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Targeting ALK in neuroblastoma—preclinical and clinical advancements

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Abstract

Despite improvements in cancer therapies in the past 50 years, neuroblastoma remains a devastating clinical problem and a leading cause of childhood cancer deaths. Advances in treatments for children with high-risk neuroblastoma have, until recently, involved addition of cytotoxic therapy to dose-intensive regimens. In this era of targeted therapies, substantial efforts have been made to identify optimal targets for different types of cancer. The discovery of hereditary and somatic activating mutations in the oncogene *ALK* has now placed neuroblastoma among other cancers, such as melanoma and non-small-cell lung cancer (NSCLC), which benefit from therapies with oncogene-specific small-molecule tyrosine kinase inhibitors. Crizotinib, a small-molecule inhibitor of ALK, has transformed the landscape for the treatment of NSCLC harbouring ALK translocations and has demonstrated activity in preclinical models of ALK-driven neuroblastomas. However, inhibition of mutated *ALK* is complex when compared with translocated ALK and remains a therapeutic challenge. This Review discusses the biology of ALK in the development of neuroblastoma, preclinical and clinical progress with the use of ALK inhibitors and immunotherapy, challenges associated with resistance to such therapies and the steps being taken to overcome some of these hurdles.

Introduction

Neuroblastoma is an embryonal tumour of the autonomic nervous system that is most commonly diagnosed in early childhood and accounts for 10% of paediatric cancer mortality.[1] It is the most frequent form of malignancy diagnosed within the first year of age, and represents a spectrum of diseases with diverse and often dramatic clinical behaviour, as well as distinct biological features in different subsets of patients.[2,3] Neuroblastoma constitutes the highest proportion of human cancer cases that undergo spontaneous regression even when metastasis forms,[4–6] but it also accounts for a disproportionate amount of childhood cancer morbidity and mortality.

High-risk neuroblastomas have a near-diploid or near-tetraploid karyotype and are characterized by complex chromosomal aberrations. A subset of tumours are characterized by deletions in chromosomes 1p and 11q,[7] but to date, no tumour suppressor genes have

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been identified in these regions. Another major subgroup of high-risk neuroblastomas have a high level of amplification of the *MYCN* oncogene, a biomarker of poor prognosis[8,9] that when aberrantly expressed in neuroblastomas, is challenging to target pharmacologically.

Neuroblastoma is one of the few solid cancers in which a randomized clinical trial has shown that myeloablative consolidation therapy with autologous stem-cell rescue results in substantial improvement in event-free survival (EFS).[10] In addition, findings from the 1980s show that neuroblastoma cell lines can be induced to terminally differentiate when exposed to retinoid compounds.[11,12] This observation prompted a randomized clinical trial in which isotretinoin (a retinoid compound and derivative of vitamin A) was used after myeloablative therapy and reduced the risk of relapse among children with high-risk neuroblastoma.[10] Efficacy of stem cell transplant and isotretinoin together improved survival by ~20% compared to patients who received chemotherapy alone. These findings have motivated studies with increased dose-intensity in both induction and consolidation therapies during the past 15 years; one such study is the ongoing phase III trial testing whether tandem myelo-ablative chemotherapy improves EFS for children with high-risk neuroblastoma.[13]

Survivors of neuroblastoma are often left with considerable long-term adverse effects, many of which can be life-threatening.[1] While increasing the intensity of therapies could improve outcomes, it can be contended that no substantial changes in survival rates of children with neuroblastoma will be observed until new treatment strategies can be developed targeting fundamental molecular alterations in the tumour cells.

Until recently, survival of high-risk patients has been around 35%, with only modest improvements in the past few years.[10] The Children's Oncology Group recently reported the results of a randomized clinical trial of a new dose-intensive immunotherapeutic regimen using ch14.18, a monoclonal antibody against disialoganglioside GD2, in combination with alternating cycles of cytokines GM-CSF or IL-2 added to a regimen of isotretinoin.[14] The 2-year EFS was dramatically improved from 46% to 66% in immunotherapy-treated patients compared with those who received isotretinoin alone. Unfortunately, no other innovative treatment approaches have been used in frontline therapy. For several years, multiple tractable molecular targets have been investigated in neuroblastoma, including the neurotrophic tyrosine kinase receptor pathways,[15–17] c-Kit and PDGFR,[18,19] angiogenic factors such as VEGF,[20–22] histone deacetylases,[23,24] and programmed cell death pathways;[25] however, there is limited biological rationale and evidence of preclinical efficacy to help prioritize drug development targeting these molecules. To improve the overall survival in patients with neuroblastoma demands additional novel treatment approaches targeting validated molecular and genetic abnormalities underlying tumorigenesis and/or disease progression.

