REVIEW ARTICLE

New perspectives for targeting therapy in ALK-positive human cancers

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Anaplastic lymphoma kinase (ALK) is a member of the insulin receptor protein-tyrosine kinase superfamily and was first discovered in anaplastic large-cell lymphoma (ALCL). ALK alterations, including fusions, over-expression and mutations, are highly associated with cancer initiation and progression. This kinase plays an important role in different cancers, from very rare to the more prevalent non-small cell lung cancers. Several ALK inhibitors have been developed and received Food and Drug Administration (FDA) approval. However, like other drugs used in targeted therapies, ALK inhibitors inevitably encounter cancer cell resistance. Therefore, monoclonal antibody screening based on extracellular domain or combination therapies may provide viable alternatives for treating ALK-positive tumors. In this review, we discuss the current understanding of wild-type ALK and fusion protein structures, the pathological functions of ALK, ALK target therapy, drug resistance and future therapeutic directions.

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INTRODUCTION

The targeting of key oncogenic alterations, such as activating mutations or chromosomal rearrangements, is considered as one of the most important breakthroughs in cancer research and therapy over the past decades [1, 2]. Among somatic alterations, the anaplastic lymphoma kinase (ALK) fusion mutation is called the "diamond mutation" due its low mutation rate and the significant effect of target therapy. ALK mRNA and protein express extensively on the Central Nervous System (CNS) and the Peripheral Nervous System (PNS) during mice embryonic development, this suggests ALK might play an important role in the normal brain development and function on specific neurons of the nervous system [3, 4]. Extensive evidence has shown that non-small cell lung cancers (NSCLC) harboring rearranged ALK demonstrate remarkable sensitivity to ALK target therapy. Like other tyrosine kinase inhibitors, ALK inhibitors primarily target the kinase domain of ALK. Currently, three generations of ALK inhibitors have been developed. These drugs have shown efficacy in treating ALK-recombined NSCLC, however, like other targeting drugs, ALK inhibitors also inevitably develop tumor resistance. In this review, we explore recent advances in the understanding of ALK structure, discuss inhibitors developed against the ALK kinase domain, and highlight drug resistance encountered upon prolong treatment with ALK inhibitors. We also indicate methods used to develop new ALK inhibitors based on structural features in order to overcome tumor resistance caused by current inhibitors and provide more helpful therapeutic strategy through combination therapy.

The structure of ALK

ALK was first described in 1994 as a tyrosine kinase receptor found in anaplastic large-cell lymphoma (ALCL) cell lines [5]. So far, ALK has been considered as one of the most promising targets to overcome cancer. ALK is a classical member of the insulin receptor protein-tyrosine kinase superfamily and is comprised of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain [6].

The structure of ALK extracellular domain

The extracellular domain of ALK includes two MAM (Meprin, A5 protein, and receptor protein tyrosine phosphatase mu) domains (amino acids 264-427 and 480-626), one LDLa (low density lipoprotein class A) domain (amino acids 453-71), and a glycine-rich portion (amino acids 816-940). The receptor protein tyrosine phosphatase (PTP) mu belongs to the MAM-containing subclass of PTP and was reported to promote cell-to-cell adhesion [7]. The LDL-A modules mediates the receptor binding with lipoproteins [8] (Fig. 1A). The glycine-rich domain (GRD) is sufficient for ligand binding. AUG-α (FAM150B), a reported ligand, was shown to induce ALK activation through binding to the GRD of ALK [9, 10]. Analysis of the ALK-GRD crystal structure showed that the GR core has three glycine rich polyglycine type II (pGII) helices which may tightly associate with other pGII helices to form a honeycomb structure [11] (Fig. 1B). The pGII array provides the structural basis for ligand anchoring in the GRD region. To explore the ligand bound ALK-GRD structure, fusion proteins were constructed that bridged the carboxy terminus of ALK with the amino terminus of the ligand through a 14-residue linker. The crystal structure

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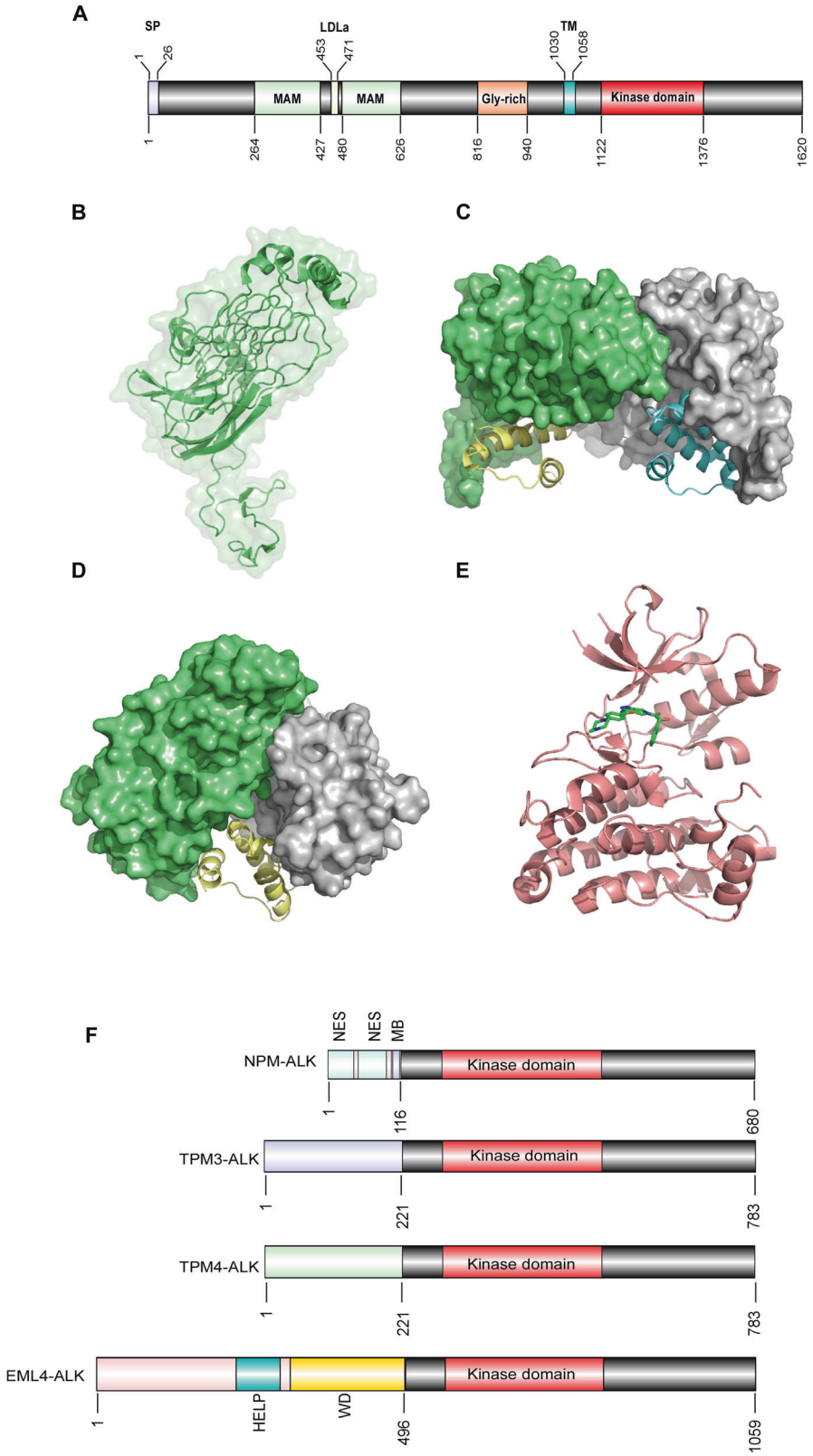

