ORIGINAL ARTICLE

ALK-positive Lung Cancer: Molecular Mechanisms and Treatment Strategies

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A note from the Author

Dear Readers,

This Essay has been written with an intention to help all the forces combating ALK-positive lung cancer to understand what we are dealing with. These forces include us, the patients, our families, our friends and perhaps members of our clinical teams. I hope you will find the content useful and I aim to update this essay regularly with new information.

Thank you ~ ***Elena Klenova***

Contents

Glossary/Abbreviations

Adenocarcinoma - type of cancer originating from epithelial cells with secretory function, for example, in the lungs these are epithelial cells secreting mucus that are lining alveoli.

***ALK** - Anaplastic Lymphoma Kinase.

ADP - Adenosine diphosphate (contains only two phosphate groups, a nitrogenous base (adenine) and a ribose sugar; it forms after a phosphate group is released from ATP).

ATP - Adenosine triphosphate (an energy rich molecule, contains three terminal phosphate groups, a nitrogenous base (adenine) and a ribose sugar.)

Cancer (malignancy) - A term for diseases in which abnormal cells divide without control and can invade nearby tissues. Cancer is always malignant.

***EML4** - Echinoderm microtubule-associated protein-like 4.

***Kinase** - a class of molecules able to add a phosphate group to other molecules (substrates); the properties and functions of the latter change after this modification.

NSCLC - Non-small cell lung cancer.

***Receptor** - a molecule, usually present at the cell surface, which receives and then further transmits a chemical signal from other cells to achieve a particular biological effect.

Tumour (neoplasia, neoplasm) - an abnormal mass of tissue that results when cells divide more than they should or do not die when they should. Tumours may be benign (not cancer), or malignant (cancer).

Substrate - the substance on which the kinase acts.

***TK** - Tyrosine Kinase, a kinase able to add a phosphate group to an amino acid, Tyrosine; (Many TKs are also RTKs!)

***RTK** – Receptor Tyrosine Kinase.

TKI - Tyrosine Kinase Inhibitor.

* According to Guidelines for Formatting Gene and Protein Names, symbols for genes are italicized (e.g., *ALK*), whereas symbols for proteins are not italicized (e.g., ALK). Symbols denoting both gene and protein are not italicized. These Guidelines will be followed in this essay.

ALK-positive Lung Cancer: Molecular Mechanisms and Treatment Strategies

1. INTRODUCTION

The main aim of this essay is to explain the molecular events underlying the development of ALK-positive lung cancer. It is a very complex process and to help understand it, in sections 2-6, general topics about cancer and mechanisms of cell response to regulatory signals will be discussed. Sections 7-9 will be focused on molecular aspects of ALK-positive lung cancer, specific medicines (TK inhibitors) developed to treat this cancer and future treatment prospects. Facts described in the essay are based on the information obtained from well-known and respected textbooks[1-3] and original articles (references will be provided as appropriate). Supplementary Table and Figures contain additional information relevant to the topics covered in this essay.

2. BASIC FACTS ABOUT LUNG ANATOMY AND LUNG CANCER

Lungs are part of the respiratory system, which allows us to breath. The main role of this system is to bring oxygen to the body and remove carbon dioxide. The general anatomy of lungs is shown in **Figure 1**[4]. Air enters the lungs via trachea (windpipe) then goes into bronchi ending in small air sacs (alveoli). The alveoli are made of several types of cells, which are responsible for the transfer of oxygen from the air into the bloodstream. Adenocarcinoma, which belongs to the category of non-small cell lung cancer (NSCLC), arises from epithelial cells secreting mucus that are lining the alveoli. ALK-positive (ALK+) lung cancer represents about four to five percent of all lung cancers, generally appearing in adenocarcinoma of NSCLC.

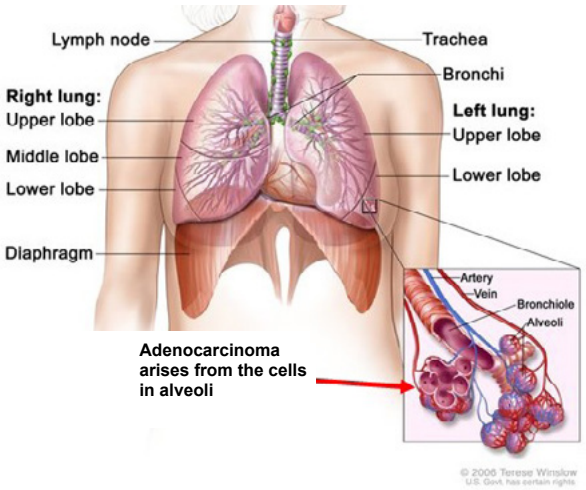


Figure 1. Structure of the respiratory system (Image obtained from[4]).

3. BASIC FACTS ABOUT CANCER

The fundamental feature of cancer is the continuous increased proliferation (division) of cancer cells. It results from cancer cells losing their ability to respond appropriately to the signals controlling behaviour of normal cells. The development of cancer is a multistep process illustrated in **Figure 2**[5]. It starts with one cell acquiring an initial mutation giving this cell the ability to multiply continuously. High rate of proliferation, which in turn increases the risk of mutations, may result in the acquisition of the second mutation in some of the descendants.

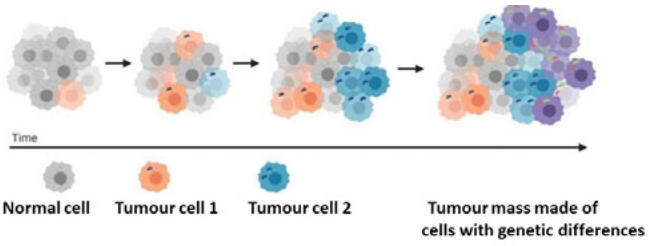


Figure 2. Multistage evolution of cancer (adapted from[5]). See the text above for explanations.

Cells with the double mutation may have selective advantages (i.e. they can proliferate faster). Additional mutations can lead to further evolution of cancer, for example, cells may gain the ability to spread (metastasize). The resulting cancer cell population is heterogeneous, i.e. it consists of cells carrying different properties. Due to such variations, it is very challenging to treat cancer since some cells may not respond to the therapies.

4. SIGNALS CONTROLLING CELL PROLIFERATION

In multi-cellular organisms, decisions about an individual cell growth and no-growth are made "in consultation" with other cells in the tissue. To communicate their messages, the latter release growth regulatory signals (hormones, growth factors etc.). Normal cells receive these signals through specific molecules on their surface, called receptors. Once bound to receptor, the signalling molecule becomes known as the ligand. In the cells, signals are transmitted, processed and integrated by a succession of signalling molecules, which are organized in signalling pathways. As a result, a specific biological response will be achieved, regulating survival, division, differentiation ("maturation") or death of the cell. Deregulation of one or more of the steps in these pathways can lead to uncontrolled cell division and the formation of a tumour. These processes are illustrated in **Figure 3**.