After decades of research into the neuroblastoma predisposition genes using positional cloning approaches, the discovery of activating mutations in the tyrosine kinase domain of the *ALK* oncogene as the leading cause for most cases of hereditary neuroblastoma, and the finding that these mutations are also somatically acquired in 7–10% of sporadic cases have provided the first tractable molecular target in this disease. *ALK* is a receptor tyrosine kinase (RTK) involved in the formation of several other human cancers, most notably anaplastic large-cell lymphoma (ALCL)—a cancer typically of childhood—and non-small-cell lung cancer (NSCLC), and could have a role in the pathogenesis of other unknown types of cancers. Constitutive *ALK* signalling induces cell transformation *in vitro* and *in vivo* by controlling key cellular processes, such as cell-cycle progression, survival, cell migration and cell shaping.[26–28] *ALK*, therefore, represents an attractive target for innovative therapies that are based on selective small-molecule inhibitors and target the tyrosine kinase activity of ALK. In this Review, we describe the scientific basis for targeting *ALK* aberrations in

neuroblastoma, the emerging challenges and how research has influenced strategies to study *ALK* inhibition in the clinic.

ALK structure and signalling

ALK is an orphan RTK normally expressed only in the developing embryonic and neonatal brain,[29,30] minimizing the potential risk of ALK inhibition in young children. ALK is a member of the insulin receptor superfamily and was first discovered in an anaplastic large-cell non- Hodgkin's lymphoma in 1994 in a translocated form fused to the N-terminal of nucleophosmin resulting in constitutive activation of ALK tyrosine kinase domain.[31] Over a dozen ALK fusion partners have since been identified including RANBP2 in inflammatory myofibroblastic tumour, EML4 in 3–7% of NSCLC and renal cancer,[31,32] and TPM3 in renal cancer,[33] which make ALK one of the few RTKs implicated as an oncogene in both haematopoietic and non-haematopoietic malignancies.[34] We have shown that full-length ALK protein and mRNA are expressed in the majority of primary neuroblastoma tumours,[29] and others have suggested an oncogenic role for full-length ALK in lung cancer,[35] thyroid cancer,[36] glioblastoma[37] and rhabdomyosarcoma.[38]

ALK is a 220 kD, 1,620 amino acid single-chain receptor with an extracellular domain containing two MAM domains, as well as transmembrane and intracellular domains (Figure 1). Jelly belly has been identified as the ligand of *Drosophila* ALK and activates ALK through the MAPK pathway.[39] Although the extracellular low-density lipoprotein domain could have a role in ligand binding of human ALK, whether the proposed ligands midkine and pleiotrophin actually bind and activate ALK is unclear.[40–42] Constitutive ALK activation has been linked to cellular processes related to oncogenesis including cell-cycle progression, survival, and cell migration in cells expressing translocated ALK. Downstream signalling of ALK is also best characterized in these cells and involves STAT3, PI3K/AKT, and MAPK,[43–46] although signalling through these molecules has not been demonstrated for full-length ALK in neuroblastoma. Signal transduction pathways vary among different ALK aberrations; however, both translocated and full-length ALK are sensitive to crizotinib, a small-molecule inhibitor of ALK, suggesting that ALK inhibition is a relevant therapeutic strategy.[47–50]

The biology of ALK in neuroblastoma

Hereditary neuroblastoma

In 1972, Knudson and Strong predicted that neuroblastoma, similar to the analogous embryonal cancer retinoblastoma, follows a two-hit model of tumorigenesis explaining hereditary and sporadic cases.[51] This model is correct for the majority of childhood and adult cancers, and the susceptibility genes are generally tumour suppressors, in which two genomic events constitute sequential inactivation of both alleles. The discovery of germline mutations in the oncogenes *RET*, *MET* and *KIT* in the aetiology of multiple endocrine neoplasia type 2 cancer, papillary renal carcinoma and gastrointestinal stromal tumours, respectively, challenged this paradigm, but it is now clear that somatically acquired duplication or amplification of the mutant allele provides the second genetic event.[52] The discovery of activating mutations in the tyrosine kinase domain of *ALK* as the major cause of hereditary neuroblastoma[53] provides the first example of a paediatric cancer arising from germline mutations in an oncogene. We and others have shown that *ALK* activation can be acquired somatically,[53–57] providing the first evidence for oncogenic activation of *ALK* via mutation of the kinase domain. This finding is in contrast to previous reports describing ALK rearrangements, including translocations and gene amplification, as the genetic basis for the initial observations of sensitivity to *ALK* inhibition in NSCLC, ALCL and neuroblastoma.[58]