Fig. 1 The structure of ALK. A The domain organization of ALK. ALK is comprised of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. The extracellular domain includes two MAMs, one LDLa, and a glycine-rich portion (SP signal peptide, MAM meprin, A5 protein, and receptor protein tyrosine phosphatase mu, LDLa low density lipoprotein class A); **B** Glycine-rich domain (GRD) structure of ALK (PDB: 7LIR); **C** 2:2 heterotetramer of ALK receptor with ligand (PDB: N00); **D** 2:1 heterotetramer of ALK receptor with ligand (PDB: 7NWZ); **E** the structure of ALK kinase domain (PDB: 2XBA); **F** Schematic models of four ALK-fusion proteins. NPM, TPM3, TPM4, or EML4 form fusion proteins with ALK (NES nuclear export signal, MB metal-binding, HELP hydrophobic EML protein, WD tryptophan-aspartic acid).

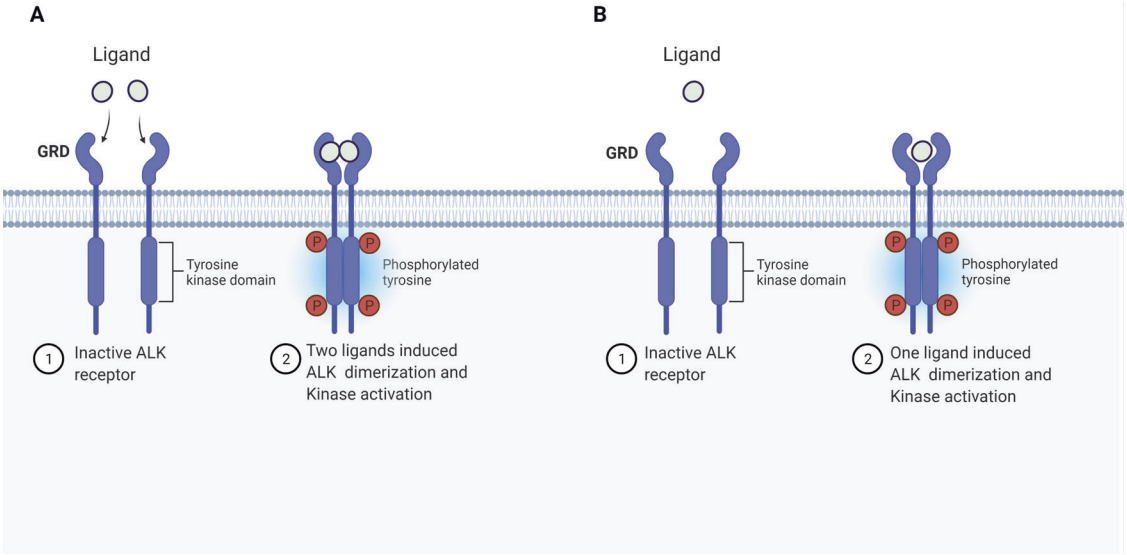

Fig. 2 Schematic model of inactive ALK and ligand-active ALK. Normal wild-type ALK is inactive. When a high affinity ligand binds with the glycine-rich domain of ALK, ALK will undergo dimerization and phosphorylation. **A** Two ligands induce ALK dimerization and activation; **B** One ligand induces ALK dimerization and activation. GRD glycine-rich domain.

showed the fusion protein formed a 2:2 heterotetramer (2 receptor: 2 ligand) complex. This structure was representative of the canonical 2:2 receptor tyrosine kinases (RTKs)-cytokine complexes. A similar study also confirmed that the complex is a 2:2 heterotetramer using cryo-electron microscopy (cryo-EM) [12] (Fig. 1C). However, another group identified that ALK and its ligand could associate in multiple ways. For instance, ALK and its ligand was shown to also form a 2:1 complex (2 receptors and 1 ligand) (Fig. 1D). Through this structure, they observed that a ligand could be recognized and bound by the GRD regions of two ALK receptors, resulting in receptor dimerization. This finding explains how a single ligand binding event could induce RTK activation [13]. Thus, ALK tyrosine kinase activity could be activated by binding with one or two ligands (Fig. 2). The structure analysis will help us understand the structure and function of the extracellular domain and develop novel targeted therapies.

