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Tumour Review

Biological and clinical perspectives of the actionable gene fusions and amplifications involving tyrosine kinase receptors in lung cancer

Maria Saigí [a,b,*], Enric Carcereny [a,b], Teresa Morán [a,b], Marc Cucurull [a,b], Marta Domènech [a,b], Ainhoa Hernandez [a,b], Anna Martinez-Cardús [b], Eva Pros [c], Montse Sanchez-Cespedes [c,*]

[a] Department of Medical Oncology, Catalan Institute of Oncology (ICO), Badalona, Barcelona, Spain
[b] Badalona·Applied Research Group in Oncology (B·ARGO). Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol (IGTP), Badalona, Barcelona, Spain
[c] Cancer Genetics Group, Josep Carreras Leukaemia Research Institute (IJC), Badalona, Barcelona, Spain

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ABSTRACT

Identifying molecular oncogenic drivers is crucial for precision oncology. Genetic rearrangements, including gene fusions and gene amplification, involving and activating receptor tyrosine kinases (RTKs) are recurrent in solid tumors, particularly in non-small cell lung cancer. Advances in the tools to detect these alterations have deepened our understanding of the underlying biology and tumor characteristics and have prompted the development of novel inhibitors targeting activated RTKs. Nowadays, druggable oncogenic rearrangements are found in around 15% of lung adenocarcinomas. However, taken separately, each of these alterations has a low prevalence, which poses a challenge to their diagnosis. The identification and characterization of novel targetable oncogenic rearrangements in lung cancer continue to expand, as shown by the recent discovery of the *CLIP1-LTK* fusion found in 0.4% of lung adenocarcinomas. While tyrosine kinase inhibitors that block the activity of RTKs have represented a breakthrough in the therapeutic landscape by improving the prognosis of this disease, prolonged treatment inevitably leads to the development of acquired resistance. Here, we review the oncogenic fusions and gene amplifications involving RTK in lung cancer. We address the genetic and molecular structure of oncogenic RTKs and the methods to diagnose them, emphasizing the role of next-generation sequencing technologies. Furthermore, we discuss the therapeutic implications of the different tyrosine kinase inhibitors, including the current clinical trials and the mechanisms responsible for acquired resistance. Finally, we provide an overview of the use of liquid biopsies to monitor the course of the disease.

Introduction

Precision oncology has revolutionized the therapeutic landscape of advanced non-small cell lung cancer (NSCLC). Over the last two decades, the deep genomic profiling of lung cancers has enabled identifying many new genes involved in the development of this disease, including oncogenic drivers that have contributed to our understanding of lung carcinogenesis and have established an era of precision medicine in advanced-stage NSCLC [1,2].

Tumors often have complex and unstable genomes that cause random mutations and genomic aberrations. While most of these alterations are unlikely to affect tumor development, some may involve essential genes that contribute to the oncogenic process. For instance, chromosomal rearrangements could result in gene fusions or gene amplifications that lead to the expression of oncoproteins. When the fusion or amplification involves a receptor tyrosine kinase (RTK), the tyrosine kinase domain (TKD) is activated—often constitutively and ligand-independent—and downstream effectors of the receptor receive constant signaling, causing uncontrolled cell growth and invasiveness [1,2]. Then, the tumor cell becomes dependent on this oncogenic RTK to maintain its malignant properties. This dependency, also called "oncogene addiction," can be therapeutically approached with drugs that inhibit the activity of the oncoprotein. Currently, most RTKs inhibitors are designed to prevent either the binding of the ligand—often by using

* Corresponding authors Department of Medical Oncology, Catalan Institute of Oncology (ICO), Badalona, Barcelona, Spain (M. Saigí). Cancer Genetics Group, Josep Carreras Leukaemia Research Institute (IJC), Badalona, Barcelona, Spain. (M. Sanchez-C.)

E-mail addresses: msaigi@iconcologia.net (M. Saigí), ecarcereny@iconcologia.net (E. Carcereny), mmoran@iconcologia.net (T. Morán), mcucurull@iconcologia.net (M. Cucurull), mdomenech@iconcologia.net (M. Domènech), ahernandezg@iconcologia.net (A. Hernandez), amartinezc@igtp.cat (A. Martinez-Cardús), epros@carrerasresearch.org (E. Pros), mscespedes@carrerasresearch.org (M. Sanchez-Cespedes).

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monoclonal or bispecific antibodies, and antibody-drug conjugates—or the binding of the ATP to the catalytic domain—mostly with small molecules. Among the latter, tyrosine kinase inhibitors (TKIs), which inhibit the catalytic domain of the target enzyme by preventing phosphorylation and subsequent signaling, are the most widely used in clinics [3]. On the other hand, other personalized therapeutic strategies include immune targeted approaches such as checkpoint blockade, personalized vaccines and/or chimeric antigen receptor T-cells [4]. Slightly more than a dozen RTKs are genetically activated in NSCLC. Although gene fusions and gene amplifications are the most common mechanisms of RTKs' activation, point mutations can also occur, as in *MET*, *ERBB2*, and *EGFR* (the latter is the most frequently RTK activated in lung cancer) [2–3]. For still unknown reasons, activated RTKs prevail in lung cancer adenocarcinomas (LuADs) of relatively young females, since they are related to lower tobacco-exposure (light- or never-smokers)These patients are often diagnosed with advanced-stage cancer and have high incidences of pleural effusion and sclerotic bone and brain metastases as common patterns of tumor spread [5–7]. The recently discovered fusion *CLIP1–LTK* in LuADs, leading to the oncogenic activation of the RTK LTK [8], will likely share similar characteristics. Some exceptions are the activation of *FGFRs*, more common in lung squamous cell carcinomas (LuSCCs), and *NTRKs*, which occur in NSCLCs across gender, age, smoking history, and histopathology [7,9]. Most of the alterations that activate RTKs are mutually exclusive between them and between alterations of molecules involved in signal transduction. This feature suggests that they are all functionally connected and confer similar growth advantages. This characteristic is highlighted in Fig. 1, in which an oncoplot has been drawn to show the associations between genetic alterations that affect RTK and signal transduction molecules among lung Tumor Cancer Genome Atlas.

Currently, at least six of the different oncogenic alterations affecting RTKs in NSCLC, including activating mutations in *EGFR* and *MET (METex14),* as well as fusions in *ALK, ROS1, NTRK*, and *RET*, are eligible to be treated with compounds approved by the US Food and Drug Administration (FDA) as standard-of-care therapy. Additional compounds are under development for tumors carrying *MET* amplification (*MET*amp) or activating mutations in *ERBB2* [3]. Other fusions (e.g., *NRG1*, and *FGFRs*) areinfrequently detected in routine clinical practice owing to their rarity. Emerging drugs such as zenocutuzumab, a bi-specific antibody, have reported activity in NRG1 tumors, regardless of histologic type [7–9]. A timeline depicting the identification of selected oncogenic alterations (gene amplifications and fusions) in RTKs of NSCLC and the different treatments approved for each of the activated RTKs is represented in Fig. 2**.**

This review will discuss the most relevant gene fusions and gene amplifications activating RTK in NSCLC, with special emphasis on the genomic structure, diagnostic approaches, and available therapeutics of each case.