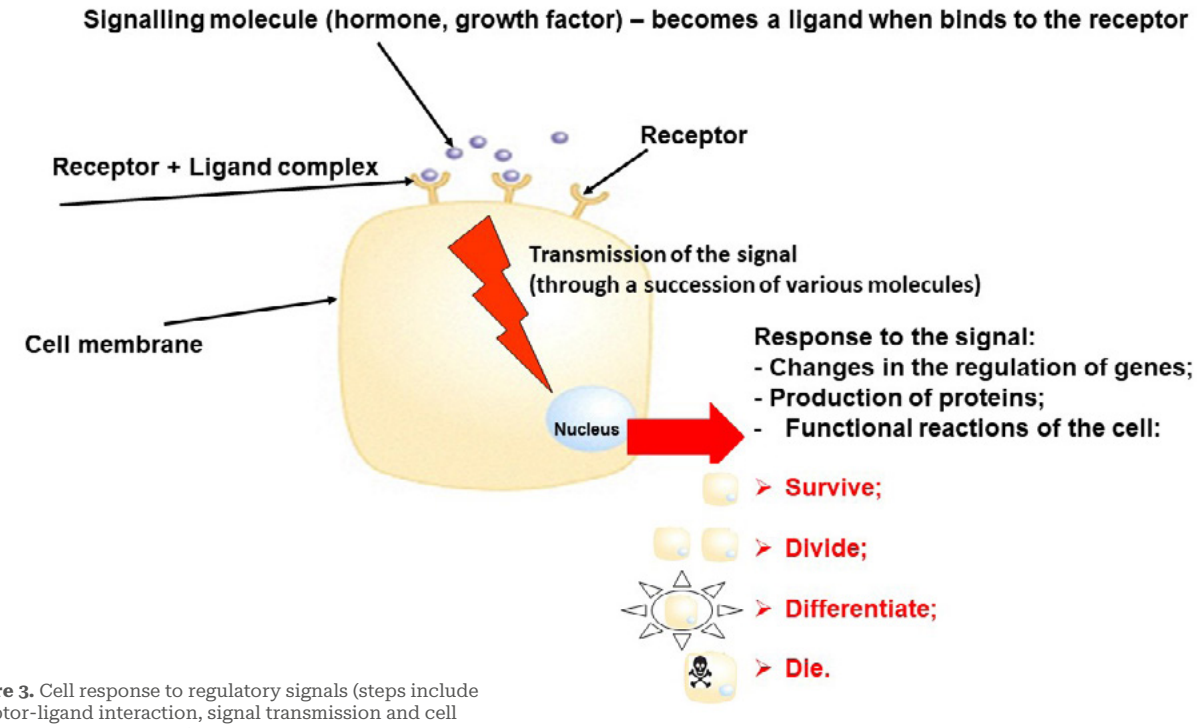


Figure 3. Cell response to regulatory signals (steps include receptor-ligand interaction, signal transmission and cell response) (see text above for details).

5. ACTIVATION OF THE SIGNALLING PATHWAYS: ROLE OF KINASES (WITH THE FOCUS ON TYROSINE KINASE)

A major role in the activation of the signalling pathway belongs to a receptor. It is usually a complex molecule, composed of several functional units (or domains). In this essay, we will focus on a class of the cell surface receptors that contain, among others, a kinase domain. What is a kinase? A kinase is a type of protein that transfers phosphate groups from high-energy donor molecules, such as ATP (Adenosine triphosphate) to specific target molecules (substrates); this process is termed phosphorylation. *A* Tyrosine Kinase (TK) is an enzyme that can transfer a phosphate group from ATP to Tyrosine residue of specific proteins (Tyrosine is one of 20 amino acids that combine to build proteins). This modification functions as an "on" or "off" switch in many cellular functions. In this reaction, Adenosine diphosphate (ADP), a molecule with two phosphate groups is also produced, after a phosphate group is released from ATP. The process of phosphorylation by TK is illustrated in **Figure 4**.

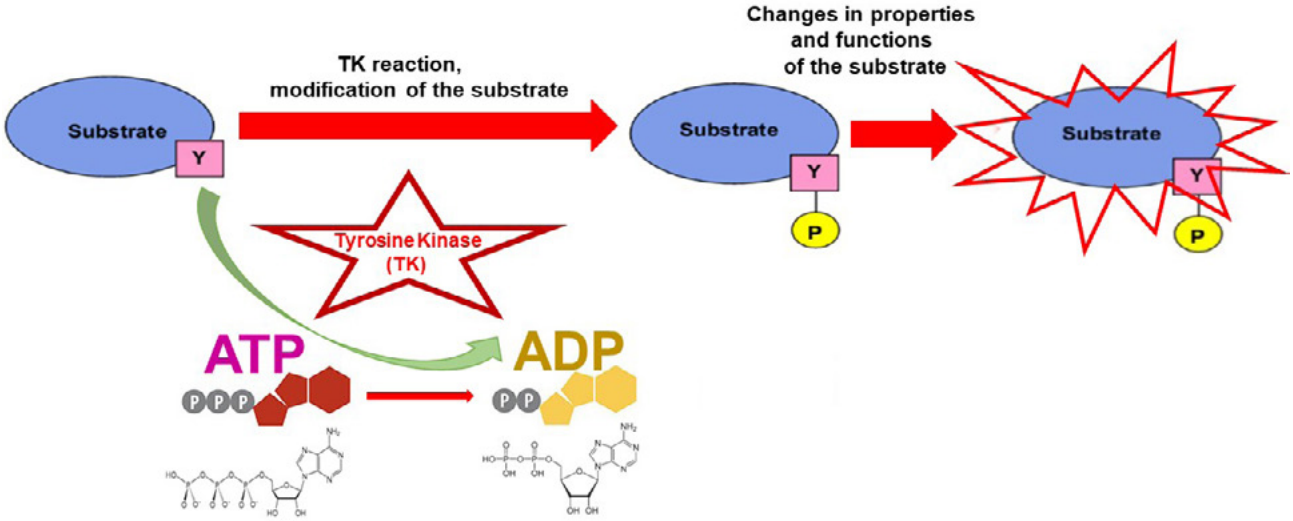


Figure 4. Modification of the substrate with Tyrosine Kinase (TK). The TK interacts with the substrate and then breaks the ATP molecule to transfer one of the phosphate groups to the amino acid Tyrosine within a protein molecule. As a result of this reaction, Tyrosine is modified, or phosphorylated, this modification changes the properties of the whole substrate molecule.

Keys: Substrate = the substance on which the kinase acts. Y = amino acid Tyrosine; P = phosphate group added to Tyrosine; ATP = an energy rich molecule with three phosphate groups; it is the donor for the phosphate group. (Other two groups are nitrogenous base (adenine) and a ribose sugar.) ADP = a molecule with two phosphate group after ATP is broken. The graphic illustrations of ATP and ADP, together with their structural diagrams, are shown.

6. IMPORTANT MOLECULAR EVENTS TAKING PLACE DURING SIGNAL TRANSMISSION THROUGH RECEPTOR TYROSINE KINASES

As discussed in the previous section, receptors are composed of several functional units (domains). As illustrated in **Figure 5A**, a typical receptor, located at the surface of a cell, contains three domains: extracellular (projected outside of the cell and responsible for binding a ligand), trans-membrane (anchoring the receptor molecule at the membrane) and kinase domain (modifying and activating the receptor). If Tyrosine is modified by the kinase domain, the latter is classified as Tyrosine Kinase (TK) domain. In the scientific literature, a receptor containing the Tyrosine Kinase domain is referred to as Receptor Tyrosine Kinase (or RTK).