The structure of ALK kinase domain

The ALK kinase domain, which is composed of 563 residues, is located in the intracellular compartment. ALK has the same characteristics as other kinases and transmits signals to downstream effector through phosphorylation. However, ALK has the unique characteristics of the IRK (insulin receptor kinase) kinase family and shares a common YXXXYY autophosphorylation motif within an activation loop (A-loop). The receptor could induce protein activation upon dimerization via trans-autophosphorylation of tyrosine residues. Between the first and second tyrosine residues of the ALK A-loop is an "RAS" triplet, but in IRK is "ETD" triplet. Replacement of the "RAS" triplet with an "ETD" triplet was shown to dramatically impair the phosphorylation of ALK peptide. Therefore, ALK exhibits narrower substrate specificity. To further understand ALK kinase characteristics, the structure of the unphosphorylated ALK catalytic domain was analyzed using X-ray crystallography by the Glen SPRAGGON group [14]. The A-loop was found to adopt an inhibitory pose, with the distal portion of A-loop obstructing part of the predicted peptide-binding region (Fig. 1E). Additional structural studies could facilitate further clarification of ALK functionality and the design of targeted inhibitors.

The structure of ALK fusion proteins

Chromosome translocation is one of the most common genetic abnormality. A gene fusion event occurs upon chromosomal rearrangement at regions between two otherwise separate genes (Fig. 1F). In 1994, ALK was first reported to exist as a fusion oncogene with nucleophosmin (NPM) in most anaplastic large-cell non-Hodgkin's lymphomas arising from activated T lymphocytes. Specifically, the *NPM nucleolar phosphoprotein* gene on chromosome 5q35 was translocated to the catalytic domain of ALK on chromosome 2p23 [5]. ALK translocations were next identified in certain 2p23 chromosomal rearrangements observed in inflammatory myofibroblastic tumors (IMTs). The fusion genes, *tropomyosin 4 (TPM4)-ALK* and *tropomyosin 3 (TPM3)-ALK*, were reported in IMTs [15]. In 2007, the *echinoderm microtubule-associated protein-like 4 (EML4)-ALK* fusion gene was detected in NSCLC patients [16]. EML4-ALK variants usually include the kinase domain of ALK and the trimerization domain (TD) of EML4 [17]. Approximately 40–60% of ALCL and 50% of IMT patients display *ALK* rearrangements [18, 19]. In contrast, *ALK* rearrangements only occur in approximately 3–5% of NSCLC patients [20]. Although the proportion of NSCLC patients with ALK rearrangements is relatively low, the absolute number of ALK fusion-positive NSCLC patients is by far the largest population. *ALK* fusion genes have also been found with low frequency in other types of cancer, including colorectal cancer [21], breast cancer [22], esophageal cancer [23], ovarian cancer [24, 25], renal cell carcinoma [26], anaplastic thyroid carcinoma [27], diffuse large B-cell lymphoma [28], liver cancer [29], gastric mesenchymal tumor [11], epithelioid fibrous histiocytoma [30], pancreatic ductal adenocarcinoma [25, 31], and glioma [32, 33]. Currently, around 30 distinct ALK fusion partners have been identified and some common features should be noted (Fig. 3). First, when ALK fusion genes are generated, the transcription of the fusion product is dependent on the promoter of the partner gene. Second, the subcellular localization of the ALK fusion protein is also dependent on the partner protein. Finally, ALK activation is dependent on dimerization that is induced by the ALK partner protein, but not the ALK ligand. Activation of the ALK fusion proteins could induce cell growth, transformation, differentiation, and suppression of apoptosis through downstream signaling pathways [34]. Overall, the various fusion proteins exhibit different biological and molecular properties. Therefore, determining the structure of the ALK fusion proteins might help in understanding their function and aid in the development of more specific and effective targeted therapies.

The pathological functions of ALK in cancer

Besides *ALK* gene fusions, ALK mutations and amplification have also shown oncogenic potential in some cancers. Many different cancer cell lines and human tumor tissues have shown