Oncogenic gene fusions involving RTK in NSCLC and their clinical implications

Gene fusions affecting the anaplastic lymphoma kinase receptor, ALK

ALK encodes a transmembrane RTK expressed in the nervous system during embryogenesis. *ALK* was first identified to fuse with *NPM1* in anaplastic large-cell lymphoma in 1994, but it was not until 2007 that *ALK* fusions were reported in NSCLC [10]. At present, this alteration is considered to affect 2–7% of LuADs, mostly women and never-smokers. Oncogenic *ALK* rearrangements fuse the intact kinase domain of ALK to the N–terminal regions of its partner, resulting in overexpression and constitutive, ligand-independent activation of ALK. Overall, more than 20 fusion partners have been identified, with *EML4* as the most prevalent (Fig. 3) [11].

Because ALK fusions involve large chromosomal inversions and translocations, fluorescent *in situ* hybridization (FISH) using break-apart

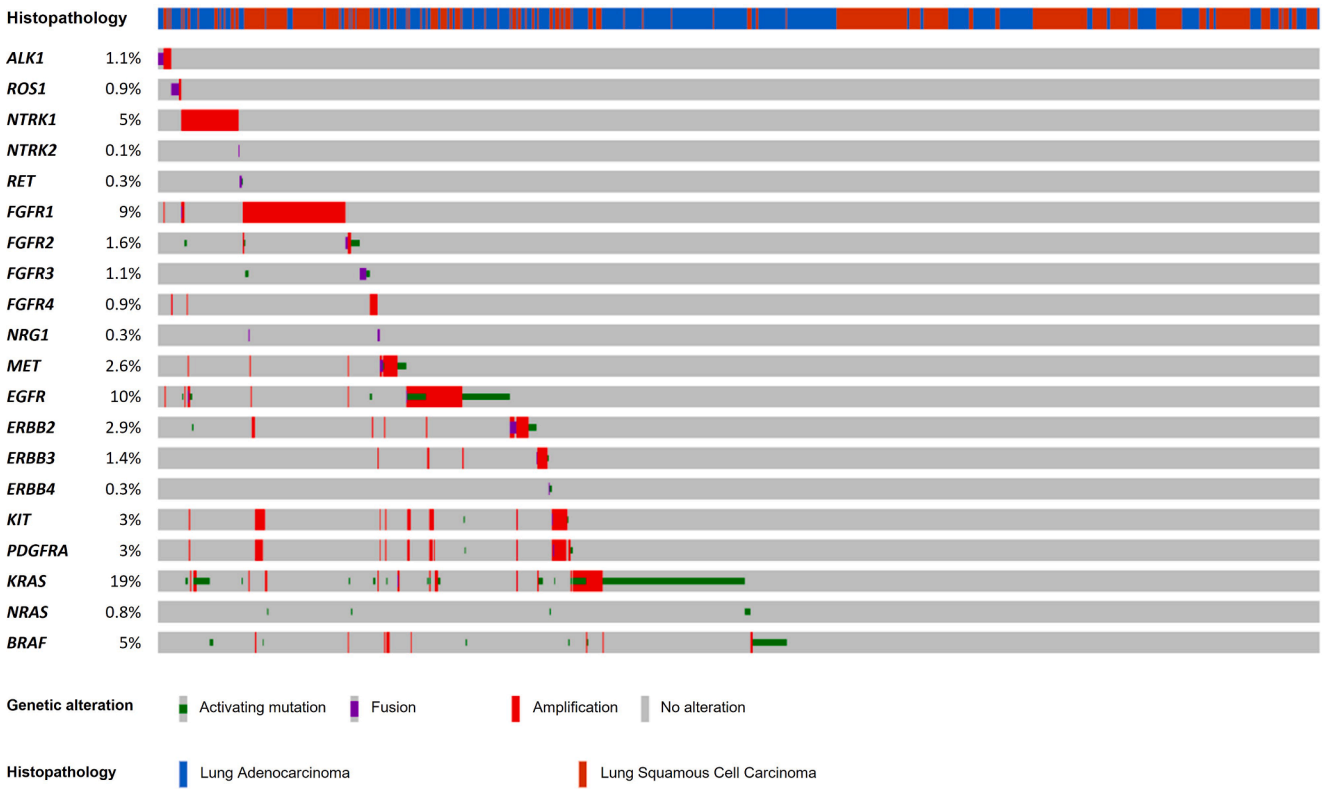

Fig. 1. Oncoplots showing the distribution of the oncogenic mutations/fusions/amplifications for each indicated gene in the LuAD and LuSCC cohorts of lung PanCancer atlas (data extracted from cbioportal, https://www.cbioportal.org/). LuAD: Lung adenocarcinoma; LuSCC: Lung squamous cell carcinomas, TCGA: Tumor Cancer Genome Atlas.

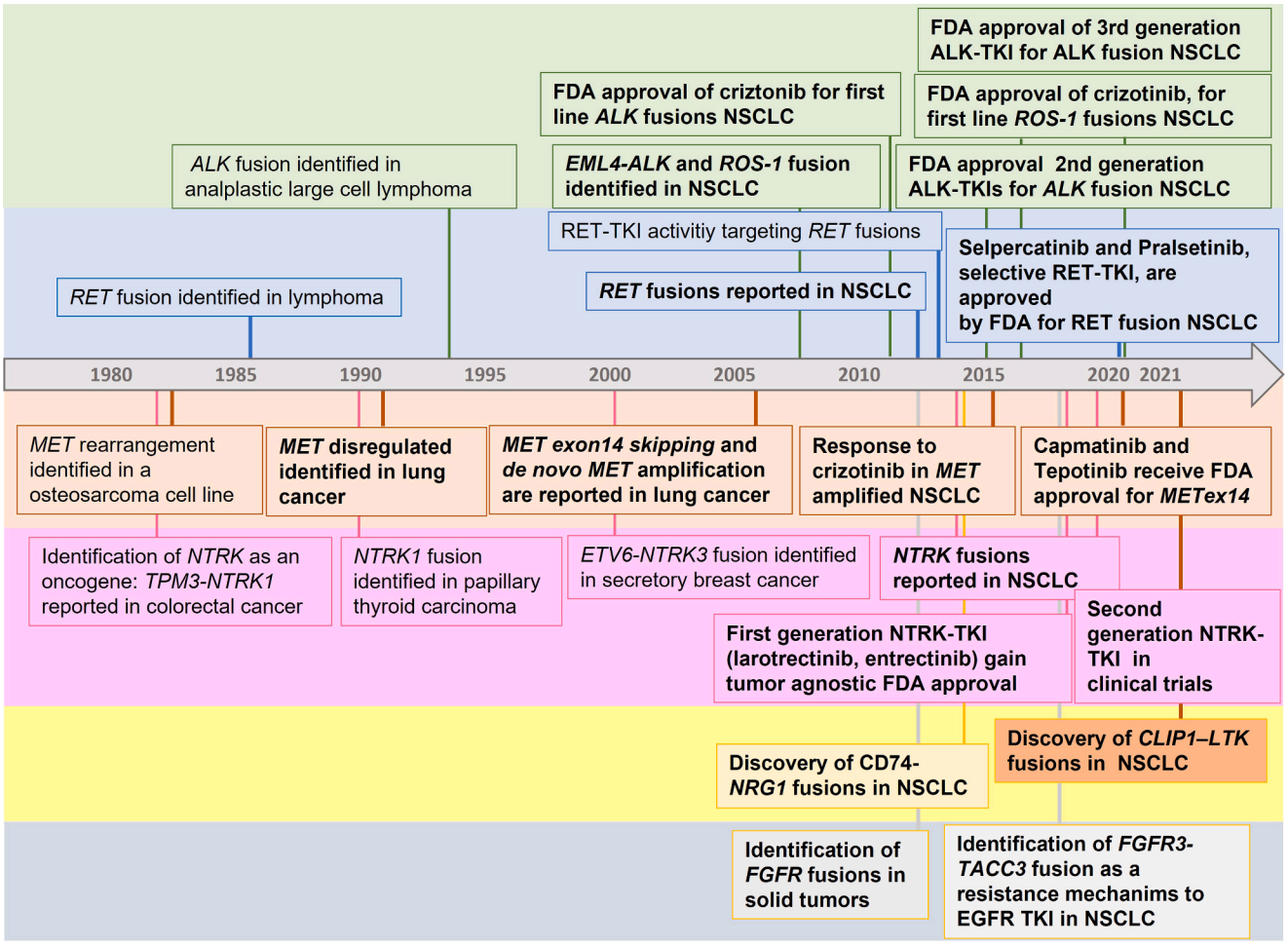

Fig. 2. Timeline depicting the identification of selected oncogenic drivers in various malignancies, including NSCLC (in bold) and targeted therapies activity and approval for each of them. FDA: US Food and Drug Administration; NSCLC: Non-small cell lung cancer; TKI: Tyrosine kinase inhibitor.

