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ALK rearrangements: Biology, detection and opportunities of therapy in non-small cell lung cancer

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ABSTRACT

The ALK receptor tyrosine kinase (*ALK*) gene encodes a transmembrane protein rearranged in 2–7% of non-small cell lung cancer (NSCLC) cases. This gene has become the second most studied therapeutic target after *EGFR* due to the implied therapeutic opportunities. While the diagnostic of ALK rearrangements is well established, small molecules targeting *ALK* are in constant evolution because tumor cells eventually will develop mechanisms of resistance. In this review we describe the biology of the *ALK* gene, alterations, epidemiology, diagnostic tests as well as strategies of treatment.

1. Background

Non-small cell lung cancer (NSCLC) is the most common malignancy in cancer and accounts for 80–85% of all lung tumors, representing the leading cause of cancer death in most countries of the world (Millett et al., 2018). In the last decade, the knowledge about the biology and genomics of NSCLC as well as the development of better therapeutic strategies for this malignancy has growth faster than the knowledge of other cancers, including breast cancer.

These advances in NSCLC have led to the identification of targetable mutations revealing great opportunities for the development of new drugs (Li et al., 2013). *ALK* genotyping has become routinely in NSCLC because it is the second most important therapeutic target in this malignancy after EGFR (Karachaliou and Rosell, 2014).

The ALK gene was initially reported in 1994 when Morris et al., described two fusion partners in a type of aggressive anaplastic large-cell lymphoma. The genes involved in this fusion were NPM and a novel gene sharing sequence similarities to tyrosine kinase receptors, named by the authors as ALK (Morris et al., 1994). Activation of ALK is described as a primary oncogenic event in most human cancers (Solomon et al., 2009).

In this review we present the biology of ALK gene, alterations, diagnostic tests, therapeutic alternatives in NSCLC patients bearing ALK rearrangements and its mechanisms of resistance.

2. *ALK* gene and protein

The anaplastic lymphoma kinase gene (ALK) encodes a tyrosine kinase transmembrane protein that is member of the superfamily of insulin receptors. This gene consists of 30 exons spanning on the short arm of chromosome 2 (2p23) (Hallberg and Palmer, 2013). Other synonyms to this gene include antigen CD246 and NBLST3.

The ALK protein is composed by 1620 amino acids with a molecular weight of 220 kD of the mature protein. During embryogenesis, ALK plays an important role in brain development by exerting its effects on the neurons of the central nervous system, expressing itself in the development of central and peripheral nervous tissue, but not in adult tissue, except for some neurons, glial and endothelial cells (Pulford et al., 1997). In adults, it presents higher expression in testis, pituitary and hypothalamus (GTEx Consortium et al., 2017; Iwahara et al., 1997).

Pleiotrophin (PTN) and midkine (MK) are known ligands for this receptor. After the binding to the extracellular domain of ALK, a dimerization occurs activating the intracellular tyrosine kinase domains producing a signaling cascade through canonical pathways such as MAPK, PI3K/mTOR, JAK-STAT, SHH among others. (Hallberg and Palmer, 2013; Souttou et al., 2001). The downstream activity of ALK activation in pathologic conditions lead to an increased cell proliferation and metabolism, cytoskeleton remodeling, migration, survival and apoptosis avoiding (Palmirotta et al., 2017). Consequences of ALK signaling are determined by the fusion partner and the type of cancer

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(Hallberg and Palmer, 2013; Palmirotta et al., 2017)

Currently, there is a gap in the knowledge of transcriptional regulation of ALK.

3. *ALK* rearragements and role in NSCLC

Rearrangements involving ALK are characterized by the control of the kinase domain of ALK under the promoter of the fusion partner. The resulting chimera protein presents constitutive tyrosine kinase activity (Rikova et al., 2007). In 2007, Soda et al. identified the first ALK rearrangement in NSCLC, occurring between this gene and the echinoderm microtubule associated protein like 4 (EML4) implying a large inversion or translocation (Soda et al., 2007).