The discovery of highly penetrant, heritable *ALK* mutations as the cause of hereditary neuroblastoma is of immediate relevance to patients with a family history of this disease and opens a new field that will likely become intrinsic to the cancer risk assessment for individuals and families with neuroblastoma. Although hereditary cases of neuroblastoma are rare relative to sporadic cases, efforts to sequence matched germline DNA for each tumour with a somatic mutation will help to understand the frequency of hereditary predisposition to neuroblastoma even in the absence of a family history. The ability to identify individuals at high risk for developing this disease brings with it the responsibility to devise effective surveillance strategies, and some fundamental questions need to be considered: what are the processes that control the genetic penetrance of mutations in *ALK*? Can understanding the genetic basis help predict clinical severity and biological grade of neuroblastoma? What is the risk of developing the disease given that there is incomplete penetrance? Answers to these questions will have a major affect on the future management of patients with neuroblastoma. This work will hopefully lead to effective early detection strategies resulting in favourable clinical outcomes, much like those achieved in Li-Fraumeni syndrome.[59]

Functional expression of ALK in neuroblastoma

Full-length *ALK* expression was first linked to neuroblastoma in 2000,[60] followed by a report showing consistent expression of activated *ALK* in tumours of neural origin,[61] but no correlation was evident between the levels of *ALK* expression and prognostic factors for neuroblastoma, such as age, *MYCN* amplification, tumour stage, tumour histology and DNA content. Rare cases of *ALK* amplification have also been described,[62] but the definitive role of this oncogene has only recently been reported.

Reports of crystal structures of the unphosphorylated human ALK tyrosine kinase domain of human ALK[63,64] revealed unexpected similarities to the EGFR tyrosine kinase domain, which could be useful for predicting the consequences of *ALK* mutations in neuroblastoma. Activating mutations in *ALK* occur as single-base mis-sense alterations in the tyrosine kinase domain;[53] no disease-causing mutations have been reported within the extracellular domain. *ALK* mutations are heterozygous and, to date, no direct evidence has pointed to acquisition of a second genetic event, such as amplification of the mutant allele.

The R1275 mutation is the most frequent mutation in ALK in neuroblastoma, as it occurs in the germline of patients with hereditary predisposition,[53] and is detected in almost 50% of tumours with ALK mutation. The most common *ALK* mutations in sporadic cases of neuro-blastoma are found at positions R1275, F1174, and F1245, all of which activate ALK, are located in key regulatory regions of the RTK domain, and have transformation capabilities *in vitro* and *in vivo* (Figure 1).[55,56] Biochemical data further showed that the R1275Q mutation in ALK stabilizes the autoinhibited conformation of the tyrosine kinase domain and coincides closely with Lys837 in EGFR, where a mutation to Gln activates EGFR in NSCLC.[47] Once R1275 is replaced with Gln in ALK, the autoinhibitory interactions are disrupted, ALK is activated and, in the presence of a tyrosine kinase inhibitor (TKI), results in preferential binding of crizotinib versus ATP. F1174 in ALK contributes to a well-packed hydrophobic core near the activation loop and when mutated, the packing of this hydrophobic core is disrupted, weakening the autoinhibitory interactions and enables the tyrosine kinase domain to adopt its active configuration.[47] F1174L mutation in ALK combines the characteristics of an activating mutation and *de novo* resistance mutation as it resembles the drug-resistant variant of EGFR (harbouring Y858R and T766M double mutation), and has increased ATP-binding affinity—a feature which, in contrast to R1275Q mutation, reduces the potency of ATP-competitive inhibitors.[47] Interestingly, the F1174L mutation has emerged as an escape mechanism in adults with neuroblastoma who have an

ALK translocation treated with crizotinib,[65] highlighting the importance of findings in neuroblastoma for other types of ALK-positive cancers.