In an unstimulated cell, single molecules (monomers) of the RTKs can move freely in the cell membrane. From time to time they encounter another single molecule and form temporary association (dimer). It is usually not stable and can dissociate easily. However, if a cell receives a signal, resulting in a binding of a ligand to the RTK, the bond between the monomers strengthen and the dimer becomes stable. The processes, which follow the dimer formation are described in **Figure 5B**. The ligand-dimer RTK complex can now bind ATP molecules; this takes place within a pocket in the TK domain. This binding is very specific, so that ATP fits very accurately into the cavity of the pocket. As a result, TK function is activated; this leads to the release of the phosphate group from ATP, its transfer to Tyrosine of the partner RTK and release of ADP (the details of the kinase reaction are described in the previous section and **Figure 4**). The modified RTK dimer changes its spatial structure (conformation) and it is now able to trigger subsequent events leading to changes in gene function, production of particular proteins and, finally, adequate biological response of the cell to the initial signal.

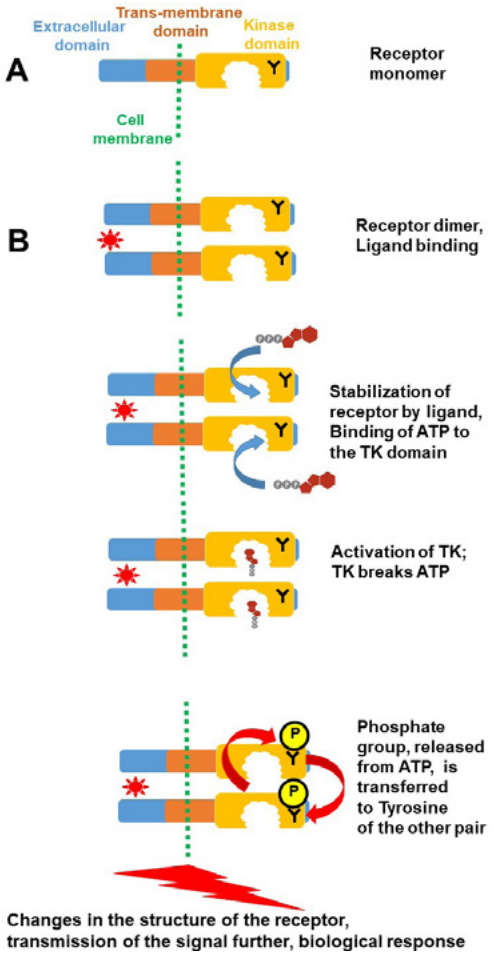


Figure 5. Figure 5. RTK/TK signalling activation. **(A)** Structure of the RTK monomer. The three functional domains are depicted in different colours (extra-cellular, blue; trans-membrane, brown; kinase, orange). **(B)** Activation of RTK/TK signalling following the ligand binding.(See the text for detail).

Keys: Octagram = ligand; Dashed green line = cell membrane; P = Phosphate: Y = Tyrosine.

7. MOLECULAR EVENTS UNDERLYING THE DEVELOPMENT OF THE ALK-POSITIVE LUNG CANCER

ALK stands for anaplastic lymphoma kinase and it is a Receptor Tyrosine Kinase (RTK). The *ALK* gene was originally identified and implicated in the development of the anaplastic large cell lymphoma[6]. Later, the rearrangements in the *ALK* gene, discovered in a subgroup of NSCLC and called ALK-positive[7], were demonstrated to be responsible for the development of lung cancer[8]. ALK belongs to the RTK family, and is composed of three domains (extra-cellular, trans-membrane and kinase) (**Figure 5A**)[9-11(and references therein)]. ALK is not normally detected in human cells and tissues, except in embryos where it controls the development of the nervous

system (**Figure 6**). There, ALK is regulated by specific ligands, and, typically for the RTKs, two ALK monomers bind to form the ALK dimer and the subsequent events follow as described in the previous section and **Figure 5**.

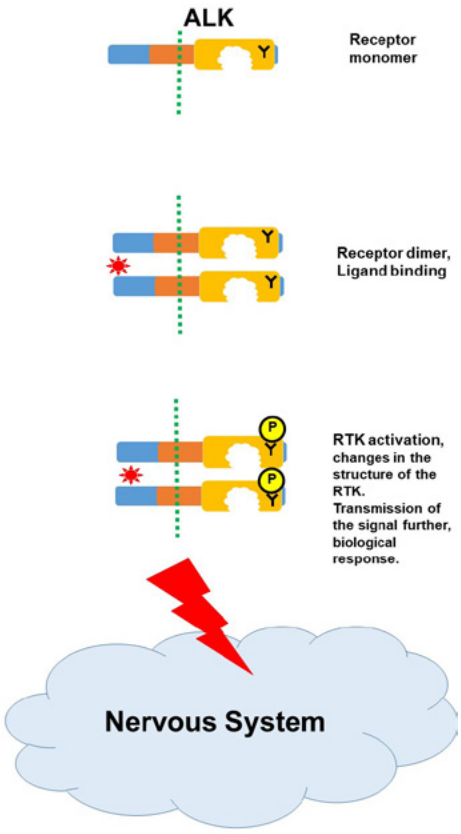


Figure 6. ALK signalling during normal embryonic development (a few key steps are shown). Specific ligands activate ALK leading to regulation of biological functions important for the growth and development of the nervous system. (Adapted from[10]). (Keys are as in **Figure 5**).

In ALK-positive lung cancer cells an abnormal configuration in the DNA was discovered, which resulted in the aberrant production of ALK in these cells[12]. Molecular analysis of the DNA from these cells showed that the *ALK* gene is fused to another gene, called echinoderm microtubule-associated protein-like 4 (*EML4*). This abnormal gene fusion results in the production of a fusion protein (EML4-ALK) that is the main driver of the malignant behaviour (**Figure 7**).

The *EML4-ALK* fusion gene always contains the kinase domain from the *ALK* gene, but the size of the fragment derived from the *EML4* gene can differ, depending on the breakage point in *EML4*. Indeed, in NSCLC, 15 different *EML5-ALK* fusion variants have been reported[14, 15 and references therein]. Different variants of *EML4-ALK* fusion partners and their frequencies are shown in the diagrams in **Supplementary Figure 1**. The fusion genes encode a hybrid protein, EML4 ALK, which can form dimers even without the presence of the extracellular ligand[12, 16, 17]. This is due to the fact that the protein regions derived from EML4 carry the features that facilitate interaction between proteins. The phosphorylation of the EML4-ALK follows, leading to activation of signalling pathways promoting cell proliferation, metastasis and inhibiting cell death. These events result in the occurrence of the ALK-positive NSCLC (**Figure 8**).