The EML4-ALK rearrangement presents a potent oncogenic activity as EML4 enhances the constitutive oligomerization of ALK by altering its kinase activity. Many fusion variants have been found involving different breakpoints in several EML4 exons (2,6,13,14,15,18,20) and exon 20 of ALK. Other fusion pairs with ALK in non-small cell lung cancer (NSCLC) are TFG, KIF5B, KLC1, STRN, TPR, HIP1, GCC2, DCTN1, SQSTM1, LMO7, BIRC6, PHACTR1 and PTPN3 form (Fang et al., 2014; Hallberg and Palmer, 2013; Iyevleva et al., 2015; Jiang et al., 2018; Jung et al., 2012; Noh et al., 2017; Shan et al., 2015; Soda et al., 2007) (Table 1).

There are several epidemiologic studies around the globe reporting 1%–10% frequencies of ALK rearrangements in NSCLC (Table 2). In the studies found in the cBioPortal platform the presence of ALK rearrangements occurs in 0.99% of cases of the TCGA PanCancer Atlas of Lung Adenocarcinoma project and in 3.06% (n = 28) in the MSK-IMPACT dataset of NSCLC (http://www.cbioportal.org/) (Cerami et al., 2012; Gao et al., 2013; Jordan et al., 2017).

ALK rearrangements are more common in younger, never or light-smoker patients with adenocarcinoma (Camidge et al., 2010; Shaw et al., 2009). Notably, EML4-ALK appears to be mutually exclusive with EGFR and KRAS mutations (Inamura et al., 2008).

4. *ALK*-rearrangements and testing methods

The detection of rearrangements of the ALK gene is essential for the choice of the best therapy for patients with advanced non-small cell lung cancer (Shaw et al., 2009). Below we describe a series of techniques routinely used to evaluate ALK abnormalities:

Table 2
Incidence of *ALK* rearrangements in NSCLC in populations from different countries.

Country of origin	N	n (%)	Reference
Latin-America	188	19 (10.1)	(Corrales-Rodríguez et al., 2017)
Latin-America	7600	487 (6.4)	(Martín et al., 2018)
Mexico	200	18 (9)	(Cruz-Rico et al., 2017)
Argentina	131	8 (6.11)	(Verzura et al., 2018)
Brazil	62	2 (3.23)	(Lopes and Bacchi, 2012)
Chile	49	3 (6.12)	(Fernandez-Bussy et al., 2017)
Italy	96	1 (1.04)	(Lee et al., 2018)
Spain	97	2 (2.06)	
Germany	97	2 (2.06)	
Australia	92	4 (4.35)	
Japan	100	2 (2.00)	
Korea	94	9 (9.57)	
Taiwan	80	3 (3.75)	
China	1387	71 (5.12)	(Tian et al., 2017)
China	1160	94 (8.10)	(Hong et al., 2014)
USA	1387	49 (3.53)	(Dai et al., 2012)
Australia	296	25 (8.45)	(Tan et al., 2018)
Korea	3767	270 (7.17)	(Lee et al., 2016)

4.1. Immunohistochemistry (IHC)

This technique is based in the use of a primary antibody (monoclonal or polyclonal) intended to detect an antigen in the sample. The result is the product of an immune enzymatic reaction producing colored precipitates. IHC detects the overexpression of aberrant ALK protein occurring when there is an ALK fusion not present in normal tissues. It has now become a very effective method for screening patients with NSCLC for subsequent FISH analysis and very useful for uncertain FISH results. ALK IHC evaluation is qualitative according to the intensity of staining of membrane and classified as negative (0), +, ++ and +++. The threshold is ambiguous between + and ++

Table 1
ALK fusion partners in NSCLC.