probes was the first method developed for detecting all *ALK* rearrangements and the first to receive approval by the FDA [12–13]. Since *ALK* fusions trigger an increase in the mRNA and protein levels, immunohistochemistry (IHC) can also be used. Owing to its high sensitivity and cost-effectiveness, IHC with the Ventana ALK D5F3 antibody gained FDA approval [14]. RNA-based next-generation sequencing (NGS) is also a valid technique to reveal *ALK* fusions as long as the quality of RNA is optimal [15] (Table 1).

Currently, five TKIs (i.e., crizotinib, ceritinib, alectinib, brigatinib, and lorlatinib) have been approved by the FDA to treat NSCLCs harboring *ALK* translocations (Table 2). Crizotinib is a first-generation TKI that inhibits ALK but also targets ROS1 and MET and was the first to show efficacy in previously treated patients [16–17]. Almost one-third of crizotinib-treated patients acquire resistance by mutations affecting the kinase domain, with p.Leu1196Met and p.Gly1269Ala as the most prevalent [18]. Ceritinib, alectinib, and brigatinib are second-generation ALK-TKIs that bind to the receptor with higher affinity than crizotinib. These TKIs show activity against multiple mutations acquired during the treatment with crizotinib and have an improved penetrance into the central nervous system [18–20]. The results of the phase III ALEX trial evidenced better clinical outcomes for alectinib than for crizotinib in terms of median progression-free survival (mPFS) (34.8 vs 10.9 months; hazard ratio [HR]: 0.43) -investigator assessed- and median overall survival (mOS) (not reached [NR] vs 57.4 months; HR: 0.67). On the basis of this study, alectinib became the most common first–line treatment for patients with *ALK*–rearranged tumors [21,22] (Table 2). Similarly, the ALTA1L trial demonstrated a superior mPFS for brigatinib over crizotinib in previously untreated LuAD patients with ALK-rearranged tumors [23]. Since both trials showed similar outcomes and toxicity profiles, alectinib and brigatinib represent a reasonable option for first-line treatment. Finally, lorlatinib, a selective and third-generation TKI that inhibits ALK and ROS1, has activity against most known *ALK* drug-resistant mutations [24]. Based on the results of the CROWN trial, lorlatinib also gained FDA and EMA (European Medicines Agency) approval as a first-line treatment [25]. Since no trial has compared the efficacy of all ALK-TKIs in first-line, lorlatinib also seems a reasonable option, particularly when considering its activity in the brain. More recently, the small-molecule ensartinib (X-396) demonstrated greater efficacy than crizotinib in both systemic and intracranial disease, thus representing another first-line therapeutic option for patients with ALK-positive NSCLC [26] (Table 2).

Despite the therapeutic efficacy of these TKIs, the emergence of acquired resistance may limit their long-term benefits. The solvent-front p.Gly1202Arg substitution impairs the correct binding of the drug to ALK and constitutes a common resistance mechanism to ALK-TKIs [27]. Besides, ALK-independent (or off-target) resistance mechanisms such as *MET*amp, detected in almost 15% of cases [28], loss of *NF2*, or histopathologic transformation into a neuroendocrine subtype have also been reported [29].

Gene fusions affecting the c-ros oncogene 1 receptor, ROS1

ROS1 is a receptor with tyrosine kinase activity that shares structural homology, within the TKD, with ALK and can also undergo genomic

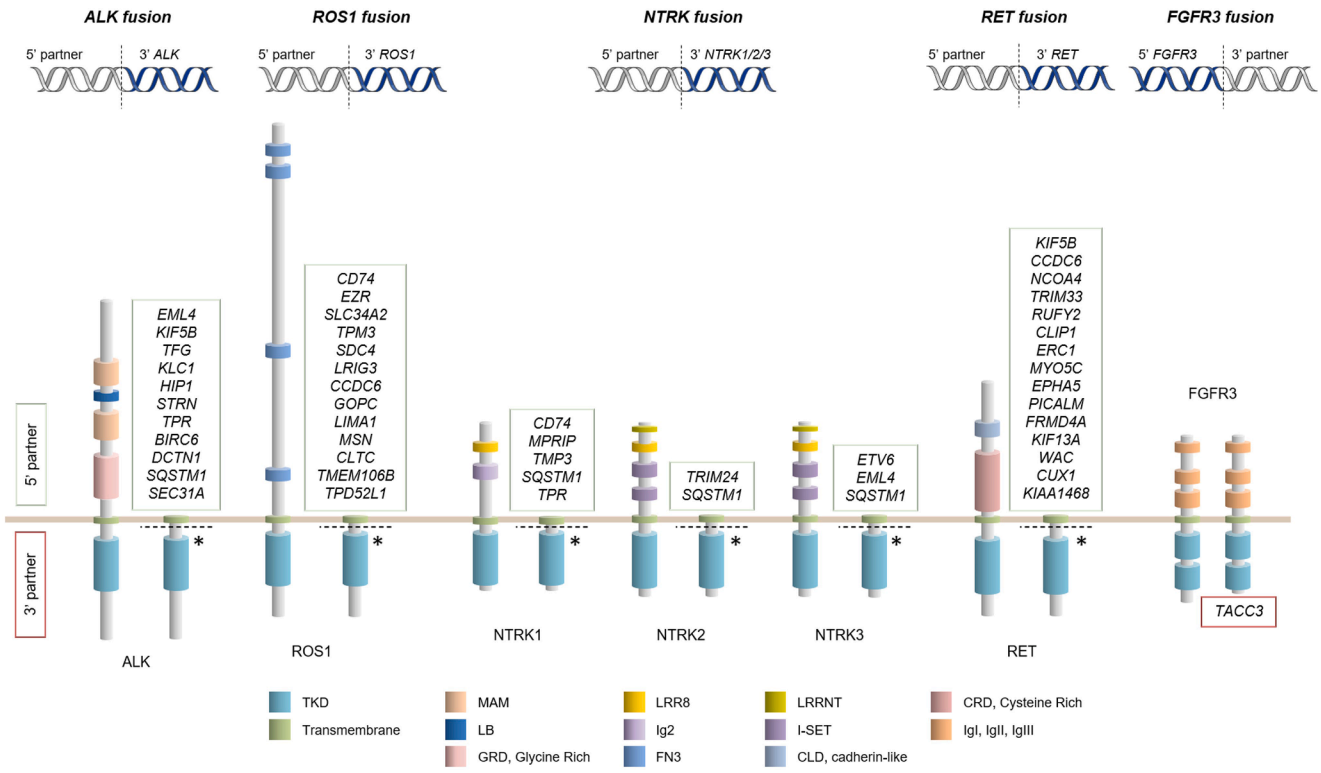

Fig. 3. Molecular structure of oncogenic fusions in NSCLC involving RTK and the most common fusion partners in each case. The upper panel describes the fusion at the DNA level and the lower panel shows the wild-type and fusion proteins. The different domains of the RTKs are indicated; (*) indicates the absence of transmembrane domain which can occur in some cases. NSCLC: Non-small cell lung cancer; RTK: Receptor tyrosine kinase.TKD: Tyrosine kinase domain; MAM: MAM domain (meprin/A5/mu); LB: Ligand binding domain; LRR**8**: Leucine rich repeat; Ig2: Immunoglobulin domain; FN3:Fibronectin type III domain; LRRNT: Leucine rich repeat N-terminal domain; I-SET: Immunoglobulin I-set domain; IgI, IgII; IgII: Immunoglobulin domains

Table 1
List of oncogenes altered by gene fusions and amplification in NSCLC.