Targeted therapy with ALK inhibitors

Through systematic resequencing of 1,600 sporadic neuroblastoma tumour samples obtained at diagnosis that are representative of the natural spectrum of the disease, we confirmed[66] that ALK is mutated in 8% of diagnostic tumour samples, and mutations are distributed across the range of phenotypes, as shown in a previous meta-analysis.[67] All three mutations at amino acid locations R1275, F1174 and F1245 account for 86% of mutations found in neuroblastoma and correlate with the constitutively active form of ALK.[47,68] Whether rare mutations occurring at other locations within the tyrosine kinase domain, such as the germline mutation at I1250T (a kinase-dead mutation),[69] are disease causing and respond to pharmacological ALK inhibition is under investigation, signifying the importance of tumour sequencing in the clinic. The prognostic importance of ALK alterations needs to be evaluated in a wide variety of diagnostic tumour samples taken from a large number of patients representative of the whole spectrum of neuroblastoma. This characterization is paramount for the identification of high-risk patients who are most likely to benefit from therapeutic stratification. Elucidation of the target population is crucial to the success and clinical efficacy of treatment, as was shown for RAF inhibition in BRAF-mutant melanoma,[70] as well as ALK inhibition in ALK-translocated NSCLC.[49]

Notably, ALK mRNA expression[71] and native ALK protein expression measured by immunohistochemistry,[72,73] have been proposed as indicators of ALK inhibition in neuroblastoma, suggesting these measurements as biomarkers of mutation-independent ALK activation. Although overall ALK mRNA expression levels in neuroblastoma are associated with a poor prognosis (independent of genomic ALK status[67]) and are significantly higher in tumour cells expressing mutated ALK, no direct correlation was found between endogenous ALK mRNA levels and constitutive activation of ALK protein. Interestingly, one study demonstrated that while ALK protein levels do not always correlate with ALK genetic alterations or mRNA abundance, both mutated and wild-type *ALK* can exert oncogenic activity in neuroblastoma cells above a threshold expression level, suggesting that wild-type *ALK* is a potential therapeutic target when overexpressed.[72]

For the clinical application of ALK-targeted therapy in neuroblastoma, a diagnostic test that can be performed using small amounts of tumour tissue is essential to screen patients and identify potential responders. ALK mRNA and native protein expression could be necessary, but insufficient, for predicting sensitivity to pharmacological ALK inhibition. While constitutive ALK activation could be a suitable biomarker, immunohistochemistry is associated with both technical and interpretive challenges, highlighting the need to develop a sensitive and reliable clinical assay for detection of constitutively active ALK. Kinases that are intimately involved in processes leading to tumour proliferation and survival become oncogenic by genetic mutation, amplification or translocation, leading to constitutive activation and transforming capacity. The success of ALK kinase inhibition therapy in neuroblastoma, therefore, relies on the identification of appropriate predictive bio-markers, and genetic alterations (mutation and amplification) remain the hallmark that render the cancer cells susceptible to kinase inhibition.

Preclinical and early phase clinical trials

The integration of cancer genomics and chemical compound screening is important for the development of patient-based cancer therapeutics.[74] One study investigated sensitivity to TAE684, a tool compound inhibitor of ALK, in a panel of 602 established cancer cell lines derived from a wide variety of tumour types.[58,75] Whereas the majority of cell lines were

refractory to the treatment, a small subset of cells displayed substantial growth inhibition. Notably, the drug-sensitive cells were derived from NSCLC, ALCL and neuroblastoma. While no correlation was found between genotypic alterations and phenotypes in the neuroblastoma cells, these findings serve as further proof-of-concept that selective kinase inhibitors can elicit dramatic responses in tumour cells harbouring specific genetic lesions.

The development of ALK-targeted therapy has benefitted from previous studies of agents targeting other druggable kinases, such as BCR–ABL in chronic myeloid leukaemia and EGFR in NSCLC. Rapid clinical development of ALK-targeted therapy in neuroblastoma and other tumours has been greatly accelerated, in part, because crizotinib (originally developed as a MET inhibitor) was developed before the discovery of ALK translocations in NSCLC and activating ALK mutations in neuroblastoma.[76] In 2010, one study showed that crizotinib yielded dramatic response rates in pre-treated patients with advanced NSCLC containing ALK-rearrangements.[49] In addition, mechanisms of resistance associated with mutations in the ALK tyrosine kinase domain were elucidated, further implicating ALK as a valuable target (Figure 2).[65]

ALK genomics, biochemical studies and preclinical drug development have firmly established ALK as a tractable molecular target in neuroblastoma. We have established that neuroblastoma cells and xenografts harbouring an ALK aberration (mutation or amplification) are significantly more sensitive to growth inhibition when treated with crizotinib than cells with wild-type ALK,[47] although each mutation confers different levels of sensitivity to crizotinib. Nevertheless, these data provide the preclinical rationale for ALK inhibition as a useful therapeutic strategy in neuroblastoma.