ALK cr.2	FUSION PARTNER	LOCALIZATION	REARRANGEMENT	BREAKPOINT
exon 20	***EML4*** (Echinoderm microtubule associated protein like 4)	**cr.2**	inv(2)(p21p23)	exon 2 exon 6 exon 13 exon 14 exon 15 exon 18 exon 20
	KIF5B (Kinesin family member 5B)	**cr.10**	t(2;10)(p23;p11)	exon 15 exon 17 exon 24
	TFG (TRK-fused gene)	**cr.3**	t(2;3)(p23;q21)	exon 3 exon 4 exon 5
	KLC1 (Kinesin light chain 1)	**cr.14**	t(2;14)(p23;q32)	exon 9
	STRN (Striatin, calmodulin binding protein)	**cr.2**	del(2)(p22p23)	exon 3
	TPR (Translocated promoter region)	**cr.1**	t(1;2)(q31.1;p23)	exon 15
	HIP1 (Huntingtin Interacting Protein 1)	**cr.7**	t(2;7)(p23;q11.23)	exon 21 exon 28
	GCC2 (GRIP and coiled-coil domain containing 2)	**cr.2**	t(2;2)(p23;q12)	exon 12
	DCTN1 (Dynactin subunit 1)	**cr.2**	t(2;2)(p13;p23)	exon 26
	SQSTM1 (Sequestosome 1)	**cr. 5**	t(2;5)(p23;q35)	exon 5
	LMO7 (LIM domain 7)	**cr. 13**	t(2;13)(p23;q22)	exon 15
	BIRC6 (Baculoviral IAP repeat-containing 6)	**cr. 2**	t(2;2)(p22;p23)	exon 10
	PHACTR1 (Phosphatase and Actin Regulator 1)	**cr. 6**	t(2;6)(p23;p24)	exon 7
exon 10 or 11	***PTPN3*** (Protein tyrosine phosphatase, non-receptor type 3)	**cr.9**	t(2;9)(p23;q31)	exon 2 exon 3

(Thunnissen et al., 2013).

Technical parameters of ALK IHC are robust and reliable, with 90% sensitivity, 95% specificity, and 93% of accurate relative in regard to the ALK FISH results (Wynes et al., 2014). ALK IHC is approved to be used without orthogonal tests and usually used to discard negative cases (Letovanec et al., 2018).

One disadvantage of ALK IHC is the lack of an internal positive control which renders difficult to determine if a negative result is actually negative for the expression of the ALK fusion protein. Considering that lung tissue under normal conditions has undetectable ALK by IHC, a diffuse expression of ALK in lung cancer cells is associated with expression of the aberrant ALK fusion protein (Takamochi et al., 2013; Takeuchi, 2013). Blocks from cell lines NCI-H3122 and NCI-H2228 (bearing ALK rearrangements) could be used as positive controls for IHC (Thunnissen et al., 2013). ALK IHC depends on the skill of the pathologist.

4.2. In situ hybridization with fluorescence (FISH)

FISH is considered as the gold standard method for detecting *ALK* rearrangements because ALK can present rearrangements with several genes and a single FISH study is able to detect all the possible fusions (Kim et al., 2011). This technique allows the visualization of specific regions of the chromosome by using fluorochrome-labeled probes of complementary sequences after a process of denaturation and hybridization (Nath and Johnson, 2000).

For the detection of *ALK* rearrangements, a break-apart probe is used. It labels with different fluorochromes the 3′region (telomeric) and fluorochrome and the 5′ region (centromeric) of ALK gene. A tumor cell bears the ALK rearrangement when the red and green signals are separated (in a distance two or more times the diameter of the greatest signal). Cells showing uniquely the 3′ signal are considered positive, because it is considered the ALK gene is under regulation of other promoter. Cells are considered negative for ALK rearrangements when both signals are close or overlapping signals (seen as a yellow signal) is displayed. Considerations for ALK FISH interpretation is described in Fig. 1.