Driver fusion/amp	Histopathologic predominance pattern	Incidence	Cancer type for FDA approval technique	IHC	FISH	NGS
ALK fusion	LuAD solid, mucinous cribiform, and signet ring cells	2–7%	NSCLC	Ventana ALK D5F3 IHC CDx assay*	Vysis ALK break Apart FISH Probe Kit (Abbott Molecular)*	NGS FoundationOne CDx*
ROS1 fusion	LuAD solid, mucinous cribiform, and signet ring cells	2%	NSCLC	ROS1 D4D6 IHC assay* Cell Signaling Technology	Vysis ROS1 break-apart FISH Probe Kit	NGS FoundationOne CDx*
NTRK (1,2,3) fusion	LuAD	3.5% <1% <1%	Solid tumors	Loxo/Ventana Pan-TRK IHC CDx*	NA	NGS FoundationOne CDx*
RET fusion	LuAD solid, mucinous cribiform, and signet ring cells, LuSCC	1–2%	NSCLC, thyroid	NA	NA	Oncomine Dx Target Test*
FGFR3 fusion	LuSCC/LuAD	<1%	NA	NA	NA	DNA/RNA-based NGS
NRG1 fusion	LuAD	<1%	NA	NA	NA	DNA/RNA-based NGS
LTK fusion	LuAD	<1%	NA	NA	NA	DNA/RNA-based NGS
FGFR1 amp	LuSCC	15–25%	NSCLC-SCC	NA	NA	DNA-based NGS
MET amp	LuAD, LuSCC, PSC	1–5%	NA	NA	NA	DNA-based NGS
ERBB2 amp	LuAD	2–5%	Breast, gastric cancer	HercepTest (Dako)*	HER2 FISH pharmDx Kit (Dako)*	DNA-based NGS
KIT/PDGFRA amp	LuAD, LuSCC	1–2%	NA	NA	NA	DNA-based NGS

* FDA approved and validated in clinical trials.
CD: Companion diagnostic; FDA: US Food and Drug Administration; FISH: Fluorescence *in situ* hybridization; IHC: Immunohistochemistry; LuAD: Lung adenocarcinoma; LuSCC: Lung squamous cell carcinoma; NA: None approved; NGS: Next-generation sequencing; NSCLC: Non-small cell lung cancer; PSC: Pulmonary sarcomatoid carcinoma.

Table 2
List of most relevant clinical trials and clinical efficacy of targeted therapies for NSCLC with tumors with driven oncogenic fusions and amplifications.

Biomarker	Targeted Drug (type)	Trial	Study type	ORR (95% CI)	mPFS months HR (95% CI)	mOS months HR (95% CI)	FDA approval	*Ref.*
ALK	Crizotinib	Profile 1001, ≥1st L	Phase I (ALK cohort n = 143)	60.8% (52.3–68.9)	9.7 (7.7–12.8)	12; OS rate: 74.8% (66.4–81.5)	Accelerated approval (2011)	*16*
		Profile 1007, ≥2nd L (vs CT)	Phase III (n = 173)	65% vs 20%	7.7 vs 3; HR: 0.49 (0.37–0.64)	20.3 vs 22.8 HR: 1.02 (0.68–1.54)	Approved (2013)	*17*
		Profile 1014, 1st L (vs CT)	Phase III (n = 343)	75% vs 45%	10.9 vs 7; HR: 0.45 (0.35–0.60)	NR vs 47.5; HR: 0.76 (0.55–1.05)		*18*
	Ceritinib	ASCEND 4, 1st L (vs CT)	Phase III (n = 376)	72.5% (65.5–78.7) vs 26.7% (20.5–33.7)	16.6 vs 8.1; HR: 0.55 (0.42–0.73)	NA	Approved (2014)	*19*
		ASCEND 5, ≥2nd L (vs CT)	Phase III (n = 231)	39.1% (30.2–48.7) vs 6.9% (3.0–13.1%)	5.4 vs 1.6; HR: 0·49 (0.36–0.67)	NA		*20*
	Alectinib	ALEX (vs crizotinib) 1st L	Phase III (n = 303)	82.9% (75.0–88.5) vs 75.5% (67.0–82.0)	34.8 vs 10.9; HR 0.43 (0.32–0.58)	NR vs 57.4; HR: 0.67 (0.46–0.98)	Approved (2017)	*22*
	Brigatinib	ALTA 1L (vs crizotinib), 1st L	Phase III (n = 275)	71% (62–78) vs 60% (54–66)	24 vs 11; HR 0.49 (0.35–0.68)	NA	Approved (2020)	*23*
	Lorlatinib	NCT01970865, ALK-TKI pre-tt	Phase I/II (n = 275)	48% (42–55)	NA	NA	Accelerated approval (2018)	*24*
		CROWN (vs crizotinib), 1st L	Phase III (n = 296)	76% (68–83) vs 58% (49–66)	NR vs 9.3; HR 0.28 (0.19–0.41)	NA	Approved (2021)	*25*
	Ensartinib or X-396 (multi-TKI)	EXALT-3 (vs crizotinib), 1st L	Phase III (n = 290)	75% vs 67%	25.8 vs 12. 7; HR 0.51 (0.35–0.72)	NA		*26*
ROS1	Crizotinib	Profile 1001, ≥1st L	Phase I (ROS-1 cohort n = 50)	72% (58–84%)	19.3 (15.2–39.1)	51.4 (29.3-NR)	Approved (2016)	*33*
		EURCROSS, Europe, TKI-naïve	Phase II (n = 34)	70% (51–85)	10.1 (20-NR)	NA		*34*
	Lorlatinib	NCT01970865, TKI-naïve and pre-tt	Phase II (n = 69)	62% (TKI-naïve)/ 35% (pre-tt)	NA	NA	Approved (2019)	*35*
	Entrectinib (multi-TKI)	ALKA 372–001, STARTK-1, TKI-naïve	Phase I/II (n = 53)	77.4%	24.6 (11.4–34.8)	NA	Approved (2019)	*36*
	Ceritinib (multi-TKI)	NCT 01964157, TKI-naïve and pre-tt	Phase II (n = 32)	62%	9.3 (0–22) all /19.3 (1–37) tt-naïve	24 (5–43)		*37*
	Repotrectinib or TPX-0005 (multi-TKI)	TRIDENT-1, TKI-naïve	Phase II (n = 7)	86%	NA	NA		*38*