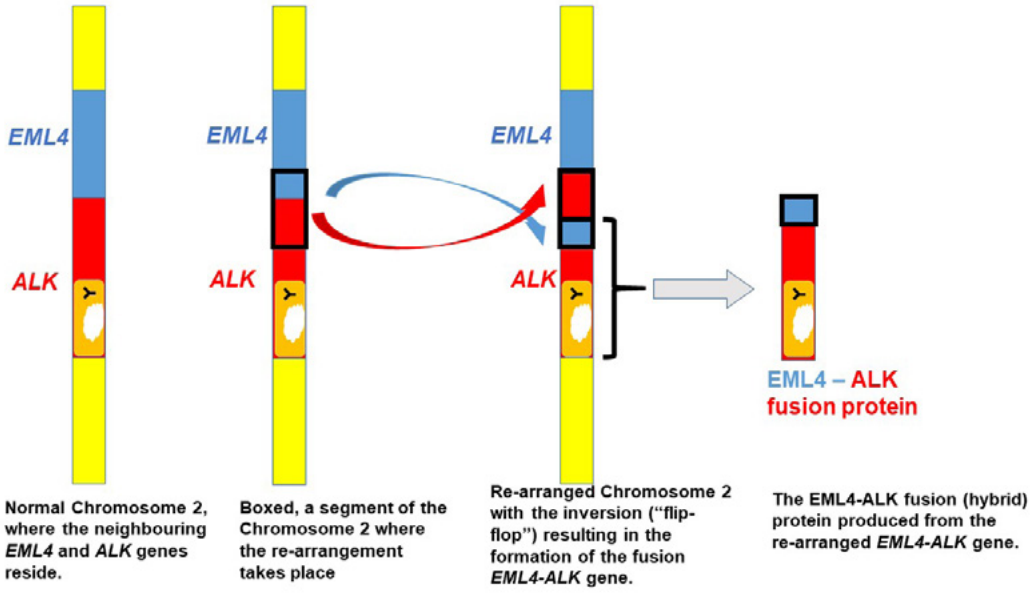


Figure 7. Generation of the *EML4–ALK* hybrid gene due to an inversion within chromosome 2. The segment with the kinase domain from ALK is now fused to a part of *EML4*, resulting in the generation of the *EML4-ALK* fusion protein.

Keys: Blue, EML4; Red, ALK; Orange, the kinase domain of ALK; Yellow, parts of Chromosome 2. Adapted from[13].

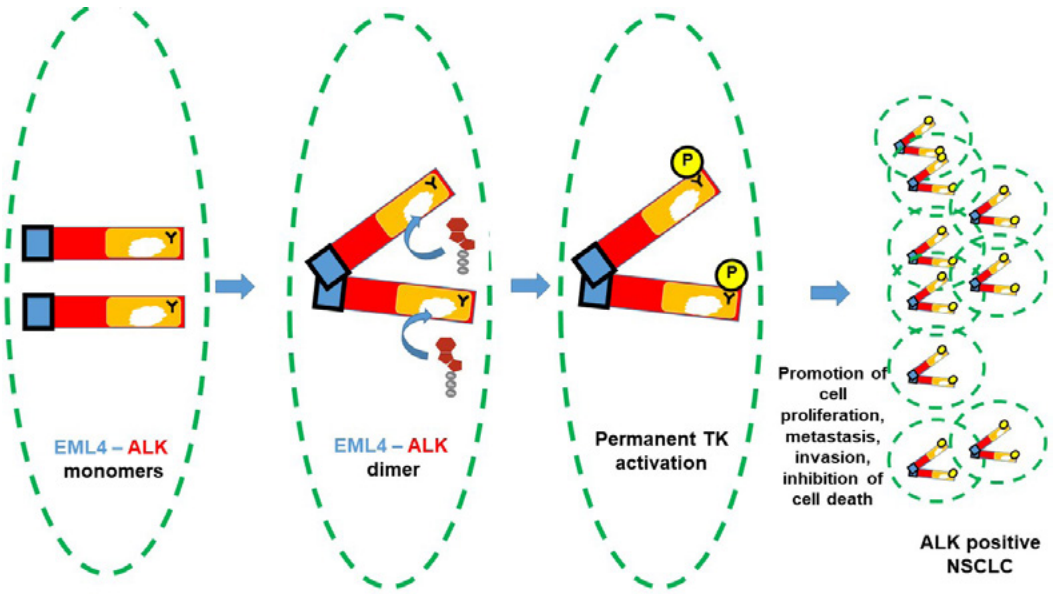


Figure 8. ALK signalling in cells producing EML4-ALK hybrid protein. The EML4-ALK protein does not have the extra-cellular domain and is located inside the cells. It does not require a ligand, it can form dimers using the EML4 surfaces, recruit ATP and be directly activated thereby triggering pathways supporting NSCLC development.

Keys: Blue, EML4; Red, ALK; Orange, the kinase domain from ALK. Green dashed line depicts the cell membrane.

8. DEVELOPMENT OF TYROSINE KINASE INHIBITORS (TKIs) AS THERAPEUTIC MEDICINES TARGETING THE ALK-POSITIVE NSCLC

Understanding of the molecular event taking place during the development of the ALK-positive NSCLC, and in particular, identification of the EML4-ALK protein as a driver of this type of cancer, paved the way to develop targeted therapies. The rationale behind these is to create molecules (inhibitors) that can block TK function in the fusion EML4-ALK protein. This will lead to its inactivation, inhibition of signalling processes, block of proliferation and even death of cancer cells. In addition, since EML4-ALK protein is only present in cancer cells and absent in normal cells, the TK inhibitors (TKIs) will only target cancer cells thereby minimizing side effects of the treatment. The TKIs resemble the ATP molecule and their role is to "trick" the TK by outcompeting ATP and preferentially binding to the TK. The small size of the TKIs is also an important factor as it helps these molecules to pass easily through the cell membrane. Structures of several TKIs used for treatment of ALK-positive NSCLC are shown in **Supplementary Table 1**; it is notable that their shapes and sizes are comparable to ATP. The TK, once TKI is bound, will not be able to process the signal since the TKI does not have correct functional groups, and hence TK will remain in the "frozen" state. As the activity of the TK is blocked, further events will not take place and there will be no functional response to the signal. This is important because cells will stop proliferating, they may even enter the death pathway and be eliminated. These processes are illustrated in **Figure 9**.

Crizotinib, the "first generation" (1G) TKI used for treatment of ALK-positive NSCLC, was approved by US FDA in 2011[18] and by UK NICE in 2014[19]. The development and approval of the second (Ceritinib, Alectinib, Brigatinib) and third (Lorlatinib) generations (2G and 3G, respectively) of TKIs followed (see the timeline in **Supplementary Figure 2**). Ensartinib is awaiting approval by US FDA (it has been approved in China[20]). These medications are highly effective targeted therapies, however, cancer cells evolve by acquiring new mutations and developing resistance leading to disease progression. A growing body of genetic data is being generated by ongoing research to understand molecular changes taking place during treatment with TKIs. One of the recent examples of molecular analysis from patients developed resistance to Crizotinib and patients developed resistance to second generations TKIs is shown in **Supplementary Figure 3**. Whereas in the initial EML4-ALK fusion protein the ALK TK domain is the same as in the original, whole ALK protein (also called "wild-type"), during treatment with TKIs single mutations accumulate. Furthermore, after the second and third TKIs the compound

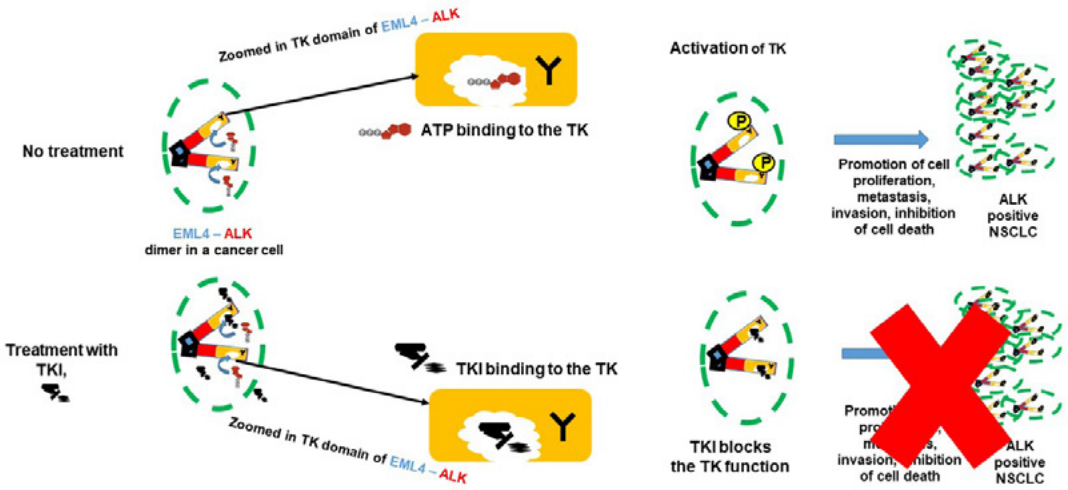


Figure 9. Inhibition of TK function by TKI. In cells, not treated with TKI, ATP binding occurs in the active site (pocket) of the TK, leading to TK activation and functional effects. Introduction of TKI leads to TKIs compete with ATP for binding to the TK active site (pocket). Keys are as in **Figure 8**.

