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Review Article

Oncogenic mutations of *ALK* in neuroblastoma

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Neuroblastoma is one of the most common solid cancers among children. Prognosis of advanced neuroblastoma is still poor despite the recent advances in chemo/radiotherapies. In view of improving the clinical outcome of advanced neuroblastoma, it is important to identify the key molecules responsible for the pathogenesis of neuroblastoma and to develop effective drugs that target these molecules. Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase, initially identified through the analysis of a specific translocation associated with a rare subtype of non-Hodgkin's lymphoma. Recently it was demonstrated that *ALK* is frequently mutated in sporadic cases with advanced neuroblastoma. Moreover, germline mutations of *ALK* were shown to be responsible for the majority of hereditary neuroblastoma. ALK mutants found in neuroblastoma show constitutive active kinase activity and oncogenic potentials. Inhibition of ALK in neuroblastoma cell lines carrying amplified or mutated *ALK* alleles results in compromised downstream signaling and cell growth, indicating potential roles of small molecule ALK inhibitors in the therapeutics of neuroblastoma carrying mutated ALK kinases. (*Cancer Sci* 2011; 102: 302–308)

Neuroblastoma is a malignant embryonal neoplasm arising from developing neural crest tissues.[1] It commonly affects younger children, where the median age of diagnosis is 17 months and approximately 90% of the patients are <4 years old. In the United States, the incidence of neuroblastoma is estimated to be one in 7000 births, although the incidence calculated from the mass screening program in Japan was as high as 29.80 cases per 100 000 births, which is significantly higher than the estimation in the prescreening cohort (11.56 cases per 100 000 births).[2] It is the third most common cancer in childhood after leukemia and brain tumors, accounting for 7–11% of all pediatric cancers.[3] The presentation and following clinical courses of neuroblastoma are highly variable, ranging from a solitary localized mass with no apparent clinical symptoms to widely disseminated diseases presenting with severe systemic illness.[1] While some tumors undergo spontaneous regression without therapy, approximately 60–70% of high-risk neuroblastoma patients are resistant to any therapies currently available and succumb to death,[4–6] even though a substantial improvement in 5-year survival rates has been obtained for a subset of advanced tumors through the development of multimodal chemo/radiotherapies during the past several decades.[1] Thus, one of the urgent problems in the current neuroblastoma treatment would be to develop rational and effective therapeutic strategies for the high-risk neuroblastoma cases based on their molecular pathogenesis.

On the other hand, during the past three decades, little advancement has been made in the understanding of neuroblastoma pathogenesis in terms of critical gene targets, except for the identification of frequent *MYCN* amplification.[7] Amplification of the *MYCN* gene is found in approximately 20% of neuroblastoma, especially in advanced diseases, and has been consistently associated with poor prognosis.[8,9] Although *MYCN* amplification is a critical genetic event in neuroblastoma development,[10] it encodes a transcription factor and thus may not be a plausible pharmacological target for therapeutics. Recently, several groups independently discovered activating mutations of the *ALK* gene in the majority of familial neuroblastoma and also in a subset of sporadic neuroblastoma cases.[11–14] Given that the mutated ALK kinases are well-tractable targets for small-molecule kinase inhibitors, the discovery draws attention in the field of neuroblastoma research. In this review, we provide a brief overview of the role of *ALK* mutations in neuroblastoma pathogenesis and their implication in future therapeutics.