Biomarker	Targeted Drug (type)	Trial	Study type	ORR (95% CI)	mPFS months HR (95% CI)	mOS months HR (95% CI)	FDA approval	*Ref.*
NTRK	Larotrectinib (Selective-TKI)	LOXO-TRK-14001, SCOUT & NAVIGATE	Phase I & II (n=159)	79% (72–88)	28.3 (22.1-NR)	44.4 (36.5-NR)	Approved for solid agnostic tumors (2018)	*45, 46*
	Entrectinib (multi-TKI)	ALKA-372–001, STARTRK-1 & STARTRK-2	Phase I & II (n=54)	57% (43.2–70.8)	11 (9–14.9)	21 (14.9-NA)	Approved for solid agnostic tumors (2019)	*47*
	Selitrectinib or LOXO-195 (selective-TKI)	NCT03215511 (TKI pre-tt)	Phase I (n=20)	36%	NA	NA	Orphan drug designation	*49*
	Repotrectinib or TPX-0005 (multi-TKI)	TRIDENT-1 (TKI pre-tt)	Phase II (n=6)	50%	NA	NA		*50*
RET	Cabozantinib (multi-TKI)	NCT01639508, pre-tt	Phase II (n=25)	28%	5.5 (3.8–9.4)	9.9 (8.1-NR)		*56*
	Vandetanib (multi-TKI)	LURET, pre-tt	Phase II (n=19)	53%	6.5 (2.8–9.5)	13.5 (9.8–29.1)		*57*
	Lenvatinib (multi-TKI)	NCT01877083, pre-tt	Phase II (n=25)	16%	7.3 (3.6–10.2)	NA		*58*
	Selpercatinibor LOXO-292 (selective-TKI)	LIBRETTO-001,1st L or pre-tt	Phase I/II (n=105)	68% (pre-tt, n=105)/85% (tt-naïve, n=34)	18.4 (13.8–24) /NR (9.2-NR)	NA	Accelerated approval (2020)	*59*
	Pralsetinib or BLU-667 (selective-TKI)	ARROW, 1st L or pre-tt	Phase I/II (n=114 NSCLC)	58% (pre-tt n=48)/ 71% (tt-naïve, n=7)	NA	NA	Accelerated approval (2020)	*60*
MET amp	Crizotinib	Profile 1001, ≥1st L	Phase I (MET cohort=37)	GCN≥4: 44%; GCN 2-<4: 14%; GCN<2: 33%	6.7/1.9/1.8	NA		*81*
	Capmatinib	GEOMETRY, ≥1st L	Phase II, GCN≥10 (n=14/55)	40%/29%	4.2/4.1	9.6/10.6		*82*

95% CI: 95% confidence interval; FDA: US Food and Drug Administration; GCN: Gain copy number; HR: Hazard ratio; L: Line (of treatment); mOS: Median overall survival; mPFS: Median progression-free survival; NA: Not assessed; NR: Not reached; NSCLC: Non-small cell lung cancer; ORR: Overall response rate; pre-tt: Pre-treated; TKI: Tyrosine kinase inhibitor; tt-naïve: Treatment naïve.

rearrangements to create fusion proteins [30]. For ALK and other RTK fusions, the fusion partner provides a dimerization domain that induces constitutive oligomerization and, thus, activation of the kinase. However, since most *ROS1* fusion partners lack this dimerization domain, the mechanism for ROS1 oncogenic activation remains unknown [31]. In the ROS1 fusions, the kinase domain of ROS1 is paired with a wide range of partners, the most common being CD74 (Fig. 3) [30]. Chromosomal rearrangements of the *ROS1* proto-oncogene occur in about 1–2% of LuADs and more often in never-smokers [30]. The diagnosis of *ROS1* fusions can be performed by IHC—the most cost-effective method—and confirmed by FISH, but NGS can also be used (Table 1) [32].

Crizotinib was the first TKI to demonstrate activity in *ROS1*-rearranged tumors, showing promising overall response rates (ORR) (mPFS of 19.3 months and mOS of 51.4 months (95% CI 29.3- NR) in a subset of mostly pretreated patients with advanced-stage NSCLC [33]. At present, crizotinib remains the recommended first-line therapy for these patients [34]. Lorlatinib has also demonstrated efficacy in both TKI-naïve and TKI-pretreated patients with *ROS1*-rearranged tumors [35]. Entrectinib, which also targets TRKA/B/C and ALK, has shown activity against these tumors as well, inducing meaningful intracranial responses in patients with brain metastases [36]. While these three drugs have gained FDA approval, other agents such as ceritinib, brigatinib, repotrectinib (TPX-0005), and DS-6051b are currently being evaluated in phase I and II trials [37–39] (Table 2).

The genetic mechanisms that confer resistance to crizotinib in tumors with *ROS1* fusions are equivalent to those in *ALK*-rearranged NSCLC. The p.Gly2032Arg mutation, which is structurally analogous to the p.Gly1202Arg mutation in *ALK*, is the most common, although other mutations (e.g., p.Asp2033Asn and p.Ser1986Phe) have also been reported [40]. Since not all on-target mutations are equally sensitive to the new generation of inhibitors, tumor stratification according to the type of mutation will be required.

Gene fusions affecting the neurotrophic tropomyosin tyrosine kinase receptor, NTRK

The *NTRK*-family of genes comprises three members (*NTRK1, NTRK2,* and *NTRK3*) that encode tropomyosin receptor kinases (TRK) A, B, and C, respectively. These kinase receptors are physiologically involved in neuronal development and differentiation. The aberrant activation of *NTRK*, which results in the constitutive activation of downstream pathways, appears mainly through gene fusions and several partners have been described (Fig. 3) [41]. These alterations have a prevalence of 0.2–0.4% across several solid tumor types in adult cancers, regardless of gender and smoking habit, with higher prevalence in pediatric population (1.34%). Among adult patients, *NTRK* fusions are more prevalent in salivary gland cancers (2.43%), soft tissue sarcoma (1.27%) and thyroid cancers (1.28%),albeit their frequency in LuADs is low (<1%) [42]. Notably, the rare fusion *ETV6–NTRK3* is detectable in nearly all patients with secretory carcinoma of the breast and with mammary analogue secretory carcinoma of the salivary glands and is, thus, considered pathognomonic in these two histologically identical tumor types [43]. IHC screening confirmed by FISH or by NGS are appropriate methods for diagnosing these fusions [44] (Table 1).

The first generation of TKIs that blocked the activity of RTKs were larotrectinib (a selective TRK inhibitor) and entrectinib (a multikinase inhibitor also targeting ROS1 and ALK), and both conferred durable responses in patients with metastatic disease (ORR of larotrectinib: 75% and ORR of entrectinib: 61.2%, with a mPFS of 13.8 months (95% CI, 10.1–19.9), regardless of tumor histology, age, or fusion partner [45–47]. The activity of larotrectinib have also been reported in 15 patients with NSCLC harboring *NTRK* fusions, showing a ORR of 73%, mPFS of 35.4 months (95% CI, 5.3–35.4), and mOS of 40.7 months (95% CI, 17.2 to not estimable) [46]. These two TKIs gained tumor-agnostic regulatory accelerated approvals by the FDA to treat cancers harboring *NTRK*-fusions (Table 2) [45,47]. However, like others RTKs, resistance to these TKIs can eventually arise by the emergence of secondary mutations at the kinase domain of NTRK [48]. Next-generation agents, such as selitrectinib (LOXO-195) and repotrectinib, were designed to address on-target resistance mechanisms and have shown promising activities in clinical trials (Table 2) [49–51]. Given the potent clinical activity of the TRK inhibitors, the current clinical guidelines recommend testing all NSCLCs for *NTRK* fusions.