("double") mutations occur[18, 21]. In addition to ALK mutations ("on-target"), other mechanisms, including the activation of bypass signalling pathways ("off-target"), can cause resistance. Such complex mutational landscape creates a substantial challenge for identifying optimal treatment strategies. These aspects will be briefly discussed in the next section.

9. FUTURE TREATMENT PERSPECTIVES

9.1. FUTURE TYROSINE KYNASE INHIBITORS (TKIs)

The understanding of the mechanisms of resistance to TKIs is important to design future therapies, and the accumulated knowledge has already revolutionized treatment of ALK-positive NSCLC with TKIs[18, 21, 22 and references therein]. Although the debate among clinicians, which is the best first line TKI treatment of *ALK*-positive NSCLC is still ongoing, the use of current TKIs, regardless of the sequence of their application, will eventually lead to the "double" *ALK* resistance mutations. Therefore, there is a strong rationale to identify new TKIs, and many new compounds with the desired properties have been reported in the literature[23-26]. Phase 1/2 clinical trials of fourth generation (4G) "double mutant active" TKIs, TPX-0131[27] and NVL-65[28], are currently taking place and these TKIs are anticipated to be approved.

Importantly, different genomic ALK variants and mutations demonstrate different sensitivity to therapies[29, 30 and references therein], therefore in the future it will become common practice to obtain molecular profiles of the ALK-positive NSCLC from patients throughout their treatment pathways to identify the best treatment options. New technologies, such as molecular analysis of liquid biopsies will therefore become gold standards in clinical practice[31] and provide comprehensive information about specific "on-target" and "off-target" events that cause resistance. One of the proposed future treatment algorithm for ALK-positive NSCLC is presented in **Supplementary Figure 4**[18].

A very encouraging example of clinical value of TKIs is treatment of one of blood cancers, chronic myeloid leukaemia (CML). The Philadelphia (PH) chromosome, a genetic alteration generating the fusion protein, BCR-ABL1, characterizes CML. BCR-ABL1 is a constitutively activated TK responsible for the pathogenesis of CML. Approved in 2001, Imatinib (or Gleevec), representing the prototype TKI of targeted therapy, has had spectacular success in increasing the life expectancy of CML patients, which now approaches life expectancy of the general population[32, 33 and references therein]. Until recently, therapy of CML with TKIs was not considered curative, however, various recent trial results demonstrated that a proportion of patients can stop treatment with TKI without experiencing a disease

relapse and can potentially be cured[34]. It should be noted, however, that the direct comparison of CML (blood cancer) and ALK-positive NSCLC (solid tumour) is not accurate in many aspects. Nevertheless, the knowledge gained by scientists and clinicians over more than two decades of TKIs application for CML treatment will hopefully guide and facilitate the progress in the targeted treatment of ALK-positive NSCLC.

9.2. SALVAGE SURGERY (I.E. SURGICAL RESECTION OF PERSISTENT OR RECURRENT PRIMARY LUNG CANCER AFTER PREVIOUS LOCAL TREATMENT WITHOUT SURGERY)

Local consolidative therapy (LCT), surgery or radiotherapy, for patients with stage IV NSCLC and limited metastatic disease burden has been used successfully in clinical practice[35, 36]. Could salvage surgery be an option for ALK-positive NSCLC patients treated with TKIs? The rationale for this is that despite the efficacy of TKIs, acquired drug resistance inevitably occurs, and, under current treatment guidance, chemotherapy is usually offered after all TKIs are exhausted. Recent reports demonstrated that salvage surgical resection of primary lung cancer performed before resistance to TKIs develops can improve clinical benefits for these patients[37]. In another paper, an ALK-positive NSCLC patient with multiple organ metastases who had chemotherapy and targeted therapy, was successfully treated with surgical resection and obtained treatment-free remission (TFR) for more than 3 years[38]. In these studies the NSCLCs were down-staged following chemo- and/or targeted therapies, so that active cancer cells were detected only in the primary site. Recently, a case was reported, when surgery was performed after a patient had progression on Alectinib. Lorlatinib was used as the second-line treatment after surgery, but it was discontinued due to toxicity. However, the patient still had no lung cancer recurrence 14 months after discontinuation[39]. These data show promise in using surgery for treating ALK-positive NSCLC and give hope to the patients.

9.3 IMMUNOTHERAPY AND ALK VACCINE

The role of the immune system in cancer development and treatment has been increasingly recognized. Various immunotherapies assisting the immune system in fighting cancer cells have been designed and successfully employed. Immunotherapy with monoclonal antibodies (mAbs) can block programmed death 1 and programmed death ligand 1 (PD-1/PD-L1) -dependent negative regulation of the immune response and has been important in the treatment of particular types of lung cancer. In pre-clinical studies, the presence of EML4-ALK was found to be associated with increased levels of PD-L1[40] and, therefore, treatment of ALK-rearranged NSCLC with immune checkpoint inhibitors (ICI) was expected to be an effective therapeutic strategy. In clinical studies, however, levels of PD-L1 in ALK-positive NSCLC tissue samples varied broadly and treatment with ICI was inefficient in ALK-positive NSCLC[41]. In fact, the poorest response to ICI was observed in patients with the highest levels of PD-L1. The reason for this phenomenon is likely to lie in the immunosuppressive tumour microenvironment in ALK[42, 43]. Research and pre-clinical studies are currently ongoing to understand mechanisms by which ALK-positive NSCLC escape host immunity and are thereby do not respond to immunotherapies[42].

The chimeric antigen receptor (CAR)-T cell therapy, which has been effective in treatment of hematologic malignancies, encountered significant challenges when applied to solid tumours, including ALK-positive NSCLC. Factors such as the necessity for ALK-positive NSCLC to carry “potent” tumour-specific proteins to provide response to CAR-T cells, complexities of the tumour microenvironment, obtaining appropriate CAR structure together with difficulties for CAR-T cells to enter a solid tumour, contributed to the significant delay in the development of these immunotherapeutics[42].

The concept of ALK vaccines for treatment of ALK-positive NSCLC is to trigger a specific immune response against ALK-fusion proteins[44, 45]. Since ALK

is not normally present in human cells and tissues (except in embryos), but appears in cancer cells, it makes it a very promising tumour-specific target for immunotherapy in ALK fusion-positive NSCLC. In pre-clinical studies using mouse models, two different ALK vaccines were shown to be effective[46, 47]. A phase I clinical trial is currently ongoing to test an ALK vaccine for ALK-positive NSCLC[48].