A minimum of 50 cancer cells should be evaluated. If < 5 cells results positive, then the result is negative. In contrast, when > 25 cells are positive, the overall result is considered positive. If 5–25 cells are positive, the result is considered equivocal and a further analysis is conducted in 100 additional cells. In this step a clear cutoff of 15% is established. A presence of ≥15% of positive cells is interpreted as positive; otherwise, the result is considered negative (Camidge et al., 2010; Yoshida and Varella-Garcia, 2013).

FISH is a reliable diagnostic method in pathology due to its easy reproducibility in formalin-fixed and paraffin-embedded tissue samples (FFPE) (Yoshida and Varella-Garcia, 2013).

4.3. Chromogenic in situ hybridization (CISH)

This technique is based on the combination of DNA probes and the use of chromogens for the visualization of specific genetic targets. The architecture of the tumor and the cytomorphology almost remains intact allowing the evaluation of the signals by conventional bright field light microscopy (Kim et al., 2011; Yoshida and Varella-Garcia, 2013).

Currently, the *ALK* Break apart CISH test has been implemented for NSCLC, using individual probes labeled with different haptens that can be visualized by antibody reactions similar to those used in the IHC test. A black dot in the nuclei represent to normal gene while ALK rearrangements are visualized as two separated signals (red and blue) (Yoshida and Varella-Garcia, 2013).

Results obtained using CISH ALK-break-apart show a great concordance with FISH and / or RT-PCR results (Kim et al., 2011; Nitta et al., 2013; Schildhaus et al., 2013; Yoshida et al., 2011). CISH is still under validation for use in the detection of ALK rearrangements in NSCLC (Yoshida and Varella-Garcia, 2013).

4.4. Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR is a variant of the polymerase chain reaction, where an RNA strand is retro-transcribed into complementary DNA using the reverse or reverse transcriptase enzyme. The result is amplified by a conventional PCR (Costa et al., 2013).

This technique could be more sensitive than other methods due to the lower amount of tumor cells needed and less subjective than IHC and FISH. RT-PCR has some disadvantages, such as the requirement for high-quality RNA and lack of flexibility to detect additional fusions according to the used primers. In this case a multiplex RT-PCR should be performed (Costa et al., 2013; Takeuchi et al., 2009).

Because it is not possible to screen unknown ALK rearrangements, this test is not recommended to the select patients candidates to ALK inhibitors; however, it is useful to confirm ALK alterations detected by IHC or FISH (Yatabe et al., 2013).

4.5. Next-generation sequencing (NGS)

NGS is a high-throughput technology capable of massively process multiple DNA sequences following four basic steps, including preparation of the sequencing library, clonal amplification, sequencing and finally the data analysis. This technology is used to provide more efficient sequencing and a large amount of data stream. The isolated sequences that are obtained need the use of different computer tools for their assembly and expertise in the interpretation of results (Patel and Jain, 2012). Although NGS provides massive data of mutations and molecular mechanisms of cancer; however, it should be considered as a complementary test associated with FISH and IHC (Uguen et al., 2015).

5. *ALK* inhibition

The transforming potential and the “addiction” of ALK-rearranged lung cancer cells on ALK signaling for survival and growth were established shortly after the identification of the fusion EML4-ALK (Soda et al., 2008, 2007); triggering the development of TKIs targeting ALK. Current drugs with anti-ALK activity include first-generation drugs, crizotinib; second generation drugs, ceritinib, alectinib and brigatinib and the third-generation agent lorlatinib (Table 3) (Fig. 2).

The first molecule to enter the clinic was crizotinib, a multi-targeted TKI with activity against ALK, MET and ROS1 (Yasuda et al., 2012). In 2011, crizotinib received accelerated approval for metastatic lung cancer with ALK rearrangements based on the results from 2 small single-arm studies which demonstrated an ORR ≥ 50% and a median duration of response of around 12 months (Kazandjian et al., 2014).