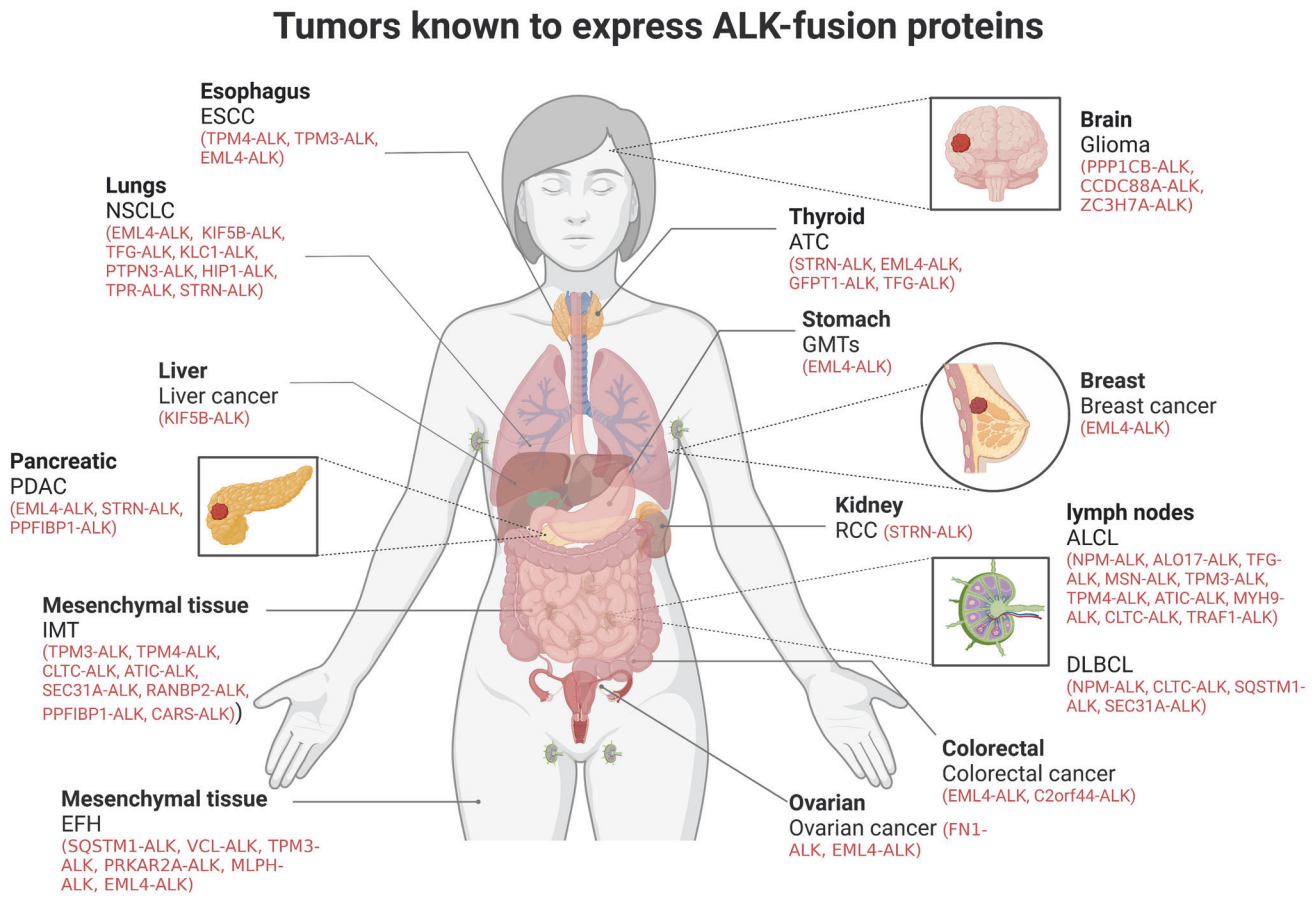

Fig. 3 Body diagram of all tumors currently linked to ALK-fusion proteins. ESCC Esophageal squamous cell carcinoma, GMTs Gastric mesenchymal tumors, PDAC Pancreatic ductal adenocarcinoma, IMT Inflammatory myofibroblastic tumor, EFH Epithelioid fibrous histiocytoma, ATC Anaplastic thyroid carcinoma, NSCLC Non-small cell lung cancer, RCC Renal cell carcinoma, ALCL Anaplastic large cell lymphoma, DLBCL Diffuse large B cell lymphoma.

amplification of the *ALK* locus and over-express ALK. Cell lines from tumors derived from the ectodermal layer showed widespread mRNA expression of the *ALK* receptor (53/63), especially tumors originating from the neural crest (21/22) [35]. Transcriptional repression was observed in tumor cell lines that originated from the endoderm, including colorectal carcinoma (CRC) cell lines. Interestingly, *ALK* amplification and gain in copy number were observed in 3.4% (26 out of 756) of CRC patients and were associated with poor patient outcome [36]. *ALK* gain-of-function mutations were observed at a frequency of 6.9% in neuroblastoma tumors. These "hot-spot" mutations occur in the kinase domain (R1275, 43%; F1174, 30%; and F1245, 12%) and have been identified in around 85% of cases [37]. The *ALK* F1174L mutation, along with *N-myc* proto-oncogene (*MYCN*) over-expression, could synergistically induce the development of neuroblastomas [38]. Currently, many *ALK* mutations have been reported and fall into three classes: (1) ligand-independent activation mutations (e.g., F1174I/S/L); (2) ligand-dependent activation mutations (e.g., T1087I, D1091N, A1099T, T1151M, A1234T) [39]; and (3) kinase-inactivating mutations (e.g., I1250T) [40]. Different ALK alterations can promote ALK activation, inducing downstream signaling pathways, including AKT/mTOR, MEK/ERK, PLC-γ, JAK3-STAT3 and JAK2-STAT5B, which ultimately lead to cell proliferation, angiogenesis, cell invasion, and tumor formation [12, 13, 41, 42] (Fig. 4).

TARGET THERAPY BASED ON ALK STRUCTURE

Target therapy based on ALK kinase domain

FDA approved drugs

Crizotinib: After the initial discovery of the EML4-ALK rearrangement, ALK inhibitors began to be rapidly developed, evaluated in pre-clinical studies, and subsequently translated to the clinic. Crizotinib (PF-02341066), the first FDA-approved drug for ALK-positive NSCLC developed in 2011, is a selective small-molecule competitive inhibitor that affects ALK, hepatocyte growth factor receptor (MET), and *c-ros* oncogene 1 receptor kinase (ROS1) [43]. A multicenter phase II trial testing the efficacy of crizotinib (NCT00932451) for treating ALK-positive NSCLC patients was conducted; the median progression-free survival (PFS) in the central-testing and local-testing subgroups were 8.4 months and 6.9 months, respectively [44]. Next, a phase III trial (NCT00932893) was initiated to compare crizotinib (250 mg, twice daily) with chemotherapy (500 mg per square meter of body-surface area pemetrexed or 75 mg per square meter docetaxel every 3 weeks) in 347 ALK-positive lung cancer patients who had received one prior platinum-based regimen. The median PFS was 7.7 months in the crizotinib group compared to 3.0 months in the chemotherapy group. Sixty-five percent of patients administered crizotinib responded favorably compared to the 20% response rate of patients treated with chemotherapy [45]. Additionally, another phase III trial (NCT01154140) was initiated to evaluate the efficiency of crizotinib (250 mg, twice daily) as first-line treatment compared with chemotherapy (pemetrexed, 500 mg per square meter of body surface area, plus either cisplatin, 75 mg per square meter, or carboplatin, target area under the curve of 5–6 mg per milliliter per minute, every 3 weeks for up to six cycles) for advanced ALK-positive NSCLC. The PFS in the crizotinib-treated group (median,10.9 months) was significantly longer than the