9.4 DRUG COMBINATION THERAPIES

It is important to acknowledge that the quest to discover novel therapeutic strategies to treat ALK-positive NSCLC is ongoing. One of them is to develop combinatorial approaches, whereby ALK TKI is combined with another drug attacking the secondary target to enhance the treatment efficacy[49, 50]. One example is the combination of ALK TKI with immune checkpoint inhibitors (ICI), which demonstrated encouraging preliminary results, although the issue of toxicity remains to be resolved[18, 42]. Another approach is to use ALK TKI together with inhibitors of angiogenesis that prevent tumour growth by blocking the signals responsible for development of blood vessels supplying tumour with oxygen. This strategy provided excellent outcome when applied to patients with the EGFR-positive NSCLC. Several ongoing clinical trials are being carried out to determine the efficacy of this combination in treatment of ALK-positive NSCLC[51]. Chemotherapy with ALK TKI is yet another example of a combined treatment; the phase II trial to evaluate the efficacy of Carboplatin, Pemetrexed and Brigatinib in ALK-positive NSCLC is ongoing in Japan[18]. Combining ALK TKIs with radiotherapy is also being explored and these studies show promise[50]. Finally, ALK TKI in combination with drugs targeting other members of activated ALK-dependant and also ALK-independent (bypass) signalling pathways is an exciting treatment avenue that attracts attention from scientists, clinicians and pharmaceutical industry[50]. In this context, detection of the molecular changes occurring in ALK-positive NSCLC becomes imperative to design the most appropriate, patient-tailored treatment options.

10. CONCLUDING REMARKS

The ALK-positive NSCLC represents a unique cancer type with very distinct molecular and clinical-pathological characteristics. These features attract more and more attention of various communities: scientific, medical, health, and industry. Success in the development of the medicines to treat and potentially cure this cancer depends on active collaboration between these communities. In addition to existing treatments, a number of promising novel therapeutic agents are in the pipeline and more are more are coming from research and innovation. Genetic profiling will become a common tool to design the most effective treatment and achieve the best therapeutic outcome for each individual patient. The progress made in recent years in the understanding and treating ALK-positive NSCLC brings promise and hope to all people affected by this condition.

11. SUPPLEMENTARY TABLE 1. CHARACTERISTICS AND STRUCTURES OF FIRST-, SECOND-, AND THIRD-GENERATION OF ALK INHIBITORS (ADAPTED FROM BRENNER AND GUNNES, 2021[52]).

Note that these inhibitors structurally mimic ATP (graphic illustration and chemical diagram are shown below).

Abbreviations: ATP - Adenosine triphosphate ; ROS1: ROS pro-oncogene 1; c-Met: tyrosine-protein kinase Met or hepatocyte growth factor receptor;CNS: central nervous system; IGFR1: insulin-like growth factor; ACK: activated CDC24 kinase; EGFR: epidermal growth factor receptor; NBL: neuroblastoma; FAK: focal adhesive kinase; JAK2: Janus kinase 2; Trk: tropomyosin receptor kinase.

ATP - Graphic illustration

ATP - Chemical diagram

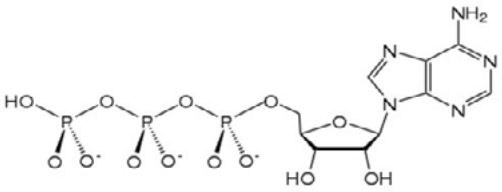
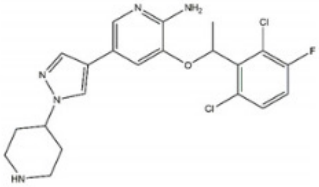
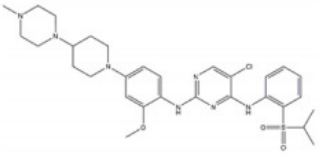
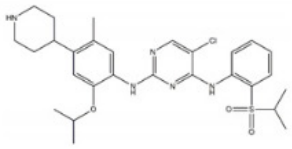
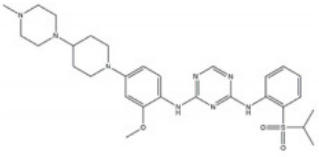
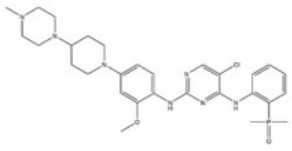
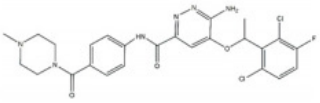
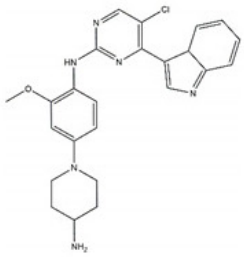
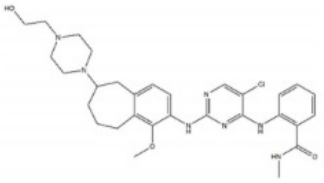
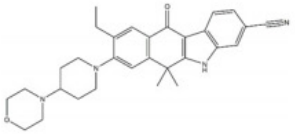
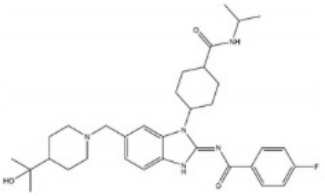
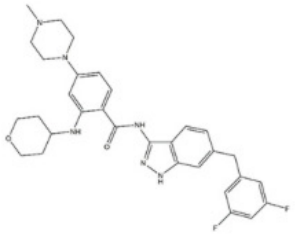
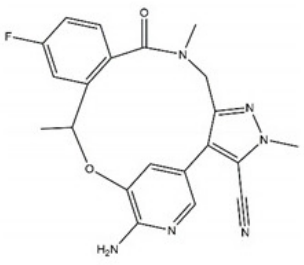
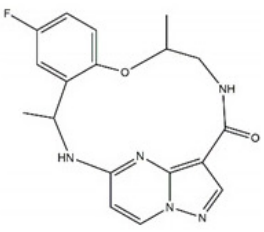


Table 2

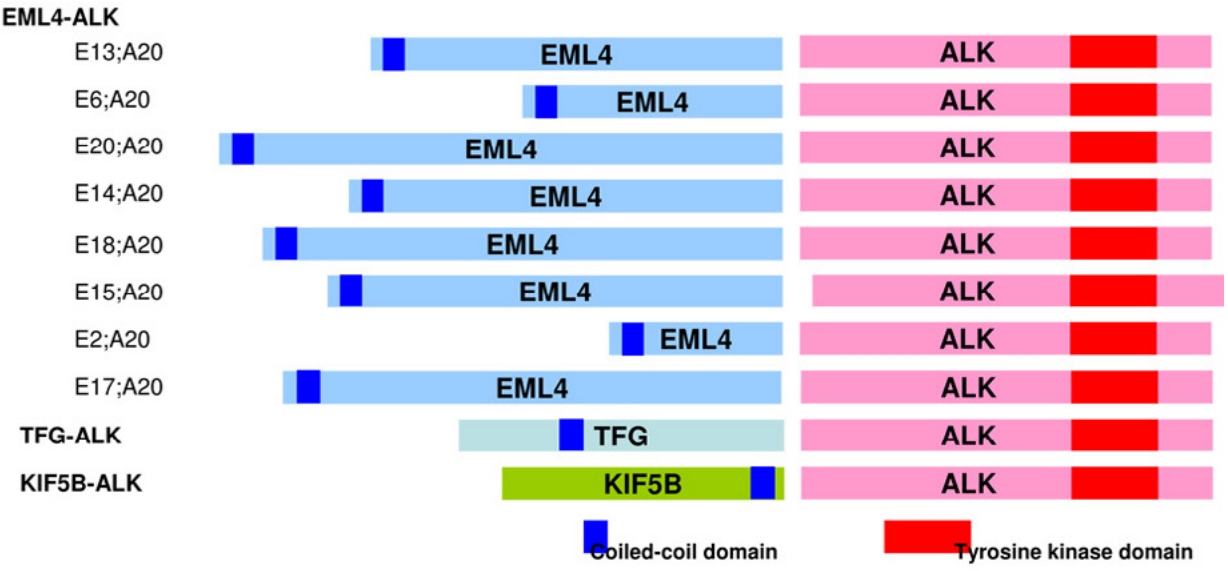
Characteristics and structures of first-, second-, and third-generation of anaplastic lymphoma kinase inhibitors.

