NSCLC.** Sum of progression-free survival (PFS) durations in different trials of frontline tyrosine-kinase inhibitors (TKIs) in patients with non-small-cell lung carcinoma (NSCLC) harbouring *EGFR* mutations (with first-generation, second-generation and next-generation TKIs) (part **a**) or *ALK* rearrangements (with first-generation and next-generation TKIs) (part **b**). mo, months; NR, not reported; T790M$^-$/T790M$^+$, negative/positive for the T790M mutation in *EGFR*.

Conclusions

At present, the optimal approach for the selection of a frontline EGFR or ALK TKI for patients with advanced-stage NSCLC remains a matter of debate, while results and post-progression survival analysis at longer follow-up durations from ongoing comparative trials are awaited. Both strategies have advantages and disadvantages that need to be carefully weighed (BOX 1). The currently available evidence suggests that patients with *EGFR*-mutated NSCLC could benefit from frontline

osimertinib over first-generation EGFR TKIs in terms of tolerability and efficacy, especially patients without targetable T790M mutations. Similarly, patients with *ALK*-rearranged NSCLC would derive a greater benefit from frontline alectinib than with first-line ALK TKIs in terms of tolerability, activity in the CNS, and PFS. For these patients, lorlatinib might be a favourable option for second-line treatment upon regulatory approval. Nonetheless, analysis of long-term survival outcomes of ongoing and future randomized trials, including the effect of post-progression treatments, will be key to settle what the most beneficial treatment strategy for patients with NSCLC according to the molecular profile of their tumours in order to adapt therapies to tumour dynamics.

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Title:

Resistance mechanisms to ALK tyrosine kinase inhibitors in NSCLC

Keywords:

Resistance to targeted therapies, ALK, Lung cancer

Abstract:

The molecular study and classification of lung adenocarcinomas has led to the development of selective targeted therapies aiming to improve disease control and survival in patients. The anaplastic lymphoma kinase (ALK) is a tyrosine kinase receptor from the insulin tyrosine kinase receptor family, with a physiologic role in neural development. Gene rearrangements involving the ALK kinase domain occur in ~3-6% of patients with lung adenocarcinoma. The fusion protein dimerizes leading to transactivation of the ALK kinase domain in a ligand-independent and constitutive manner.

Lorlatinib is a third generation ALK inhibitor with high potency and selectivity for this kinase in vitro and in vivo, and elevated penetrance in the central nervous system. Lorlatinib can overcome resistance mediated by over 16 secondary kinase domain mutations occurring in 13 residues upon progression to first- and second- generation ALK TKI. In addition, treatment with lorlatinib is effective for patients who have been previously treated with a first and a second generation or a second generation ALK TKI upfront and is currently approved for this indication.

The full spectrum of biological mechanisms driving lorlatinib resistance in patients remains to be elucidated. It has been recently reported that the sequential acquisition of two or more mutations in the kinase domain, also referred as compound mutations, is responsible for disease progression in about 35% of patients treated with lorlatinib, mainly by impairing its binding to the ALK kinase domain. However, the effect of these compound mutations on the sensitivity to the repertoire of ALK inhibitors can vary, and other resistance mechanisms occurring in most patients are unknown.

My PhD thesis aimed at exploring resistance to lorlatinib in patients with ALK-rearranged lung cancer through spatial and temporal tumor biopsies and development of patient-derived models. Within the institutional MATCH-R study (NCT02517892), we performed high-throughput whole exome, RNA and targeted next-generation sequencing, together with plasma sequencing to identify putative genomic and bypass mechanisms of resistance. We developed patient-derived cell lines and characterized novel mechanisms of resistance and personalized treatment strategies in vitro and in vivo.

We characterized three mechanisms of resistance in five patients with paired biopsies. We studied the induction of epithelial-mesenchymal transition (EMT) by SRC activation in two patient-derived cell lines exposed to lorlatinib. Mesenchymal cells were sensitive to combined SRC and ALK co-inhibition, showing that even in the presence of an aggressive and challenging phenotype, combination strategies can overcome ALK resistance. We identified three novel ALK kinase domain compound mutations, F1174L/G1202R, C1156Y/G1269A, L1196M/D1203N occurring in three patients treated with lorlatinib. We developed Ba/F3 cell models harboring single and compound mutations to study the differential effect of these mutations on lorlatinib resistance. Finally, we characterized a novel mechanism of resistance caused by NF2 loss of function at the time of lorlatinib progression through the development of patients derived PDX and cell lines, and in vitro validation of NF2 knock-out with CRISPR/CAS9 gene editing. Downstream activation of mTOR was found to drive lorlatinib resistance by NF2 loss of function and was overcome by providing treatment with mTOR inhibitors.