In the second line setting, crizotinib showed an ORR of 65% and 4 months of PFS-benefit in comparison with docetaxel or pemetrexed (Shaw et al., 2013). As first line treatment in the PROFILE-1014 study, crizotinib obtained an impressive ORR of 74% and a median PFS of 10.9 months (Solomon et al., 2014). With a median follow-up of 46 months, median OS for crizotinib has not yet been reached. Crossover to crizotinib was permitted after disease progression. After adjusting for crossover, the HR for OS reached statistical significance [HR 0.346 (0.081-0.718)] (Solomon et al., 2018b). Crizotinib is usually well tolerated, being gastrointestinal toxicity and vision changes the most common adverse events.

Inevitably and like EGFR inhibition with EGFR TKIs, resistance to ALK inhibition develops in an average of 1 year. As such, newer potent inhibitors of ALK have been developed to overcome the resistance mechanisms. Ceritinib, a second-generation agent, showed in the phase I ASCEND-1 trial, an ORR of 56% and median PFS of 6.9 months in crizotinib pretreated patients (Kim et al., 2016; Shaw et al., 2014). The ASCEND-4 trial evaluated ceritinib vs chemotherapy in treatment-naïve patients, obtaining an ORR of 72%, and a median PFS of 16.6 months

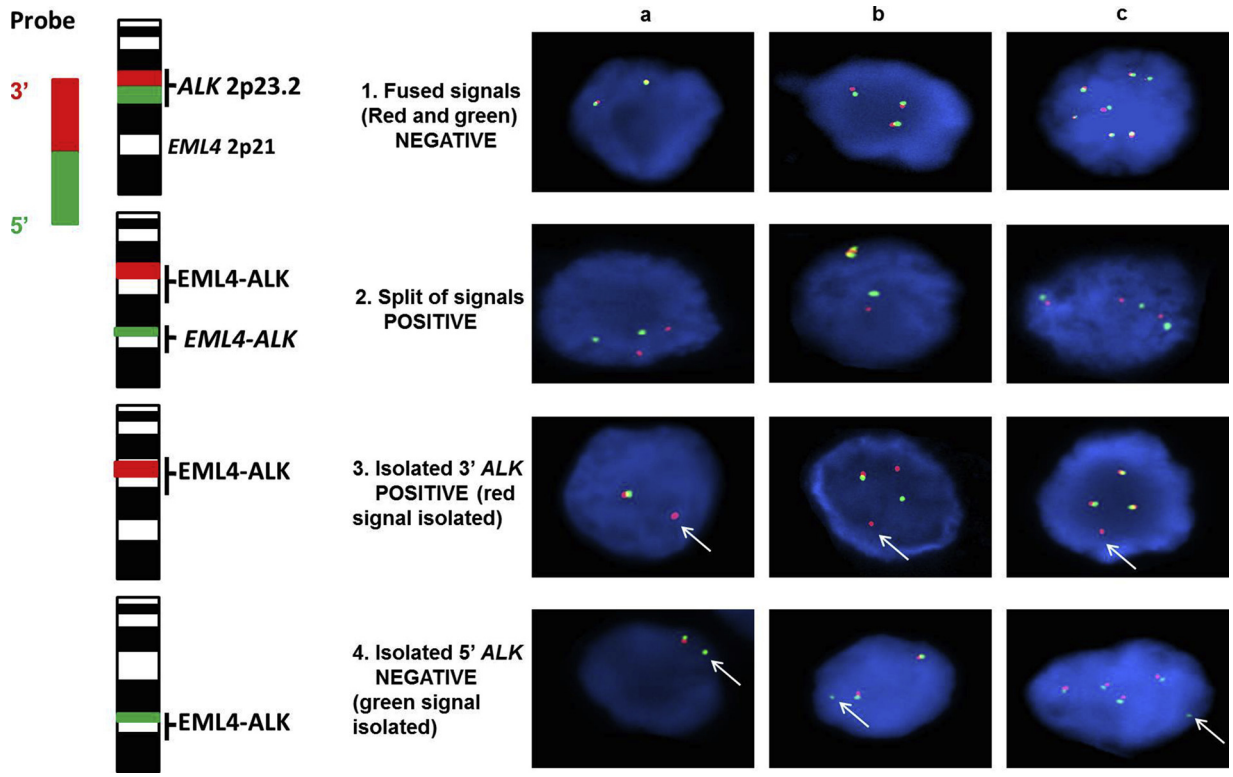

Fig. 1. Interpretation of FISH Results. **(1)** The ALK gene in its native state, must appear as two adjacent signals green and red or fused in a yellow signal (1a). Tumor cells can be polysomic, and may exist more than two fused signals (1b,1c). **(2)** When there is a ALK rearrangement, the signals green and red are observed separate from each other, the distance in between should be at last two times the diameter of the bigger signal. The presence of fused signals is not relevant for the classification of patterns (2b). **(3)** A tumor cell is considered positive when we only observed a red signal (green signal deleted). Positive tumor cell with additional two fused signals (3a). Tumor cell can have fused signals, signals separated by rupture and deletions (3b). Tumor cell can be polysomic, with fused signals and red signal (3c). **(4)** Fused signals plus a green signal without the corresponding red signal indicates a deletion of the red portion of the ALK gene. It is considered negative. Tumor cells can be polysomic, the tumor cell may have fused signals and deleted signal red (4c) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