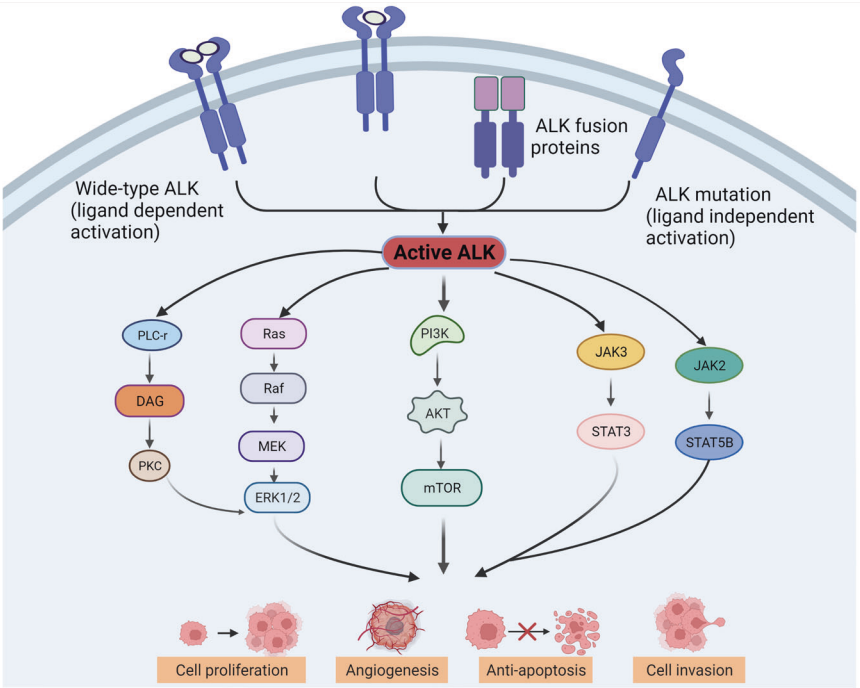

Fig. 4 Signaling pathways of ALK. ALK alteration could induce cell proliferation, angiogenesis and cell invasion by activating AKT/mTOR, MEK/ERK, PLC-γ, JAK3-STAT3 and JAK2-STAT5B pathways.

chemotherapy group (median, 7 months); the response rates were 74% in crizotinib treated group and 45% in chemotherapy group [46]. These trials provided further proof that crizotinib was superior to standard second-line chemotherapy and first-line chemotherapy (Fig. 5).

Ceritinib (LDK378): Ceritinib, a next-generation ALK inhibitor, was the first drug to have efficacy against several crizotinib-resistant mutations in patients with ALK-positive NSCLC and exhibited a 20-fold greater potency than crizotinib [47]. Pre-clinical data showed that ceritinib effectively inhibited crizotinib-resistant mutations, including L1196M, G1269A, I1171T, and S1206Y, but failed to overcome two other crizotinib-resistant ALK mutations, G1202R and F1174C [48]. With the support of the pre-clinical and the ASCEND-1 clinical trial data, ceritinib received FDA approval in April 2014 for patients with advanced disease or intolerance to crizotinib. The result of a multicenter phase II study that aimed to assess the efficacy of ceritinib in patients with ALK-rearranged NSCLC showed that the disease control rate was 69.3–83.8%, the time to response was 1.6–5.6 months, the duration of response was 7.1–11.1 months, and PFS was 5.4–7.6 months [49]. Next, the ASCEND-4 phase III clinical trial (NCT01828099) was conducted to evaluate the efficacy and safety of ceritinib compared with platinum-based chemotherapy as the first-line treatment for patients with advanced ALK-positive NSCLC. The PFS was higher in the ceritinib group (median, 16.6 months) compared with the chemotherapy group (Median, 8.1 months) [50].

Alectinib (CH5424802): Alectinib is a second generation ALK inhibitor that was granted FDA approval on November 6, 2017. Alectinib blocks the gatekeeper gene mutation (L1196M) that confers resistance against crizotinib [51]. Patients with stage IIIB-IV ALK-positive NSCLC that were resistant to crizotinib treatment were administered alectinib (600 mg twice daily) and achieved an objective response of 48% (NCT01871805) [52]. ALK-positive NSCLC patients with CNS metastases who were treated with alectinib achieved a median PFS of 8.9 months; the CNS disease control rate was 83% and the median CNS duration of response was 10.3 months (NCT01801111) [53]. Based on the two clinical trials, alectinib is highly effective for patients with advanced ALK-positive NSCLC who were resistant to crizotinib or had CNS metastasis.

Brigatinib (AP26113): Brigatinib, which received FDA approval in April of 2018, is a selective next-generation ALK inhibitor. Brigatinib showed high efficacy against all crizotinib-resistant mutants and a superior inhibitory profile compared with crizotinib, ceritinib, or alectinib. Brigatinib was active against the ALK resistant mutation, G1202R [54]. The randomized phase II trial (NCT02094573) was conducted to evaluate the efficacy and safety of brigatinib at 90 mg once daily and 180 mg once daily in patients with crizotinib-resistant advanced ALK-positive NSCLC; the median PFS for the indicated concentrations were 9.2 months and 12.9 months, respectively. The efficacy and safety of the trial supported the 180-mg regimen (with lead-in at 90 mg) for future trials [55].

Lorlatinib (PF-06463922): Lorlatinib, a third generation ALK/ROS1 inhibitor, received accelerated FDA approval on November 2, 2018. In preclinical studies, lorlatinib prolonged survival in mice by regressing EML4-ALK-driven brain metastases and showed superior potency against acquired ALK mutations, including the highly resistant G1202R mutation [56]. In a phase I/II clinical trial (NCT01970865) assessing the efficacy of lorlatinib in ALK or ROS1 positive NSCLC patients, the partial response was 42% and overall response rate was 50% [57]. Another clinical trial (NCT03727477) which aimed to assess the efficacy of lorlatinib in ALK-positive NSCLC showed that the median PFS and median overall survival of patients that received lorlatinib treatment were 9.9 months (6–12.3 months) and 32.9 months (18.7 months to not reached), respectively [58]. Interestingly, a woman with metastatic NSCLC harboring an ALK-rearrangement developed a crizotinib-induced mutation (C1156Y). The tumor responded to lorlatinib but eventually relapsed upon acquiring a novel ALK L1198F mutation. Interestingly, the L1198F mutation enhanced the binding affinity to crizotinib even after negating the effect of the C1156Y