Genetic analysis of familial neuroblastoma

One of the first clues to identifying the novel genetic target of neuroblastoma was obtained from a linkage study of neuroblastoma-prone families. It was recognized that approximately 1–2% of newly diagnosed neuroblastoma cases occur within families (familial/hereditary neuroblastoma), indicating the existence of dominantly acting neuroblastoma susceptibility gene(s),[15–19] although previous linkage studies, in an attempt to identify the susceptibility locus, failed to provide a reproducible result due to the insufficient power of the studies.[20–22] Germline mutations of the paired-like homeobox 2B (*PHOX2B*) gene at 4p12 was reported to be responsible for neuroblastoma predisposition, but they were mostly related to a rare form of familial neuroblastoma associated with congenital central hypoventilation syndrome (CCHS) and/or Hirschsprung disease, with rare somatic mutations.[23–26] Recently, researchers at the Pennsylvania University analyzed 20 neuroblastoma pedigrees for linkage using approximately 6000 genetic markers, and mapped a candidate neuroblastoma susceptibility locus to the 2p region between rs18621106 and rs2008535, which contains 104 genes including *MYCN* and *ALK*.[11] Through a resequencing analysis of the *ALK* exons within the pedigrees they identified germline mutations of the *ALK* gene in >90% of the pedigrees that co-segregated with neuroblastoma development within the families, clearly demonstrating that the germline *ALK* mutations are responsible for the susceptibility to the development of hereditary neuroblastoma in the majority of the cases.[11,12] Moreover, the subsequent analysis of *ALK* mutations in sporadic neuroblastoma cases identified a subset of sporadic neuroblastoma cases carrying acquired/germline mutations of *ALK*, which was also reported independently by other groups.[12–14,27]

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Genome-wide copy number scanning of neuroblastoma

These groups conducted genome-wide copy number analyses of neuroblastoma using comparative genomic hybridization (CGH) arrays[12] or single nucleotide polymorphism (SNP) arrays.[11,14,27,28] With thousands to half-a-million genetic probes, both platforms enabled high-throughput detection of subtle genetic changes occurring in tumor genomes.[29,30] Neuroblastoma genomes show characteristic copy number changes that involve large chromosomal segments, including gains of 17q, 1q, 2p and 11p, and losses of 1p, 3p and 11q, which, like other human cancers, collectively comprise a unique genomic profile of neuroblastoma.[11,12,14] High-level amplifications, which usually involve discrete chromosomal regions <1 Mb in length, occurred in approximately 30% of neuroblastoma cases. Approximately 90% of the high-level amplifications in neuroblastoma were centered on the *MYCN* locus at 2p24, whereas other amplicons rarely mutually overlapped, except for the amplifications at 2p23, which exclusively involved the *ALK* locus in common[12,14,28] (Fig. 1).

High-level amplification of the *ALK* gene and aberrantly activated ALK signaling in neuroblastoma was first described by Osajima-Hakomori *et al.*[31] in two neuroblastoma-derived cell lines and a single case of primary neuroblastoma. The genome-wide copy number studies confirmed their finding, in which the frequency of *ALK* amplifications is reported to occur in 3–5% of primary neuroblastoma cases.[11,12,14] Subsequent resequencing studies of *ALK* coding exons disclosed non-synonymous nucleotide substitutions of *ALK* in a subset of sporadic neuroblastoma cases and also of neuroblastoma-derived cell lines with mutation rates of approximately 6–11% and approximately 30%, respectively. Amplified *ALK* alleles, as a rule, did not harbor additional mutations, although in rare cases mutated ALK alleles were amplified.

Genetic abnormalities of the *ALK* gene in human cancers

ALK was initially isolated as a partner of the fusion gene generated by t(2;5)(q23;q35) translocation, which is characteristic of anaplastic large cell lymphoma (ALCL), a rare subtype of non-Hodgkin's lymphoma.[32,33] *ALK* encodes an orphan receptor tyrosine kinase with an apparent molecular mass of 220 kDa. Jelly belly,[34] and pleiotrophin[35] and midkine[36] have been postulated as putative ALK ligands in *Drosophila* and mammalians, respectively, but a dispute about the authentic ligands of ALK still remains. ALK has an extracellular domain that is highly similar to LTK and, together with IGF-1R and c-Ros kinases, belongs to the insulin family of proteins.[37] Expression of ALK is largely restricted to neural tissues and is most abundant in the neonatal brain and, to a lesser extent, in the adult brain.[38–41] In the developing brain, the highest expression was found in the thalamus, mid-brain, olfactory bulb and selected parts of cranial and dorsal ganglia.[38,39] It is of particular note that high frequencies of ALK expression were reported in primary neuroblastoma specimens (22 out of 24 samples) and in other tumor cell lines derived from neuroectdermal tumors including neuroblastoma (13 out of 29 cell lines).[42] These expression patterns of ALK suggest its primary role in normal neural development as well as the pathogenesis of neuroblastoma, although *ALK*-deficient mice seem to show apparently normal develop.[37]