compared to 8.1 months with chemotherapy (Soria et al., 2017). Ceritinib use was mainly associated with gastrointestinal toxicities and fatigue.

Alectinib is an ALK inhibitor, 10 times more potent than crizotinib. It was initially tested in a Japanese phase I/II including ALK-rearranged NSCLC patients without previous exposure to ALK inhibitors, obtaining an ORR of 93.5% (Seto et al., 2013). Alectinib's activity in crizotinib resistant patients was confirmed in a phase II study which reported an ORR of 48% with median PFS of 8.1 months (Shaw et al., 2016). Alectinib has also shown efficacy in treatment-naive ALK-positive NSCLC patients compared with crizotinib as reported in the J-ALEX and ALEX phase 3 studies (Camidge et al., 2018b; Hida et al., 2017; Peters et al., 2017; Takiguchi et al., 2017). For patients in the alectinib arms ORR ranged from 82.9 to 91.6% and median PFS from 25.9 to 34.8

Table 3
Randomized phase 3 trials of ALK TKI-inhibitors.

TRIAL	SETTING	COMPARATORS	ORR (%)	Median PFS (m)	HR for progression (95% CI)	Median OS (m)	HR for death (95% CI)
ALK TKI-inhibitors vs chemotherapy							
PROFILE-1007 (Shaw et al., 2016; Shaw et al., 2013)	2nd line	Crizotinib	65	7.7	0.49 (0.37-0.64),	21.7	NS
		Docetaxel or Pemetrexed	20	3	p < 0.001	21.9	
PROFILE-1014 (Solomon et al., 2018b, 2014)	1 st line	Crizotinib	74	10.9	0.45 (0.35-0.60),	NR	0.346 (0.081-0.718)
		Platinum-based doublet	45	7	p < 0.001	47.5	
ASCEND-5 (Shaw et al., 2017)	2nd or 3rd line and after progression to crizotinib	Ceritinib	39	5.4	0.49 (0.36-0.67),	NA	NA
		Docetaxel or Pemetrexed	7	1.6	p < 0.0001	NA	
ASCEND-4 (Soria et al., 2017)	1 st line	Ceritinib	72.5	16.6	0.55 (0.42-0.73),	NR	NS
		Platinum-based doublet	26.7	8.1	p < 0.00,001	26.2	
First vs second or third generation ALK-inhibitors							
J-ALEX (Hida et al., 2017; Takiguchi et al., 2017)	1 st or 2nd line	Alectinib	91.6	25.9	0.38 (0.26-0.55),	NA	NA
		Crizotinib	78.9	10.2	p < 0.0001	NA	
ALEX (Camidge et al., 2018b; Peters et al., 2017)	1st line	Alectinib	82.9	34.8	0.43 (0.32–0.58)	NR	NS
		Crizotinib	75.5	10.9		NR	
ALTA-1 L (Camidge et al., 2018a)	1st line	Brigatinib	71	NR	0.49 (0.33-0.74),	NR	NA
		Crizotinib	60	NR	p < 0.001	NR	