In 2009, within 18 months of the initial discovery of a mutation in a neuroblastoma pedigree, the ADVL0912 trial,[77] a phase I–II clinical trial of crizotinib, was initiated by the Children's Oncology Group for paediatric patients with relapsed solid tumours and ALCL, and demonstrated a rapid translation of preclinical molecular findings into the clinic (Figure 2). As the focus of anticancer drug discovery has shifted to molecularly targeted drugs, this trial was designed, at least in part, to define the optimal dose of crizotinib by emphasizing therapeutic and pharmacokinetic end points. Pharmacokinetic studies, often considered to be optional when used in paediatric phase I trials, are mandatory and critical to guide selection of the appropriate dose in future trials. In parallel with the dose-escalation phase that uses a rolling-six design,[78] patients with ALK-positive tumours are allowed to enroll in the study at any time and have access to the drug that has prospect for direct benefit. In addition, the trial enriches for patients with neuroblastoma, who are typically more heavily pretreated than other patients and often do not meet the eligibility criteria for phase I trials. Such trial enrichment enables patients to enroll at one dose lower than that of the highest dose used in the study, followed by intrapatient dose escalation once the next higher dose level has been deemed safe. Correlative biology studies are an essential component of this type of trial to understand tumour heterogeneity, as well as mechanisms of response and resistance to the drug. Greaves and Maley have elegantly shown that whereas therapeutic intervention could eliminate cancer clones, it can also inadvertently lead to the expansion of resistant clones and treatment failure.[79] An important goal, therefore, is to develop a reliable method for monitoring the presence of residual cancer cells at regular stages during the treatment course to obtain a good understanding of how to approach the problem of drug resistance, either acquired or caused by selection of rare resistant clones from a heterogeneous tumour environment.

Preclinical and early phase clinical development of ALK-targeted therapy in neuroblastoma has advanced rapidly, in a manner that has enabled real-time integration of discoveries to define an optimal dose of crizotinib for phase II II trials. Our current understanding of

neuroblastoma biology suggests that near-complete inhibition of constitutively active ALK is necessary to achieve a clinical response, similar to that achieved in *BRAF*-mutant melanoma following >80% inhibition of ERK activation.[80]

Resistance to therapy

The development of resistance to targeted cancer therapy is now considered largely inevitable.[81] While TKIs targeting ALK, including crizotinib, are effective treatments in preclinical models, their clinical efficacy will ultimately be hindered by drug resistance. One study showed that crizotinib resistance in neuroblastoma cells expressing F1174L-mutated ALK arises from increased ATP-binding affinity for this mutant, thereby reducing the potency of ATP-competitive inhibitors.[47] These data also suggest that crizotinib resistance is surmountable by using higher doses of the drug or new high-affinity inhibitors.[47] This work has directly affected the clinical trial of crizotinib that is in progress by the Children's Oncology Group in an effort to circumvent the *de novo* resistance caused by the F1174L mutation (Figure 2), with ongoing dose-escalation beyond the traditional four doses to define a true recommended phase II dose. This approach is important for proper design of front-line therapy and for guiding the development of next-generation ALK inhibitors, several of which are in early phase clinical trials[48] in neuroblastoma and other cancers driven by ALK translocations.

Mechanisms of acquired resistance have been identified for BCR–ABL inhibition in acute myeloid leukaemia, EGFR inhibition in lung cancer, HER2 inhibition in breast cancer, and Smoothened (a G-protein-coupled receptor encoded by *SMO*) inhibition in medulloblastoma.[82] In light of these challenges, we and others are working to develop a model system for predicting crizotinib resistance mechanisms in the laboratory. To achieve this, neuroblastoma cell lines of known crizotinib sensitivity are treated with sustained and increasing doses of the drug for several months and resistance evaluated by the reduced sensitivity of these cells to treatment as compared to an untreated cell line. Interestingly, Sanger sequencing of resistant cell lines revealed no additional mutations in ALK tyrosine kinase domain, even after addition of the chemical mutagen N-ethyl-nitrosourea, a potent inducer of point mutations. In addition, genomic profiling also failed to detect any changes in ALK copy number.[83] Probing of a phospho-array to assess changes in the activation level of a panel of RTKs revealed significant upregulation of phosphorylated EGFR; this result is corroborated by *in situ* analysis of ALK mRNA showing a significant increase in EGFR expression exclusively in resistant cells.[83] These data suggest the importance of elucidating the upstream and downstream signalling pathways in tumours with activated oncoproteins to design effective therapies that are likely to require combining inhibitors of specific signalling pathways. Cautious extrapolation from these preclinical models will enable us to define biomarkers predictive of response and resistance to ALK-targeted therapy.