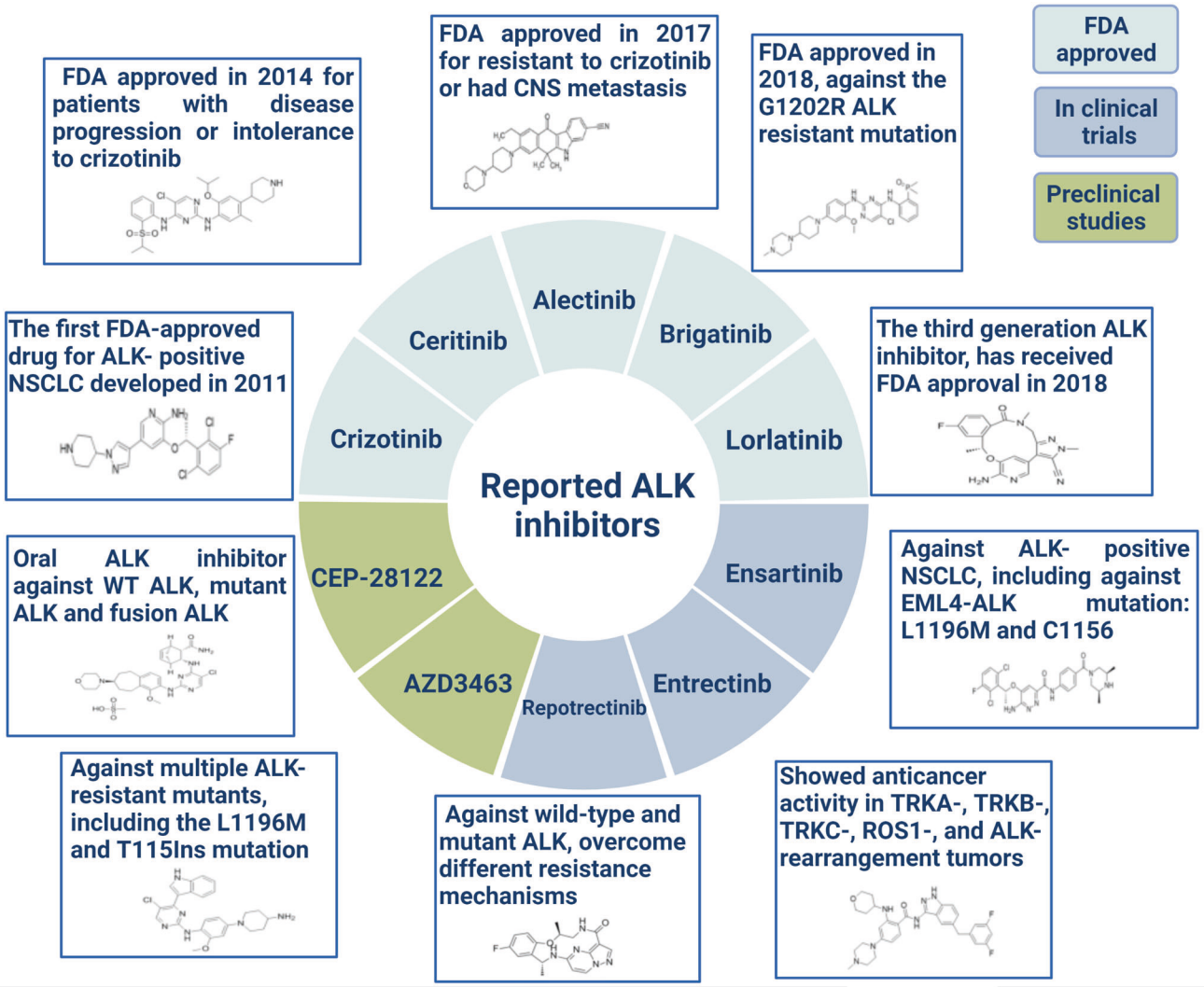

Fig. 5 Development of ALK inhibitors. ALK inhibitors include three different stages: FDA approved, in clinical trials and preclinical studies.

mutation. When the patient received crizotinib again, she exhibited an obvious clinical response [59].

Drugs currently in clinical trials

Ensartinib (X-396): Ensartinib is a novel and potent small molecule TKI acting against ALK-positive NSCLC. Preclinical data showed that ensartinib inhibits autophosphorylation of ALK and displays powerful anti-tumor activity in vitro, including against the two-point mutations in EML4-ALK (L1196M and C1156Y). In vivo, ensartinib also showed anti-tumor activity and was well tolerated in an EML4-ALK-positive lung cancer xenograft model [60]. A phase I/II clinical trial was conducted to evaluate the safety and determine the recommended dose of ensartinib in patients with ALK-positive NSCLC. Treatment of ALK-positive ASCLC patients with ≥200 mg ensartinib resulted in a response rate of 60% and median PFS of 9.2 months (NCT01625234) [61]. Currently, a phase III clinical study comparing the efficacy and safety of ensartinib with crizotinib is ongoing in ALK-positive TKI naive NSCLC patients (NCT02767804) [62].

Entrectinib (NMS-E628): Entrectinib (NMS-E628) is a small molecule that exerts anticancer activity in tumors with TRKA-, TRKB-, TRKC-, ROS1-, or ALK-rearrangements [63]. Entrectinib showed growth inhibitory activity in over 200 types human tumor cell lines and induced tumor regression in ROS- and ALK-positive tumor animal models [64]. Thus, a phase I/II study of entrectinib was conducted in children, adolescents, and young adults with recurrent or refractory solid tumors. Results indicated that preliminary anti-tumor activity was observed in gene fusion-positive patients (NCT02650401); the PFS was 17.5 months in fusion-positive patients versus 1.9 months in non-fusion patients [65]. Currently, a trial with expanded access to entrectinib is ongoing for patients harboring cancers with NTRK1/2/3, ROS1, or ALK gene fusions (NCT03066661).

Repotrectinib (TPX-0005): Repotrectinib is an ALK and SRC inhibitor that exerts its activity by targeting the ATP binding sites of its targets. Furthermore, it could overcome steric interference from mutations occurring outside the ATP binding boundary. Repotrectinib showed inhibitory effects against wild-type and mutant ALK and could overcome different resistance mechanisms [66]. A phase I/II clinical trial in ALK, ROS1, or NTRK1-3 fusion-positive advanced solid tumors was conducted to examine the safety and efficacy of this drug. Results showed that repotrectinib was well-tolerated and exerted intra- and extra-cranial clinical activity (NCT03093116) [67]. Other clinical trials (NCT05004116, NCT04772235) detecting the effect of repotrectinib treatment are currently ongoing.

Drugs currently in preclinical studies

AZD3463: AZD3463 is a novel orally-administered ALK inhibitor that has shown efficacy in vitro and in vivo. This drug also showed

Table. 1. Development of ALK inhibitors at different stages.