NA, not available; NR, not reached; NS, not significant.

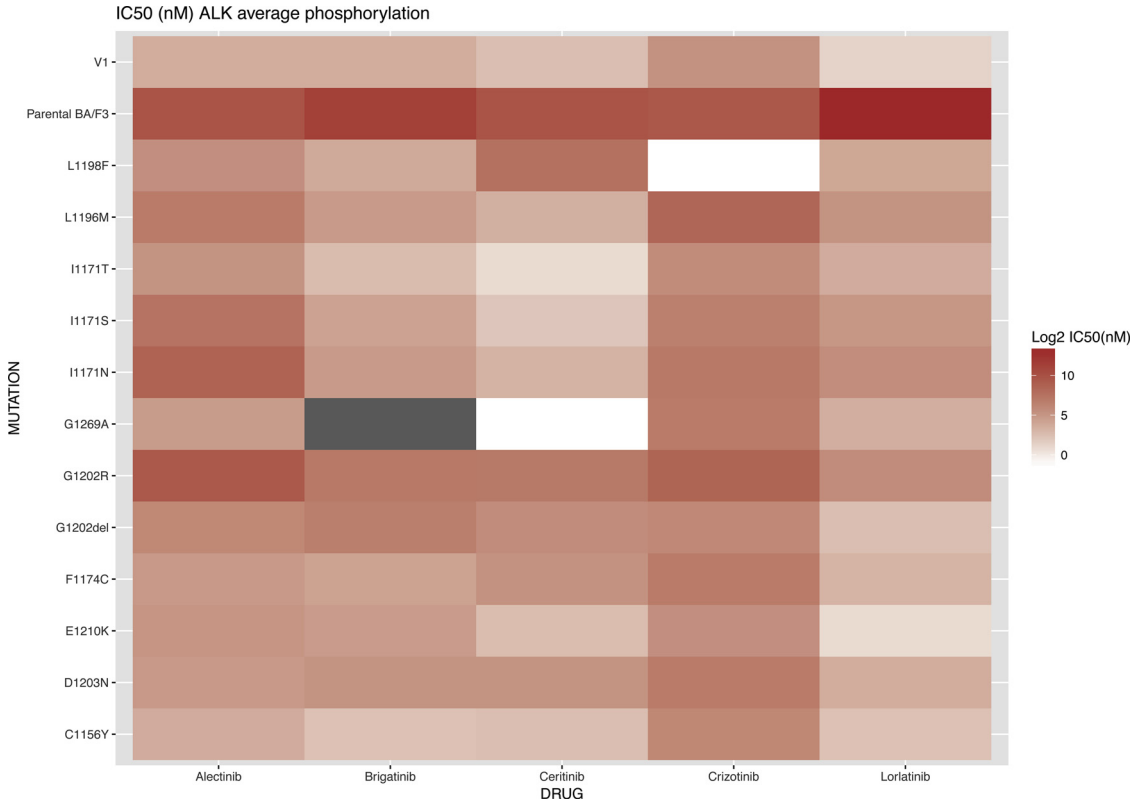

Fig. 2. Sensitivity to ALK inhibitors showed as IC50 (nM) Average cell phosphorylation of ALK. Adapted with data retrieved from (Gainor et al., 2016).

months, respectively. Importantly, alectinib has activity in the CNS; median PFS by baseline CNS metastases status was 27.7 months with alectinib vs 7.4 months with crizotinib (Camidge et al., 2018b). OS data are still immature. The improved efficacy of alectinib came in hand with reduced toxicity with a rate of grade 3–4 adverse events of 26.2% compared with 51.9% in the crizotinib arm.