ALK-targeted immunotherapy

Although small-molecule inhibition of ALK remains a promising strategy, development of parallel approaches is necessary. As mentioned above, preclinical studies have shown that *ALK* copy number and genotype strongly predict responsiveness to ALK inhibition by TKIs, with *de novo* resistant mutations, such as F1174L, rendering the tumour largely refractory to crizotinib.[47] Neuroblastoma cells almost uniformly express GD2 on their surface, which provides a tractable target for immunotherapeutic approaches and sets the stage for immunotherapy in neuroblastoma, as recently shown in the study of the Children's Oncology Group.[14]

ALK is an ideal tumour antigen for targeting treatment given that it is detectable by immunohistochemistry on the majority of neuroblastoma cells, and yet its expression is

restricted to the tumour, thus minimizing the risk of cytotoxicity in non-malignant tissue; these attributes may simplify patient selection.[29,30] Although clinically relevant ALK antibodies are being developed, so far pre-clinical analysis has been limited.[84] Our work has shown not only that such an ALK antibody can lead to growth inhibition of neuroblastoma cell lines in the absence of immune effector cells, but also that ALK antibodies are able to mediate antibody-dependent cell-mediated cytotoxicity (ADCC), suggesting two possible mechanisms of action.[29] Moreover, these effects were shown in a neuroblastoma cell line that harbours the crizotinib-refractory mutation F1174L,[29] further suggesting that ALK-targeted immunotherapy would provide a therapeutic option for patients who are least likely to respond to crizotinib treatment. Unlike GD2 antibodies, ALK antibodies bind to and inhibit an oncogenic receptor, providing an additional inhibitory mechanism independent of the immune system.

Therapeutic antibodies targeting the RTKs HER2 and EGFR were first developed in late 1990s and have since demonstrated therapeutic efficacy.[85,86] Several studies have highlighted the potential for dual targeting of oncogenic RTKs in cancer,[87,88] and in one preclinical study in lung cancer, dual targeting of EGFR and erlotinib-resistant EGFR was the only therapeutic approach that induced tumour regression.[87] TKI-induced cell surface accumulation of the targeted tumour antigen has been suggested as one mechanism for the enhanced efficacy of dual targeting with a TKI and an RTK antibody.[88] Other studies have suggested that the combination of small-molecule inhibitors with antibodies results in enhanced apoptosis of cancer cells.[89] Indeed, our results are consistent with these studies and suggest that small-molecule inhibition of ALK would sensitize neuroblastoma cells to treatment with an ALK antibody, and that the combination leads to enhanced programmed cell death.[29] Whether the direct cytotoxic effects of ALK-targeted immunotherapy are due to ligand blockade, inhibition of dimerization, or enhanced endocytosis and proteosomal degradation is under investigation. Preclinical optimization will reduce immunogenicity and antibody–drug conjugate technology will improve therapeutic index to maximize success in the clinic.

Future perspectives

An important obstacle in the development and assessment of anticancer therapies is the failure of preclinical mouse tumour models to reliably predict how a drug will perform in humans. There has been a gradual movement to use transgenic oncomouse models for anticancer drug testing. The most characteristic transgenic model of neuroblastoma is the TH-MYCN model,[90] in which the animals develop an aggressive malignancy similar to the human *MYCN*-amplified neuroblastomas. This model has been used widely for a multitude of basic biology and therapeutic sensitivity studies.[91–99] Recent data suggest that inhibition of PI3K and mTOR signalling pathways affect angiogenic blockade, in part, by degradation of MYCN *in vivo*, and should be tested in children with high-risk *MYCN*-amplified neuroblastoma.[100] Besides the TH-MYCN model, generating genetically engineered mouse models (GEMM) that overexpress various ALK mutants will require sophisticated approaches,[101] but such approaches are vital to assess the efficacy of novel ALK-targeted therapeutics. GEMM will also enable small interfering RNA screens of the druggable genome in combination with ALK inhibitors to evaluate synergy and antagonism with existing chemotherapy backbones. Transgenic zebra fish models have also emerged as an invaluable model system of human cancer to analyze underlying cellular processes. Zebra fish also present an exciting *in vivo* model for high-throughput drug screening as they enable visual assessment of both drug efficacy and toxicity,[102] and are likely to emerge as an essential tool for the study of ALK-driven neuroblastoma, complementing *in vitro* cell culture-based drug screens. The advances in GEMM and zebra fish studies will help to

address fundamental questions about the regulation of ALK signalling and mechanisms of *de novo* and acquired drug resistance.