First-Generation Inhibitor			
Crizotinib Off-targets: ROS1, c-MET No CNS penetration Sensitive: R1275Q Resistant: F1174L/V			
Second-Generation Inhibitors			
TAE-684 Sensitive: R1275Q, F1174L Discontinued		**Ceritinib** Off-targets: IGFR1, ROS1 CNS penetration Resistant: F1174L/C	
ASP3026 Off-targets: ROS1, ACK Resistant: L1196M		**Brigatinib** Off-targets: ROS1, EGFR CNS penetration Sensitive: all NBL-associated point mutations	
Ensartinib Sensitive: R1275Q, F1174L		**AZD3463** Off-target: IGFR1 Sensitive: NBL hotspot mutations	
CEP-37440 Off-target: FAK CNS penetration Sensitive: NBL hotspot mutations		**Alectinib** Sensitive: all NBL-associated point mutations, plus amplifications	
Belizatinib Off-targets: IGFR1, JAK2, TrkA/B/C, c-Src CNS penetration Sensitive: R1275Q, L1196M		**Entrectinib** Off-targets: ROS1, TrkA/B/C CNS penetration Sensitive: ALK amplifications	
Third-Generation Inhibitors			
Lorlatinib Off-target: ROS1 CNS penetration Sensitive: NBL hotspot mutations		**Repotrectinib** Off-targets: ROS1, TrkA/B/C CNS penetration Sensitive: all NBL-associated point mutations	

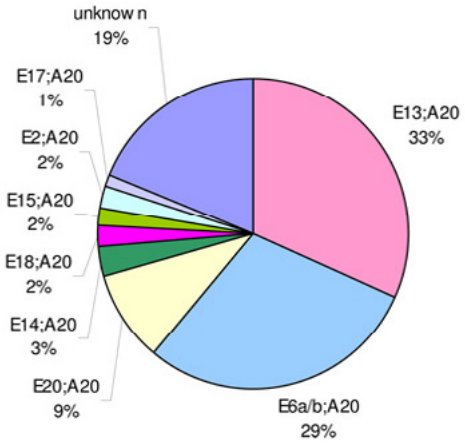
12. SUPPLEMENTARY FIGURE 1. DIFFERENT VARIANTS OF *EML4-ALK* AND NON-*EML4* FUSION PARTNERS (ADAPTED FROM SASAKI *ET AL*, 2010[15]).

Different genetic variants of *EML4-ALK* are depicted. The nomenclature refers to the exon* in *EML4* translocated to the exon in ALK. **B.** Frequency of different *EML4-ALK* variants. The most common variants are E13;A20 (variant 1) and E6a/b; A20 (variant 3). Of note not all studies list the specific *EML4-ALK* variant.

A



B



EML4-ALK Variants

E13;A20	E13;A20 (variant 1), E13;ins69 A20
E6;A20	E6a/b;A20 (variant 3a/b)
E20;A20	E20;A20 (variant 2), E20;ins18A20
E14;A20	E14;ins11del49A20(variant 4'), E14;del12A20 (variant 7)
E18;A20	E18;A20 (variant 5')
E15;A20	E15 del19;del20A20 (variant 4)
E2;A20	E2;A20 &E2;ins117A20 (variant 5a/b)
E17;A20	E17;ins68A20

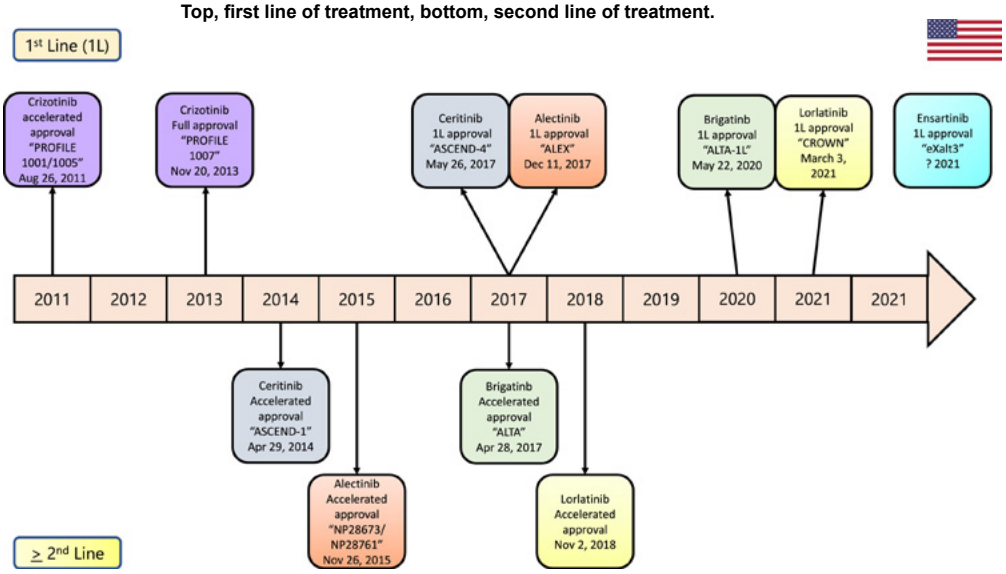
NSCLC Cell lines

H3122 and DFCI032 contain E13;A20. H2228 contain E6;A20

* Genes in the genome consist of exons and introns. Exons represent so called coding regions, which contain information for making a protein. Introns are non-coding regions, i.e. they do not contain information for making proteins.

13. SUPPLEMENTARY FIGURE 2. TIMELINE OF US FDA APPROVAL OF ALK-POSITIVE NSCLC TKIS, BASED ON SUCCESSFUL CLINICAL TRIALS (FROM OU *ET AL*, 2021[21]).

Top, first line of treatment, bottom, second line of treatment.