Gene fusions affecting the REarranged during transfection receptor, RET

The *RET* proto-oncogene encodes a transmembrane RTK involved in numerous developmental pathways and multiple malignancies, including multiple endocrine neoplasia 2, papillary thyroid cancer, and NSCLC. Unlike other RTKs, RET does not directly bind to its ligands but requires glycosyl-phosphatidylinositol-anchored (GFRalpha1-4) co-receptors. Indeed, ligands of the glial cell line-derived neurotrophic factor family bind to one of the four co-receptors, which subsequently allow RET dimerization, autophosphorylation, and activation, leading to downstream activation signaling [52]. *RET* fusions can be found in 1–3% of LuADs and are more common in young, female, never-smoker patients (Table 1). More than ten *RET* fusion partners have been described, being *KIF5B* the most frequent (Fig. 3) [52,53]. Unlike *ALK* and *ROS1* fusions, *RET* fusions cannot be properly detected by IHC because of the low sensitivity and highly variable specificity of the available antibodies. On the other hand, FISH is a sensitive but rather unspecific approach to test for RET fusions in NSCLC, owing to the frequent presence of RET rearrangements not resulting in oncogenic RET [54]. Therefore, NGS is the most appropriate option for diagnosing RET fusions [55] (Table 1).

Several multitarget kinase inhibitors, such as cabozantinib, vandetanib, and lenvatinib—approved for treating patients with advanced medullar thyroid cancer—have limited activity in *RET*-rearranged NSCLC and important adverse effects due to their off-target activity [56–58]. More recently, two highly potent and selective RET-TKI, selpercatinib and pralsetinib, have gained FDA approval for lung and thyroid cancers. In the LIBRETTO-001 trial on advanced NSCLC, selpercatinib (LOXO-292) was given to pretreated (ORR of 65%) and treatment-naïve patients (ORR of 85%) [59] (Table 2). Pralsetinib's (BLU-667) efficacy was established in the phase I/II ARROW trial with similar results [60]. Both drugs were well tolerated with an acceptable safety profile and relevant intracranial activity regardless of the *RET*-fusion partner (ORR of selpercatinib: 91% and ORR of pralsetinib: 78%). However, p.Gly810Arg/Ser/Cys mutations have been described as acquired resistance mechanisms to selective RET inhibitors [61]. Second-generation RET-TKIs (e.g., TPX-0046 [NCT04161391] and BOS-172738 [NCT03780517]) are currently being tested in clinical trials [62].

Gene fusions affecting the fibroblast growth factor receptors, FGFRs

FGFRs are a family of RTKs expressed on the cell membrane that play crucial roles in developmental and adult cells. The human family of FGFRs consists of four members: FGFR1, FGFR2, FGFR3, and FGFR4 [63]. Despite being encoded by different genes, the four members share high homology. The aberrant activation of FGFRs, caused by activating gene fusions, amplifications, or mutations involving the TKD, is a frequent oncogenic mechanism in different types of cancer, such as glioblastomas, cholangiocarcinomas, breast cancer, and bladder cancer, among others [63]. Although fusions affecting *FGFRs* are uncommon, they have been described in lung cancer, mostly in LuSCCs. The most widely recognized is the fusion between *FGFR3* and *TACC3* [9] (Fig. 3). Fusions at *FGFR1, FGFR2*, and *FGFR4* are even rarer and still poorly characterized. Alterations at *FGFR1* in lung cancer are mostly gene amplification and are found in up to 25% of LuSCCs, approximately (Table 1) [64].

In contrast to *ALK, ROS1*, and *RET*, *FGFR*-fusions are more frequent in LuSCC from smokers and in poorly differentiated tumors. NGS is the

standard technique to diagnose *FGFR*-fusions. Several drugs targeting FGFRs are currently under development and their efficacy is being evaluated in several basket trials regardless of histopathologic subtype. The early-phase clinical trial and first–in–human study (NCT01703481) tested the TKI erdafitinib (JNJ–42756493), a pan-FGFR inhibitor for patients with detectable *FGFR1-3* alterations, but yielded only a few responses with an acceptable toxicity profile [65]. Similar efficacy has been reported for the ATP-competitive broad-spectrum kinase inhibitor ARQ–087 in NSCLC patients with tumors harboring *FGFR* genetic activation [66]. Finally, infigratinib, an FGFR1–3 selective inhibitor, has proven effective in FGFR altered tumors, particularly in cholangiocarcinomas [67] (Table 2).

Other gene fusions affecting RTKs in NSCLC

A variety of other rare fusions involving receptors with tyrosine kinase activity have been identified in NSCLC, including *MET* and *NRG1* [68–69]. NRG1 is the ligand of the HER family and, despite not being an RTK itself, triggers the activation of RTKs. Although the prevalence of these fusions is very low, drugs targeting these proteins or the pathways regulated by them are under evaluation, and data from small-cohort studies and case reports have been obtained [70–71].

Fusions involving *MET* were initially discovered in an osteogenic sarcoma cell line (*TPR–MET*) and are very rare events in lung cancer (about 0.5%). A wide variety of fusion partners have been identified, including *HLA–DRB1, KIF5B, PTPRZ1, STARD3NL*, and *ST7,* although the biology of these alterations and their therapeutic implications have not been evaluated yet [69].

Finally, the fusion *CLIP1-LTK* has recently been discovered in a Japanese cohort of LuADs as an oncogenic driver, affecting around 0.4% of NSCLCs (Table 1). This fusion leads to the activation of the LTK (leukocyte receptor tyrosine kinase), an RTK and a member of the ros/insulin receptor family of tyrosine kinases whose function is still not well understood. Tumors with the *CLIP1-LTK* fusion responded to lorlatinib, although clinical validation of this new oncogenic driver and clinical development of novel therapeutic agents are warranted [8].

Neuroregulins (NRGs) are the ligands of the EGFR family of proteins and are encoded by four genes (*NRG1, NRG2, NRG3,* and *NRG4*). *NRG1* is the best characterized and is associated with the invasive mucinous subtype of LuAD, typically diagnosed in women and never-smokers (Table 1). Rearrangements of *NRG1* in solid tumors lead to aberrant activation of HER2/HER3 and, subsequently, of the PI3K-AKT-mTOR and RAS/MAPK cascade. Albeit several fusion partners have been described, *CD74* remains the most common [68–69] (Fig. 3). Afatinib, an irreversible inhibitor of EGFR, represents a therapeutic option for patients with tumors with *NRG1* fusions and several case reports have been published in this sense [70]. In addition, blocking HER3 also showed promising activity in these patients [71].

Oncogenic gene amplifications involving RTK in NSCLC and their clinical implications

Besides gene fusions, gene amplification represents another mechanism that activates oncogenes during cancer development. Gene amplification can be defined as an expansion in the number of copies of a gene or a chromosomal region that occurs during the DNA replication process [72]. The amplified DNA can be organized as extrachromosomal elements, as repeated units at a single locus, or scattered throughout the genome. Similar to gene fusions, oncogenic activation by gene amplification leads to a supraphysiological increase in the expression of the oncogene [73]. In the case of RTKs, this allows the receptor to become constitutively active. Gene amplification in cancer cells can ensue by expansion during the process of tumor development, by *de novo* occurrence, or by clonal selection, as an adaptative strategy to bypass the pressure exerted during therapeutics. The latter constitutes a common mechanism for the acquired resistance to different TKI targeting RTKs [74]. The most frequent gene amplification in lung cancer is *FGFR1,* described in up to approximately 25% of LuSCC, as above mentioned (Table 1) [64].