Another obstacle in the development of effective cancer therapies is patient selection and trial design. When first-generation anti-EGFR therapies for the treatment of NSCLC entered clinical trials in the 1990s, this approach was directed against the wild-type receptor that was shown to be overexpressed in many epithelial cancer types.[103] Mutations in the EGFR kinase domain in NSCLC were discovered in 2004, showing that this subset of tumours was linked to increased sensitivity of lung tumours to gefitinib and erlotinib.[104–106] A small proportion of patients with no detectable EGFR-activating mutations show a radiographic response when treated with TKIs targeting EGFR, suggesting that there are probably other types of genetic alterations that activate EGFR signalling in the absence of mutations.[107,108] Today, we know that clinical and biological selection of these patients is important for providing appropriate treatment, and stratification based on *EGFR* mutation status is mandatory prior to the initiation of frontline therapy. Rapid development of ALK inhibition in patients with NSCLC stems from this experience, and the same theory will hold true for neuroblastoma, in which determining the appropriate target population for treatment, the frequency of this target aberration in patients and how to combine therapeutic interventions with molecular diagnostics will all need to be considered. In addition, combining targeted therapies in both early phase and late-phase clinical drug development will raise practical issues at the bench and the bedside, including tumour resistance mechanisms, existence of redundant signalling pathways, tumour heterogeneity and the signalling feedback loops that may attenuate antitumour activity. Future phase III trials of ALK inhibition in neuroblastoma will benefit from previous studies of targeted agents, but several substantial challenges remain that will require adopting new paradigms for drug development and clinical trial design.

Conclusions

Neuroblastoma remains a debilitating disease that provokes both exasperation and excitement among clinical and laboratory investigators. For decades, dramatic intensification of cytotoxic therapy was only moderately effective at improving outcome, but substantial advances have been made that incorporate targeted immunotherapy. The discovery of ALK as a mutated oncogenic receptor in a subset of neuroblastoma tumours provides the basis for the development of innovative targeted treatment approaches. Preclinical data suggest that targeting mutated ALK in neuroblastoma is complex and that the ultimate design of a phase III clinical trial will require identification of an appropriate biomarker for therapeutic stratification. Equally important is the collaboration between the laboratory and clinical settings to facilitate the development of ALK-targeted therapy, to develop dual-targeting strategies aimed at preventing or delaying drug resistance, and to ensure that appropriate measures are in place for collecting adequate tissue samples to study drug sensitivity and resistance, thereby enabling translational research to improve the outcomes of patients.

Acknowledgments

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Key points

- The discovery of germline and somatic mutations in *ALK* provides the first tractable oncogenic target in neuroblastoma, prompting the initiation of a phase I–II trial of the ALK inhibitor crizotinib

- ALK is mutated in 8% of all neuroblastoma cases; mutations are distributed across the range of phenotypes and predict for an inferior outcome

- Full-length ALK is expressed on the surface of neuroblastoma tumours in both the presence and absence of a genetic alteration, suggesting that ALK antibody therapy is relevant in neuroblastoma

- The F1174L mutation in ALK results in *de novo* resistance in neuroblastoma and has emerged as a resistance mechanism in ALK-translocated tumours treated with crizotinib

- Structural and biochemical data suggest that the increase in ATP-binding affinity for the F1174L-mutated ALK can be overcome with higher doses of crizotinib