This study shows that mechanisms of resistance to lorlatinib are more diverse and complex than anticipated. Our findings also emphasize how longitudinal studies of tumor dynamics allow deciphering TKI resistance and identifying reversing strategies.

Titre

Mécanismes de résistance aux inhibiteurs de tyrosine kinase ALK dans le cancer bronchique non à petites cellules.

Mots-clés:

Resistance a therapies ciblées, ALK, Cancer du poumon

Résumé:

Les analyses moléculaires et la classification des adénocarcinomes bronchiques ont conduit au développement de thérapies ciblées sélectives visant à améliorer le contrôle de la maladie et la survie des patients. ALK (anaplastic lymphoma kinase) est un récepteur tyrosine kinase de la famille des récepteurs de l'insuline. Des réarrangements chromosomiques impliquant le domaine kinase d'ALK sont présents dans environ 3 à 6% des patients atteints d'un adénocarcinome bronchique. La protéine de fusion provoque une activation du domaine kinase de manière constitutive et indépendante du ligand.

Lorlatinib est un inhibiteur d'ALK de troisième génération avec une efficacité et une sélectivité optimale, ainsi qu'une pénétration élevée vers le système nerveux central. Lorlatinib peut vaincre la résistance induite par plus de 16 mutations secondaires dans le domaine kinase d'ALK acquises lors de la progression aux ALK TKI de première et deuxième générations. Le traitement par lorlatinib est donc efficace chez les patients préalablement traités par un ALK TKI de première ou deuxième génération, et est actuellement approuvé pour cette indication.

Le spectre complet de mécanismes de résistance au lorlatinib chez les patients reste à élucider. Il a récemment été rapporté que l'acquisition séquentielle de deux mutations ou plus dans le domaine kinase, également appelées mutations composées, est responsable de la progression de la maladie chez environ 35% des patients traités par le lorlatinib, principalement en altérant sa liaison au domaine kinase d'ALK. Cependant, l'effet de ces mutations sur la sensibilité aux différents inhibiteurs d'ALK peut varier, et les autres mécanismes de résistance survenant chez la plupart des patients restent inconnus.

Mon travail de thèse avait pour but d'explorer la résistance au lorlatinib chez des patients atteints d'un cancer du poumon ALK réarrangé par la mise en œuvre de biopsies spatiales et temporelles et le développement de modèles dérivés de patients. Dans le cadre de l'étude institutionnelle MATCH-R (NCT02517892), nous avons effectué un séquençage à haut débit de l'exome, de l'ARN et ciblé, ainsi qu'un séquençage des ctDNA afin d'identifier les mécanismes de résistance. Nous avons établi des lignées cellulaires dérivées de patients et caractérisé de nouveaux mécanismes de résistance et identifiés de nouvelles stratégies thérapeutiques in vitro et in vivo.

Nous avons identifié trois mécanismes de résistance chez cinq patients avec des biopsies appariées. Nous avons étudié l'induction de la transition épithélio-mésenchymateuse (EMT) par l'activation de SRC dans une lignée cellulaire, dérivée de deux patients, exposée au lorlatinib. Les cellules mésenchymateuses étaient sensibles à l'inhibition combinée de SRC et d'ALK, montrant que même en présence d'un phénotype agressif, des stratégies de combinaison peuvent surmonter la résistance aux ALK TKI. Nous avons identifié deux nouvelles mutations composées du domaine kinase d'ALK, F1174L/G1202R, C1156Y/G1269A et L1196M/D1203N survenues chez trois patients traités par le lorlatinib. Nous avons développé des modèles de cellules Ba / F3 exprimant les mutations simples et composées pour étudier leur effet sur la résistance au lorlatinib. Enfin, nous avons caractérisé un nouveau mécanisme de résistance provoqué par la perte de fonction de NF2 au moment de la progression du lorlatinib par l'utilisation de PDX et de lignées cellulaires dérivées de patients, et par CRISPR / CAS9 knock-out de NF2. Nous avons constaté que l'activation de mTOR par la perte de fonction de NF2 provoquait la résistance au lorlatinib et qu'elle pouvait être surmontée par le traitement avec des inhibiteurs de mTOR.

Cette étude montre que les mécanismes de résistance au lorlatinib sont plus divers et complexes que prévu. Nos résultats démontrent également comment les études longitudinales de la dynamique tumorale permettent de déchiffrer la résistance aux TKI et d'identifier des stratégies thérapeutiques.