Gene amplification affecting the mesenchymal-epithelial transition factor receptor, MET

The MET proto-oncogene encodes the RTK for hepatocyte growth factor and regulates a genetic program associated with cell proliferation and invasion of the extracellular matrix. MET mainly exists in epithelial cells and plays an important role in embryogenesis, tumor growth, and metastasis [75]. In NSCLC, primary *MET*amp has been reported in around 1–5% of tumors (Table 1), but, importantly, it also constitutes a mechanism for acquired resistance to EGFR-TKIs, as reported in 5–20% of the cases [76], but also in other oncogenic RTK such as *ALK* tumors treated with *ALK-TKIs* [28]. Currently, *MET*amp is defined by focal gains in *MET* copy number relative to the centromere of chromosome 7 (ratio MET/CEP7), although there is no well-established consensus on the most appropriate copy number cut-off. However, a higher ratio of MET/CEP7 copy number seems to predict a better response to MET inhibitors [77]. *MET*amp can be measured using FISH or NGS [76,77], but IHC of total MET protein is not considered a good surrogate marker for *MET*-amp tumors since strong MET immunostaining has been observed in many tumors without gene amplification, mostly in LuADs [73] (Table 1).

Notably, *MET* activation can also occur by point mutations that affect, mostly, consensus splice sites, leading to the elimination of exon 14 (*METex14*) [78]. *METex14* mutations are observed in 2–4% of NSCLCs and in both LuADs and LuSCCs, with a higher incidence in pulmonary sarcomatoid carcinomas [79,80]. These mutations have also been reported in other malignancies, such as gastric (7%) or colorectal (0–9%) cancers [78].

Crizotinib has shown activity against *MET* activated lung tumors [81]. More recently, the FDA approved two specific MET-TKIs, capmatinib and tepotinib, to treat NSCLC patients with *METex14* mutant tumors [76]. Note that for the EMA approval, patients harboring *METex14* should have progressed to prior immunotherapy and/or platinum-based chemotherapy. Capmatinib has also shown activity in *MET*amp tumors, particularly in those with a high gain copy number ($\geq$10) (Table 2**)** [82]. Furthermore, the dual EGFR and MET inhibition has proven effective in those cases in which *MET*amp was the acquired resistance mechanism to EGFR-TKI [83].

Gene amplification affecting the epidermal growth factor receptors HER

The HER family, also called EGFR family, or *ERBB* family when referring to the gene, comprises four transmembrane receptor tyrosine kinases: EGFR (or HER1), HER2, HER3, and HER4. These receptors signal through homo- and hetero-dimerization and promote cell proliferation, motility, and invasion [84]. *EGFR* amplification (*EGFRamp*), in association with EGFR protein overexpression, has been reported in both LuAD and LuSCC patients and, in LuADs, can co-exist with *EGFR* mutations [85]. *EGFRamp* has also been described as a resistance mechanism to third-generation EGFR-TKIs [85]. Despite the many available EGFR-TKIs, none of them has shown remarkable activity against *EGFRamp* tumors. Monoclonal antibodies targeting EGFR, such as cetuximab, have also provided disappointing results in NSCLC, in contrast to those in colorectal and head and neck cancer [86]. Within the HER family, *ERBB2*, encoding HER2, shows oncogenic alterations in NSCLCs through either point mutations or gene amplification and represents a novel targetable RTK. These alterations are most prevalent in LuADs from never-smoker women. Most *ERBB2* mutations are localized in exon 20, within the TKD, and the most frequent mutation is the p.Tyr772_Ala775dup (c.2313_2324dup), found in 1–2% of LuADs [87]. On the other hand, *ERBB2* amplification (*ERBB2amp*) has been described in around 3% of NSCLCs, either *de novo* or as a mechanism of acquired

resistance to EGFR-TKIs [87]. In contrast to breast and gastric cancer, where *ERBB2* overexpression is relatively common and has met with a notable success of anti-HER2 therapies, targeting ERBB2 in NSCLC remains challenging. Although *ERBB2amp* shows strong HER2 protein levels [73], initial trials of the anti-HER2 monoclonal antibody trastuzumab, alone or in combination with chemotherapy or pertuzumab, displayed only modest activity in *ERBB2*amp NSCLCs and yielded negative results in HER2-overexpressing patients [88]. In the recent phase II DESTINY-Lung01 trial, the antibody-drug conjugate trastuzumab deruxtecan showed durable anticancer activity in patients with previously treated HER2-mutant NSCLC, regardless of HER2 expression and amplification status [89].

Gene amplification affecting the growth factor receptors KIT and PDGFRA

The *c-KIT* proto-oncogene encodes a transmembrane receptor tyrosine kinase, KIT, expressed in several normal human tissues. Its ligand is the stem cell factor that mediates KIT dimerization and activation. Activating mutations at *KIT* have been documented in various neoplasms, particularly in gastrointestinal stromal tumors, among others [90]. These mutations confer sensitivity to the TKI imatinib [91]. On the other hand, the members of the platelet-derived growth factor receptor (PDGFR) family are protein-tyrosine kinases encoded by two genes: *PDGFRA* and *PDGFRB* [92]. The functional receptors consist of the PDGFRα/α and PDGFRβ/β homodimers and the PDGFRα/β heterodimer. This RTK family plays an essential role in embryonic development and wound healing in adults [92]. *KIT* and *PDGFRA* are closely located in the same arm of chromosome 4q12 and have been shown to co-amplify in about 2% of NSCLCs, both in LuADs and LuSCCs [73]. *KIT/PDGFRA* amplification has also been described as a resistance mechanism to ALK-TKIs in LuADs with *ALK* fusion [93]. To this day, these alterations have not been explored as therapeutic targets in lung cancer.

Lung cancer molecular testing in clinics: State of the art

According to clinical guidelines, an upfront genomic profiling test should be a priority to detect targetable oncogenic alterations in advanced non-squamous cell carcinomas and light/non-smokers squamous cell carcinoma patients diagnosed with lung cancer since these alterations are present in about 30% of the cases [2,3]. Different diagnostic methods, including IHC, FISH, reverse transcriptase PCR, and DNA/RNA-based NGS, can be used to detect gene fusions and amplifications. A list of benefits and limitations of these approaches and the different validated diagnostic methods for each gene fusion and amplification are listed in Table 3. Conventional methods, such as IHC or FISH, have been widely implemented and are approved methodologies to detect gene amplification and specific fusions, including *ALK* or *ROS1* [12–14,32–33]. However, based on the increasingly frequent need for a comprehensive genomic evaluation, NGS panels are becoming the preferred approach. Because NGS provides a great deal of genetic information that needs to be understood and classified according to clinical evidence within an appropriate time frame, its assessment should be provided by an expert molecular tumor board [94]. Despite the advantages of the NGS technology, access to NGS panels and treatments varies broadly among the different health systems worldwide.

Tumor biopsies are the most common source of cancer cells for genotyping and categorizing tumors for clinical decisions. Good quality RNA and DNA can be obtained from them, and biopsies preserve the morphological features of the tumor. Histopathologic transformation as a resistance mechanism to TKIs can be determined. However, in lung cancer, tumor tissue extraction requires invasive procedures, becoming a caveat when multiple re-biopsies are needed to monitor the course of the disease. For this reason, alternative sources of tumor DNA are needed. A blood-based test using cell-free circulating tumor DNA (ctDNA), also called "liquid biopsy," is a potential surrogate source of tumor DNA for diagnostic, prognostic, and therapeutic biomarkers in NSCLC patients. ctDNA has proven suitable to detect gene mutations and fusions involving RTKs with reasonable sensitivity and specificity across tumor types and is gaining interest in cancer monitoring [95]. ctDNA represents a non-invasive, rapid, and cost-effective strategy for obtaining DNA from tumor cells. However, the technique has not yet been fully translated into clinical practice, and the variability in the amount of ctDNA released by the tumors to the bloodstream may prevent the standardization of the procedure [95]. In addition, the quantity of tumor ctDNA in the whole DNA extract can be excessively low to develop high-throughput analyses and can render artefactual mutations (false positive) or, according to the sensitivity of the method, false negative results. Large-scale screening and standardization of experimental steps could resolve these problems.