- Stratification of patients based on *ALK* alteration status will likely become an integral part of frontline therapy for patients with high-risk neuroblastoma
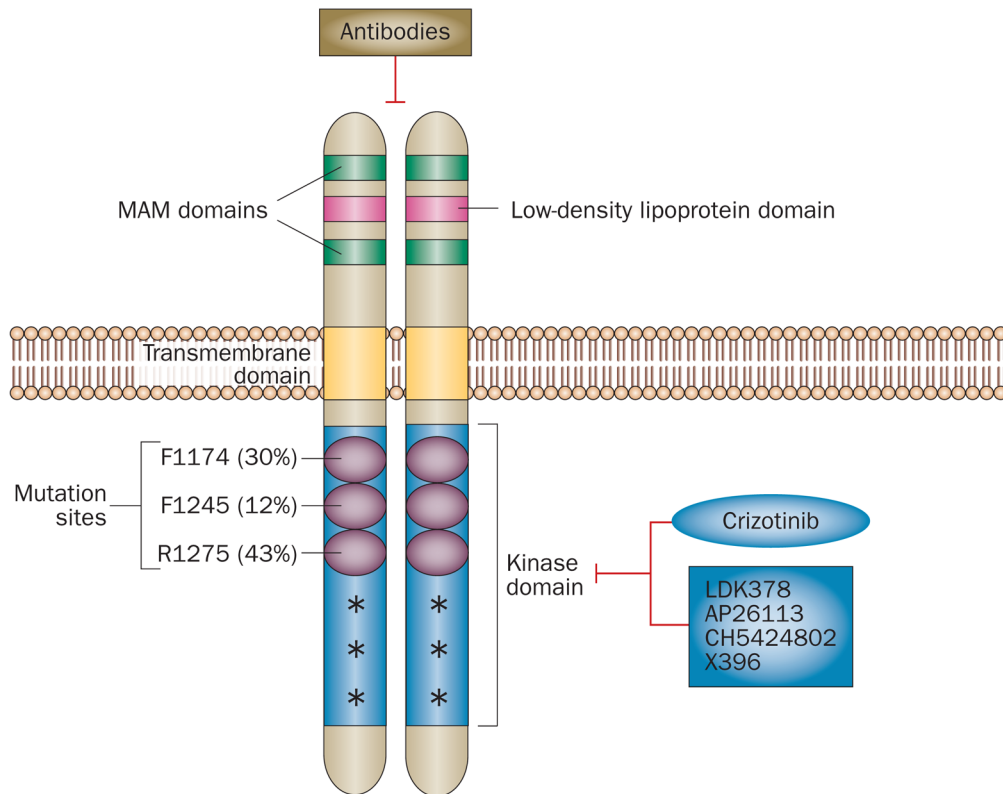
Figure 1.
Schematic representation of ALK protein structure and mutations found in neuroblastoma. The low-density lipoprotein domain, two MAM domains, and the transmembrane and kinase domains of ALK are shown. R1275, F1174 and F1245 are three most common ALK mutations in neuroblastoma; the frequency of these mutations are provided in parenthesis. Other low frequency mutations (>20) are denoted with an asterisk. The tyrosine kinase inhibitor crizotinib is in clinical trials and other second-generation ALK inhibitors are in clinical development. ALK-targeting antibodies are also being developed.
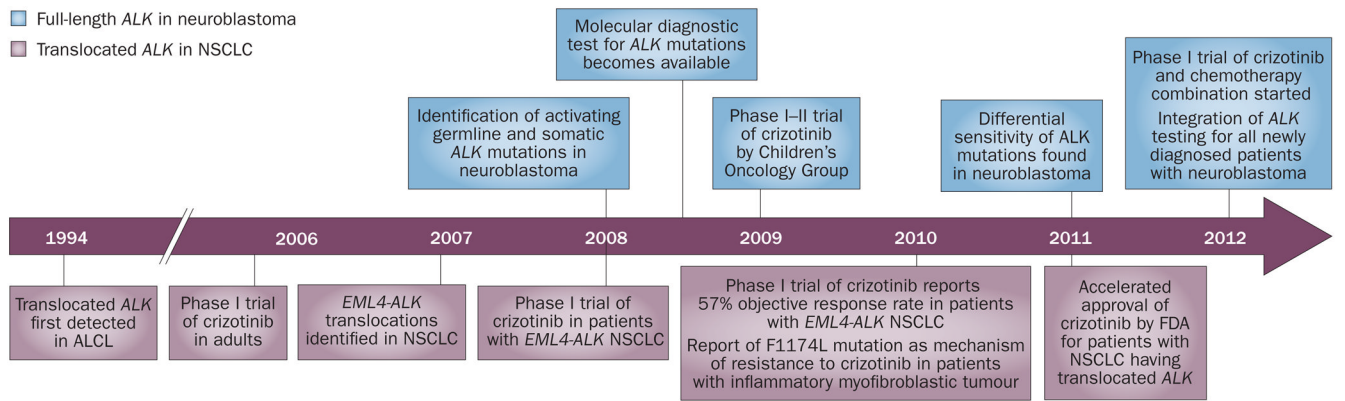
Figure 2.
Timeline depicting the milestones leading to the clinical testing of the ALK inhibitor crizotinib targeting the full-length ALK in neuroblastoma (above) and translocated ALK in non-small-cell lung cancer (below).