Inhibitor	Company	Target	Stage	Clinical trials	PFS (Months)	Ref.
Crizotinib (PF-02341066)	Pfizer	ALK MET ROS1	FDA approved	NCT00932451 NCT00932893 NCT01154140	7.7–10.9	[43–46]
Ceritinib (LDK378)	Novartis	ALK IGF-1R InsR ROS1	FDA approved	NCT01283516 NCT01685060 NCT01685138 NCT01828099	5.4–7.6	[47–50]
Alectinib (CH5424802)	Roche	ALK	FDA approved	NCT01871805 NCT01801111	8.9	[51–53]
Brigatinib (AP26113)	Takeda	ALK EGFR IGF-1R	FDA approved	NCT02094573	9.2 (90 mg) 12.9 (180 mg)	[54, 55]
Lorlatinib (PF-06463922)	Pfizer	ALK ROS1	FDA approved	NCT01970865 NCT03727477	6–12.3	[56–59]
Ensartinib (X-396)	Xcovery	ALK MET ABL AXL ROS1	in clinical trials (Phase I/II)	NCT01625234 NCT02767804	9.2	[60–62]
Entrectinib (NMS-E628)	Ignyta	ALK ROS1 TRK	in clinical trials (Phase I/II)	NCT02650401 NCT03066661	17.5 (fusion-positive) 1.9 (non-fusion)	[63–65]
Repotrectinib (TPX-0005)	Turning Point Therapeutics	ALK ROS1 TRK	in clinical trials (Phase I/II)	NCT03093116 NCT05004116 NCT04772235	NA	[66, 67]
AZD3463	NA	ALK IGF1R	in preclinical studies	NA	NA	[68, 69]
CEP-28122	NA	ALK	in preclinical studies	NA	NA	[70]

NA not applicable.

efficacy against multiple ALK-resistant mutants, including the L1196M gatekeeper mutant and T115Ins mutation [68]. AZD3463 was shown to inhibit the proliferation of neuroblastoma cell lines harboring wild-type and mutant ALK. It also displayed an inhibitory function in neuroblastoma xenograft mouse models with wild-type or F1174L mutant ALK. AZD3463 might function mechanistically through inducing apoptosis and autophagy upon targeting ALK and blocking its downstream signaling pathways [69].

CEP-28122, a highly potent, selective, oral ALK inhibitor: CEP-28122 showed a greater dose-dependent inhibitory effect on the proliferation of EML4-ALK-positive cancer cells compared with EML4-ALK-negative cancer cells. This compound also inhibited cell proliferation in neuroblastoma cells expressing wild-type and mutant ALK; however, the compound was not effective in ALK-negative neuroblastoma cell lines. In vivo, CEP-28122 showed anti-tumor efficacy in ALK-positive ALCL, NSCLC, and neuroblastoma tumor xenograft mouse models. In contrast, CEP-28122 had no effect in an ALK-negative tumor xenograft mouse model [70] (Table 1).

Drug resistance. Initially, the use of ALK inhibitors to clinically treat ALK-positive NSCLC patients resulted in remarkable responses. Unfortunately, the development of drug resistance continues to be a serious problem. Analysis of ALK-positive NSCLC patients treated with crizotinib showed that 36% (4/11) of patients developed secondary mutations in the tyrosine kinase domain of ALK [71]. When the cancers of patients treated with ALK inhibitors progressed, the C1156Y and L1196M mutations within the EML4-ALK kinase domain were identified [72]. Based on the available clinical and preclinical data, numerous ALK mutation variants with different mechanisms have been confirmed. In the first mechanism, the gatekeeper mutation (L1196M) residing in the ATP-binding pocket was shown to interfere with the binding of many tyrosine kinase inhibitors [72]. Second, various mutation sites could stabilize active ALK by promoting ATP binding (1151Tins [73], F1174L [74], L1198P [75], L1152R/P [48, 76], I1171N/T [77] and C1156Y [72]). Third, various mutation sites could impair the affinity of crizontinib for the ATP binding site (G1269A [48, 78], S1206Y [73], V1180L [79] and G1202R [73]). Another EML4-ALK mutation, D1203N, has been reported to induce resistance to ALK inhibitors through an uncharacterized mechanism [75]. *ALK* gene amplification also belongs to the on-target resistance class. Other mechanisms include the off-target alterations that can trigger downstream or parallel signaling pathways to bypass the need for kinase activation. The EGFR pathway bypass activation has previously been reported as one of the mechanisms for acquired ALK drug resistance in NSCLC patients [76]. Thus, ALK inhibitor-induced resistance is a complex issue that makes lung cancer treatment extremely challenging.

Target therapy based on ALK extracellular domain

As aforementioned, ALK dimerization facilitates kinase activation via autophosphorylation. ALK kinase inhibitors also use the ATP binding site or a similar allosteric site as the entry point to prevent or decrease the activity of the kinase domain, thereby blocking the transmission of kinase signals [80]. However, drug resistance inevitably emerges over prolonged treatment with ALK inhibitors. In addition to kinase inhibitors, there are other pharmacological approaches that can effectively inhibit RTK activity in cancer treatment. Screening of monoclonal antibodies that could bind to the extracellular portion of the receptor, which competitively bind to the ligand binding region, may also potentially block ligand-induced receptor dimerization and activation. ALK monoclonal antibodies have been previously screened in ALK-positive neuroblastoma. The study confirmed that the ALK monoclonal

antibody can inhibit the proliferation of human neuroblastoma-derived cell lines [81]. Interestingly, the simultaneous use of ALK kinase inhibitors can significantly enhance the inhibitory effect of the ALK monoclonal antibody. The ALK monoclonal antibody was also used to exploit antibody-drug conjugates (ADCs) in cancer therapy [80]. The ALK-directed ADC (CDX-0125-TEI) drug, designed through coupling anti-ALK mAb CDX-0125 with a DNA minor groove alkylating agent thienoindole (TEI), showed anti-tumor activity in ALK-positive tumors.