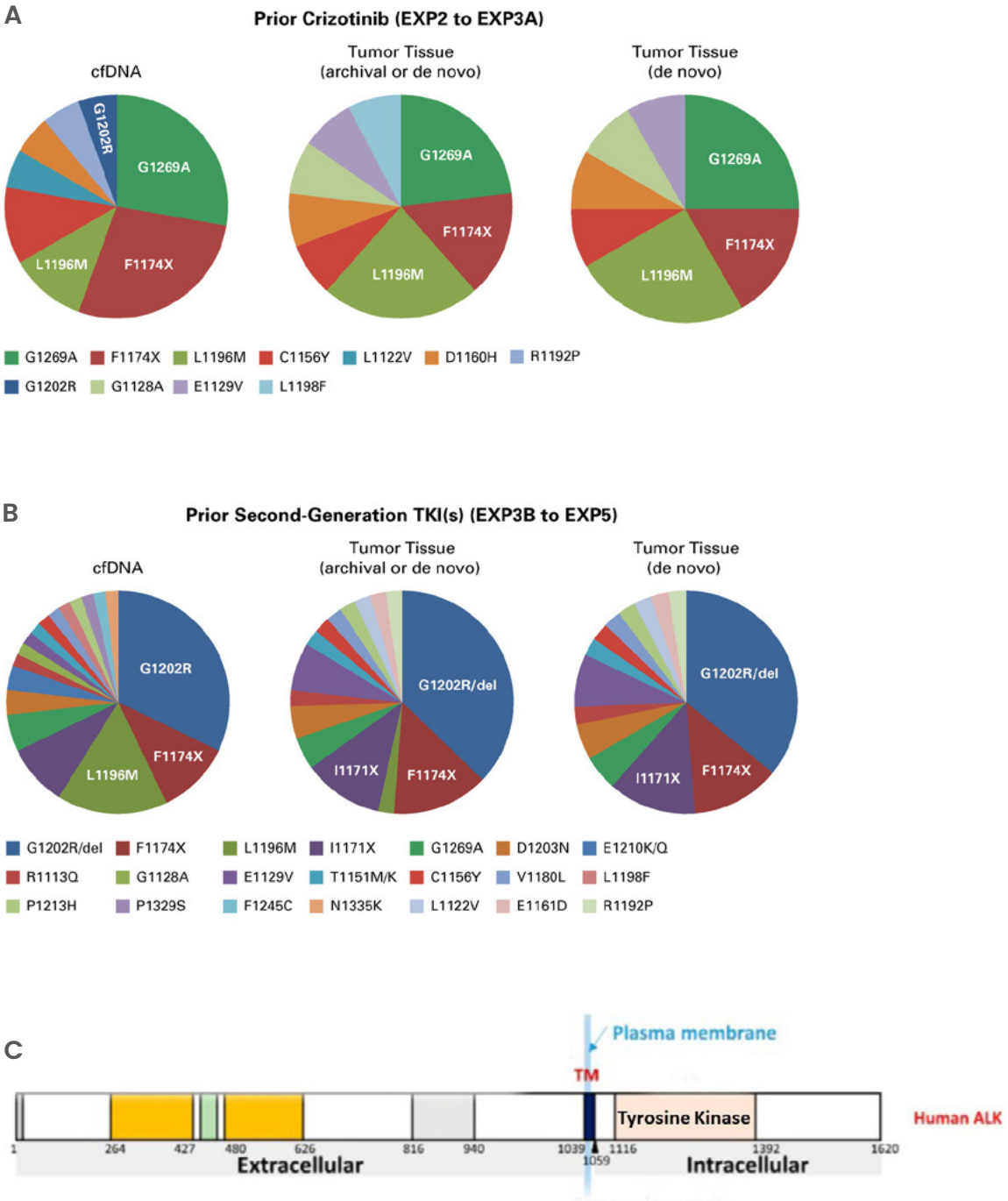
14. SUPPLEMENTARY FIGURE 3. SUMMARY OF ANAPLASTIC LYMPHOMA KINASE (*ALK*) MUTATIONS IDENTIFIED BY PLASMA AND TUMOUR GENOTYPING (FROM HUANG, 2018[53]).

(A) Post-crizotinib patients (expansion cohorts EXP2 to EXP3A). The most common ALK mutation observed in cell-free DNA (cfDNA) and tumour tissue was G1269A. *ALK* G1202R was detected in one cfDNA sample.

(B) Patients who have failed 1 or more second-generation ALK TKIs (EXP3B–5). The most common *ALK* mutation observed in cfDNA and tumour tissue was G1202R/del. (Note: only one G1202del mutation was detected.) Pie charts display the frequency of indicated *ALK* mutations as a percentage of the total number of patients with *ALK* mutations.

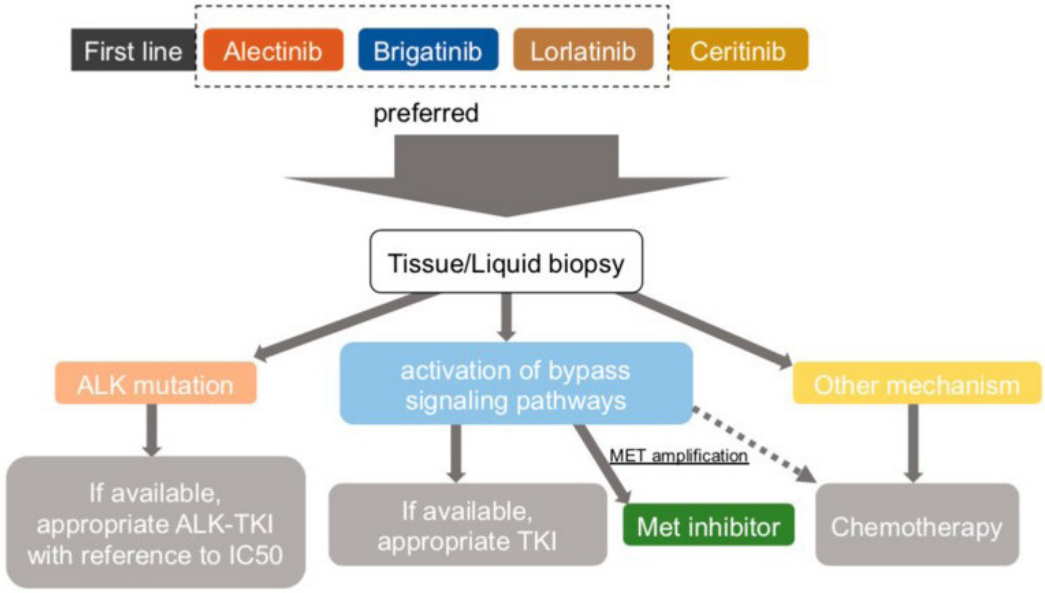
(Note that the mutation identifier contains the information about the position of the mutation in the ALK protein and type of mutations. For example, G1269A means the change at the position 1269 in the protein from Glycine to Alanine. Note, that this position is within the TK domain (see the map of the ALK protein below in Section C).

(C) ALK protein structure. Extracellular, Trans-membrane (TM) and intracellular (with TK) domains are shown. Numbers correspond to amino acids in the ALK protein (1-1620).



15. SUPPLEMENTARY FIGURE 4. PROPOSED TREATMENT ALGORITHM FOR ALK-REARRANGED ADVANCED NSCLC (FROM FUKUI *ET AL*, 2022[18]).

"When the disease progresses during first-line therapy, the identification of resistance mechanisms by performing tissue/liquid biopsy may help to guide optimal treatment. For example, in the case that EML4-ALK G1202R is the cause of resistance, lorlatinib may be a favorable subsequent therapy, and the fourth-generation ALK-TKIs to be developed in the future are effective for compound ALK mutation. When an EGFR mutation is identified, combination therapy with ALK-TKI and EGFR-TKI may be effective, and crizotinib is a reasonable option for patients with confirmed MET amplification. If the disease has converted to small cell lung cancer, a different chemotherapy regimen is required from those for NSCLC." (from Fukui *et al*, 2022[18]).



In certain circumstances, crizotinib may be a 1st line treatment option before next-generation ALK-TKIs.

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