Table 3
List of pros and cons of diagnostic techniques for the detection of gene fusions and gene amplification in clinical cancer management.

Method	Pros	Cons
FISH	Well-established approach No need for complex and expensive equipment Useful as a validation approach after positive IHC or NGS	The cut-off values should be standardized for each gene Not able to identify the fusion partner involved Limited to detect intrachromosomic translocations
IHC	Well-established approach No need for complex and expensive equipment Useful for preselecting tumors for confirmatory FISH testing Allows to describe morphologic characteristics and tumor heterogeneity Excellent sensitivity for certain antibodies More cost-effective than FISH	IHC score should be standardized Not able to identify the fusion partner involved Specificity might relay on the antibody
NGS	Large amount of genetic information is provided at once In fusions, breakpoints are characterized at single nucleotide resolution Detection of unknown translocation partners Multiple samples can be pooled and sequenced together Suitable turn-around time	Depends on the quality and the amount of sample Needs dedicated bioinformatics personnel to maintain a clinical NGS service Requires complex and expensive equipment
RT-PCR based techniques	High specificity with robust and detailed information No need for complex and expensive equipment In fusions, breakpoints can be characterized at single nucleotide resolution	Results depend on the quality of RNA Needs to be designed according to known fusion breakpoints Unable to detect unknown partners Unconclusive for the detection of gene amplification

FISH: Fluorescent *in situ* hybridization, IHC: Immunohistochemistry, NGS: Next-generation sequencing, RT-PCR: Reverse transcriptase polymerase chain reaction.

Therapeutic challenges and opportunities in NSCLC patients harboring oncogenic fusions

Despite incorporating novel and highly selective TKIs for lung tumors harboring specific oncogenic mutations, advanced-stage lung cancer remains largely incurable. Treatments facilitate the emergence of resistant clones or the selection of pre-existing resistant sub-clones, and relapsing is unavoidable. Acquired resistance, defined as progression after initial benefit, is mediated by different biologic mechanisms that allow tumor adaptation. Therefore, re-biopsy is always encouraged to identify the mechanisms underlying the acquired resistance and

evaluate whether it can be approached therapeutically. The alterations that drive resistance include acquired mutations in the target oncogene (on–target resistance) and alterations in genes coding for proteins acting in parallel or downstream signaling pathways that allow bypassing the action of the TKI (off-target resistance) [96]. Furthermore, although not very common, histopathologic transformation, mostly from LuAD to small-cell lung carcinoma or LuSCC, represents another mechanism of resistance to targeted TKI and has been described in LuADs with mutations in *EGFR* or with *ALK* and *ROS1* fusions [97–99]. In some cases, genetic alterations may be associated with this transformation, for instance, in some LuADs that have become resistant to EGFR-TKIs through the *TACC3-FGFR3* fusion [97]. The pre-existence of *RB1* inactivation, common in mutant-*EGFR* LuADs, has also been associated with a higher predisposition to develop acquired resistance by histopathological transformation [97]. Once the tumor undergoes histologic transformation, the prognosis is detrimental, and the therapeutic approach is undertaken on a case-by-case basis.

Disease progression after treatment with TKIs can also occur because of an inadequate exposure of the drug to the receptor. The low penetration of these drugs into certain organs, particularly into the brain, poses additional challenges. This is particularly important for patients with NSCLCs harboring oncogenic driver fusions, who have an increased risk of developing brain metastases (20 to 40% at the time of diagnosis, which can increase up to 80% over the course of their disease) [100]. Since brain metastases constitute an independent prognostic factor for worse overall survival and poorer quality of life, novel generation TKIs with improved intracranial activity are being designed. These TKIs need to demonstrate higher efficacy in reducing brain metastases than previous generations, to prevent the onset of new metastases, and to delay local therapeutic strategies, such as whole-brain radiotherapy and its related toxicity [101].

Of note, a small proportion of NSCLC patients with targetable fusions can present oligometastatic disease at diagnosis or undergo oligoprogression under TKI treatment [102]. This means that, while maintaining a systemic overall response to TKI, a subset of tumor clones has become resistant and has progressed into specific locations. Therefore, adding local ablative strategies should be considered to increase the disease's control rate with prognostic implications [103].

Novel and specific inhibitors are the preferred therapeutic options for NSCLC patients with actionable driver oncogenes. Consequently, most immunotherapy trials exclude these patients, and the benefits of these treatments remain unclear for them. The majority of the currently available evidence comes from subgroup analysis from real-world data and clinical trials or from small trials specifically designated to address this issue [104]. Actionable driver fusions are commonly found in never-smoker patients, which tend to be associated with a low tumor mutation burden and a less inflamed tumor microenvironment. The multicenter study Immunotarget retrospectively collected the clinical outcomes of NSCLC patients harboring driver mutations who were treated with immunotherapy. Its results showed low efficacy in patients harboring oncogenic driver fusions (e.g., *ALK* or *RET*), regardless of the levels of PD-L1 [105].

Finally, the clinical management of lung cancer patients, as for many other cancers, would need to consider genetic predisposition as another variable. Most NSCLCs with actionable oncogenic drivers, including fusions and gene amplification, are found in never-smokers and, often, in young individuals. To date, the explanation is unclear, and the possibility that these cancers arise within hereditary cancer syndromes should be considered. In this regard, the Li-Fraumeni syndrome is the most common hereditary syndrome associated with lung cancer development, especially *EGFR*-mutant tumors [97,106]. The occasional association of lung cancer with other cancer syndromes, such as BRCA1/2, among others, should not be ruled out either [97].

Conclusions

TKIs have prompted significant improvements in the outcomes of patients with lung tumors harboring tyrosine kinase driven oncogenic mutations or rearrangements (fusions or gene amplification). Despite the low frequency of each mutation or rearrangement involving RTKs, altogether, they affect about 15% of NSCLCs. Currently, identifying these patients remains crucial because they can achieve survival for up to several years when treated with the appropriate inhibitor. The magnitude of the clinical benefits achieved with targeted therapies and the increasing number of specific clinical trials has prompted the integration of molecular profiling technologies in clinical and pathological settings. The key for succeeding with targeted therapeutics is integrating an accurate diagnosis with potent and selective therapeutics together with an optimal penetration of new, low-toxicity drugs into the central nervous system. Although several promising targeted drugs have been recently approved or are under clinical evaluation, optimal strategies to overcome resistance, including combinations with immunotherapy or chemotherapy, have not been clearly established. The future of precision medicine will likely integrate comprehensive genomic tumor characterization, dynamic monitoring of liquid biopsy and/or tissue guided rebiopsies, and the enrollment of patients into innovative clinical trials.

CRediT authorship contribution statement

Maria Saigí: Conceptualization, Writing – original draft, Writing – review & editing, Supervision. **Enric Carcereny:** Writing – review & editing. **Teresa Morán:** Writing – original draft, Writing – review & editing. **Marc Cucurull:** Writing – review & editing. **Marta Domènech:** Writing – original draft, Writing – review & editing. **Ainhoa Hernandez:** Writing – review & editing. **Anna Martinez-Cardús:** Writing – review & editing, Supervision. **Eva Pros:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision. **Montse Sanchez-Cespedes:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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