FUTURE THERAPEUTIC DIRECTIONS

Combination therapy

In regards to ALK on-target resistance, reasonable and sequential utilization of different generation ALK inhibitors might improve patient outcome based on understanding the ALK mutation profile of the patient. However, for ALK off-target resistance, combination therapies might provide more opportunities for treating lung cancer patients. As indicated earlier, the acquired resistance of crizotinib includes not only second mutations, but also the activation of the EGFR pathway. Thus, a combination of both ALK and EGFR inhibitors might provide a new therapeutic strategy for NSCLC patients [82]. Recently, the dual EGFR/ALK kinase inhibitor compound 18 (CHMFL-ALK/EGFR-050) was developed against the EGFR L858R, del 19 and T790M mutants as well as the EML4-ALK R1275Q, L1196M, F1174L, and C1156Y mutants; however, further studies are required to assess its efficacy in clinical use [83]. The combination of ALK and MAPK kinase (MEK) inhibitors and the combination of ALK and SRC inhibitors showed efficacy in preclinical models [84]. A phase I/II study assessing the effect of ceritinib combined with trametinib in ALK- or ROS1-positive NSCLC (NCT03087448) patients showed that the combination exhibited no toxicity and produced clinical benefits [85]. Clinical trials combining ALK inhibitors with other target gene inhibitors are ongoing. For example, trials include alectinib combined with bevacizumab (angiogenesis agent targeting vascular endothelial growth factor-VEGF), ceritinib combined with LEE011 (CDK4/6 inhibitor), and ceritinib combined with everolimus (mTOR inhibitor) (NCT02521051, NCT02292550 and NCT02321501). The results of a phase I/II study that aimed to assess the effect of ceritinib combined with ribociclib in ALK positive NSCLC produced an overall response rate of 37.0% and a median PFS of 21.5 months. The combination of an ALK inhibitor and a CDK4/6 inhibitor showed favorable clinical activity and manageable safety [86]. Because immune checkpoint inhibitors have shown efficacy in advanced NSCLC, and EML4-ALK and mutant EGFR could induce PD-L1 up-regulation [87], the prospect of combining ALK inhibitors with immunotherapy eventually gained the attention of clinicians. Some clinical trials were performed to examine the safety and efficacy of combination strategies. A phase I/II Study of the safety and tolerability of Nivolumab plus Crizotinib (NCT02393625) in ALK-positive NSCLC could not continue due to severe hepatic toxicity [88]. However, the result of a phase I/II study for alectinib plus bevacizumab (NCT02521051) in ALK-rearranged NSCLC showed that the combination was well tolerated and did not produce any unanticipated or dose-limiting toxicities [89]. Another phase I/II clinical trial testing Avelumab (anti-PD-L1) when combined with crizotinib or lorlatinib (NCT02584634) was initiated to check the safety and efficacy of combination therapy. The results showed that the objective response rate of Avelumab combined with crizotinib was 16.7%. However, the objective response rate of Avelumab combined with lorlatinib was 46.4%, suggesting that this combination is more safe and effective.

Antibody-mediated treatment

The positive results of anticancer antibodies provide hope that monoclonal antibodies can be used in the clinic. Cetuximab, a murine monoclonal antibody against EGFR, has been approved for EGFR-expressing metastatic CRC and head and neck cancer [90]. As indicated earlier, the activation of ALK, including expression of ALK fusion protein, ALK overexpression, and ALK mutation, plays an important role in cancer initiation and progression. Anti-ALK monoclonal antibodies compete with ALK ligands for the ligand-binding region of the extracellular domain, thereby blocking ligand-induced ALK tyrosine kinase activation. As such, neutralizing monoclonal antibodies might be another useful choice for those cancers overexpressing ALK and resistant to ALK kinase inhibitors. A panel of monoclonal antibodies was designed against the extracellular domain of the human ALK receptor. mAb 30 was shown to inhibit the activation of ALK and downstream signaling pathways; however, mAb 48 could induce ALK protein activation after homodimer formation [91]. Antibodies raised against the ALK ligand-binding domain (LBD) were designed to check their efficiency in human glioblastoma; the results showed that the anti-ALK antibodies inhibited the growth of established glioblastoma cell xenografts [92]. The ALK antagonistic antibody induced antibody-dependent cell cytotoxicity and inhibited growth in human neuroblastoma cell lines with wild-type or mutant ALK. Furthermore, the combined use of crizotinib and a monoclonal antibody could facilitate the accumulation of ALK protein on the cell surface, ultimately leading to an enhanced inhibitory effect on tumor cells [81]. The ALK antibody-drug conjugate CDX-0125-TEI showed cytotoxicity against ALK-expressing neuroblastoma cells at picomolar levels and robust antitumor activity in neuroblastoma PDX models with wild-type or mutant ALK [93]. These results suggest that ALK-targeted immunotherapy might be a promising therapeutic strategy for ALK positive cancers.

Structure-based drug design

Structure-based drug design (SBDD) achieves success by using molecular docking, structure-based virtual screening (SBVS), and molecular dynamics (MD) to analyze the binding energy, molecular interactions, and induced conformational changes between small molecules [94]. However, most drugs, including ALK inhibitors, are multi-targeted drugs that could produce off-target effects. To obtain highly potent and specific treatments, it is imperative to develop single-target drugs. Advancements in X-ray free electron lasers and cryo-EM, which can obtain near-atomic resolution, can facilitate increased progress in drug design. Currently, drug design based on the reported kinase domain and extracellular domain structure is ongoing. If the structures of the various ALK fusion proteins could be solved by cryo-EM, more specific drugs could be designed to target different domains of the protein.

CONCLUSIONS

ALK is an important therapeutic target in lung cancer and several other cancers, including ALCL, IMT, and neuroblastoma. Since the initial discovery of the *ALK* fusion gene, the understanding of oncogenic ALK and ALK inhibitor development has rapidly improved. Currently, four ALK inhibitors over three generations have been FDA-approved for the treatment of ALK-positive NSCLC patients. However, three urgent issues should be considered when developing future ALK targeting agents. First, the ALK fusion protein structures still are unclear. Therefore, solving the structure of ALK fusion proteins may be helpful for understanding their respective pathological functions and assisting in the development of useful targeted therapies. The second point is the design monoclonal antibodies antagonizing the ALK protein based on its extracellular domain, with a view to solving the current problem of drug resistance of ALK kinase domain inhibitors, so as to play an important role in ALK-positive tumor treatment. The third issue pertains to the emergence of ALK inhibitor resistance. Although ALK inhibitors have improved for ALK-positive patients, the development of drug resistance continues to hamper the efficacy of treatment. However, along with a clear understanding of

resistance mechanisms, development of reasonable and sequential ALK inhibitors, combination therapy, monoclonal antibodies, and structural solutions could overcome these issues and provide enhanced prospects for treating ALK-positive cancers.

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COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION
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