Brigatinib showed an ORR of 62% and a median PFS of 13.2 months in crizotinib-pretreated patients in one phase I/II study (Gettinger et al., 2016). In the recently published first interim analysis of the ALTA-1 L study, which compared brigatinib vs crizotinib in TKI-naïve patients, the ORR and the rate of PFS were 71 vs 60% and 12-month PFS of 67% vs 43%, respectively (Camidge et al., 2018a). Like alectinib, brigatinib is very active at the SNC with a rate of intracranial response of 78% vs 29% for crizotinib.

Lorlatinib is a brain-penetrant-third generation inhibitor of ALK and ROS1 which is active against most known resistance mutations, including the highly resistant G1202R mutation. In a phase II global study this compound showed consistent overall and intracranial activity in both treatment-naive patients and in those who has progressed on crizotinib, second-generation ALK-inhibitors or after up to three previous ALK-TKI (Solomon et al., 2018a). A phase III study of lorlatinib vs crizotinib in first line treatment of patients with ALK-positive NSCLC is currently recruiting patients (NCT03052608).

6. Mechanism of resistance

Despite the initial responses are remarkable, the effectiveness of ALK-inhibitors is universally limited by the eventual occurrence of resistance. Mechanisms of resistance are classified as a) ALK-dependent "on-target" mechanism including secondary ALK mutations or amplifications in which reliance on ALK signaling persists, and b) ALK-independent "off-target" mechanisms including the activation of bypass signaling pathways and lineage transformations, in which tumoral cells are no longer dependent on ALK.

The emergence of secondary mutations in the ALK tyrosine kinase domain is the most frequent "on-target" resistance mechanism and interestingly, each ALK TKI exhibits a somewhat different profile of mutations. In patients resistant to crizotinib, secondary mutations occur in 20%–30%, being the L1196 M gatekeeper mutation (analogous to EGFR T790 M) and G1269 A the most frequent ones, while the highly resistant G1202R appears in less than 10% of cases. For patients resistant to the more potent next-generation ALK-inhibitors, the frequency of ALK secondary resistance mutations increases to 50%–70%. The emergence of the highly resistant G1202R mutation is common and represents 21%, 29% and 43% of cases in cases resistant to ceritinib, alectinib and brigatinib, respectively (Gainor et al., 2016). Lorlatinib, the third-generation ALK inhibitor has been shown to overcome resistance to this mutation (Zou et al., 2015). ALK amplification is a recognized but infrequent cause of acquired resistance to crizotinib and seem to be clinically irrelevant for second-generation inhibitors (Lin et al., 2017).

Among off-target resistance mechanisms, the activation of bypass signaling tracks such as EGFR, HER, MET, PIK3CA, KIT and IGF1R have been identified. Other resistance mechanisms including phenotypic changes such as epithelial–to-mesenchymal transition (EMT) and small cell lung cancer (SCLC) transformation, alone or in conjunction with ALK mutations have also been implicated (Lin et al., 2017).

7. Conclusion

Activating alterations of *ALK* confers an aggressive behavior and it is an important therapeutic target in NSCLC. Although there are several diagnostic methods to detect *ALK* rearrangements, FISH is the gold standard because its reliability and easy implementations in routine laboratories. On the other hand, despite the improvement of outcomes of *ALK*-positive NSCLC and although the current repertoire of ALK inhibitors include third-generation drugs, overcome the mechanisms of drug-resistance are greatest challenge.

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