In t(2;5)(q23;q35) translocation, the carbonyl terminal of ALK that contains a kinase domain is fused with nucleophosmin (NPM), generating NPM/ALK fusion protein. *ALK* was also shown to participate in the generation of different fusion genes with a variety of partner genes in ALCL,[43–47] inflammatory fibroblastic tumor,[43,48–52] squamous cell carcinoma of the esophagus[53] and non-small-cell lung cancers (NSCLC).[54,55] In NSCLC, *ALK* was reported to be fused with EML4 to generate EML4–ALK fusion protein as a result of inv(2)(p21p23), which is found in 6% of the NSCLC cases[55] (Fig. 2).

These ALK-containing fusion proteins invariably show constitutive kinase activity and transform NIH3T3 cells and/or confer growth factor independence to 32D and/or Ba/F3 cells.[56–58] When bone marrow cells were retrovirally transduced with *NPM–ALK* and transplanted into mice, they developed B-cell lymphoma within 4 months.[58] The critical role of ALK fusion proteins in neoplastic evolution has been further demonstrated

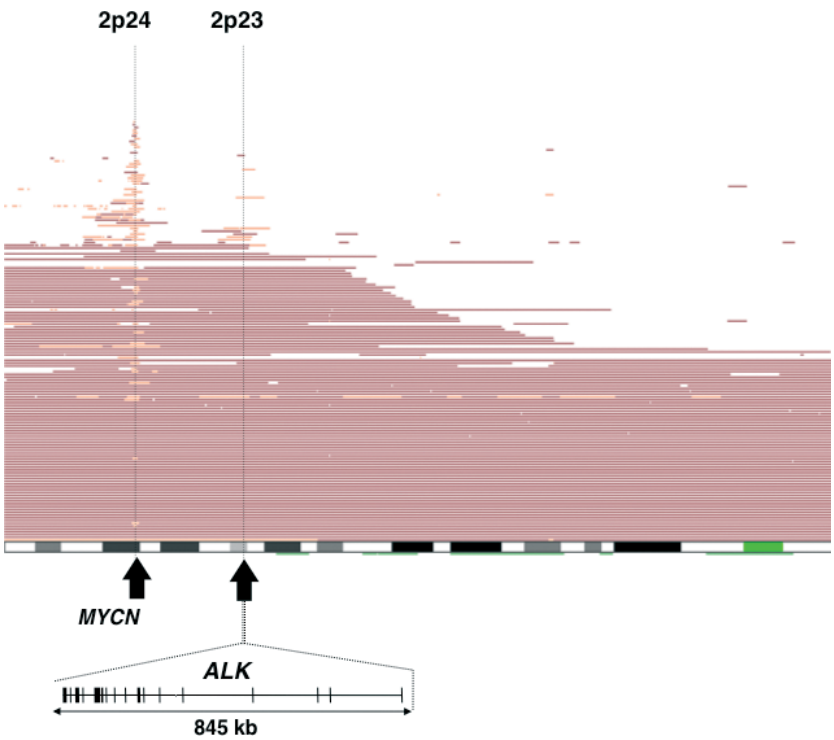

Fig. 1. Copy number gains and high-level amplifications in the short arm of chromosome 2 in neuroblastoma. Each horizontal line indicates a region showing a simple copy number (CN) gain (CN < 5; thick red) and high-level amplification (CN > 5; thin red) in each case. The majority of high-level amplifications involved the *MYCN* locus at 2p24, while the other group of amplicons is found at 2p23, which exclusively contains the *ALK* locus.

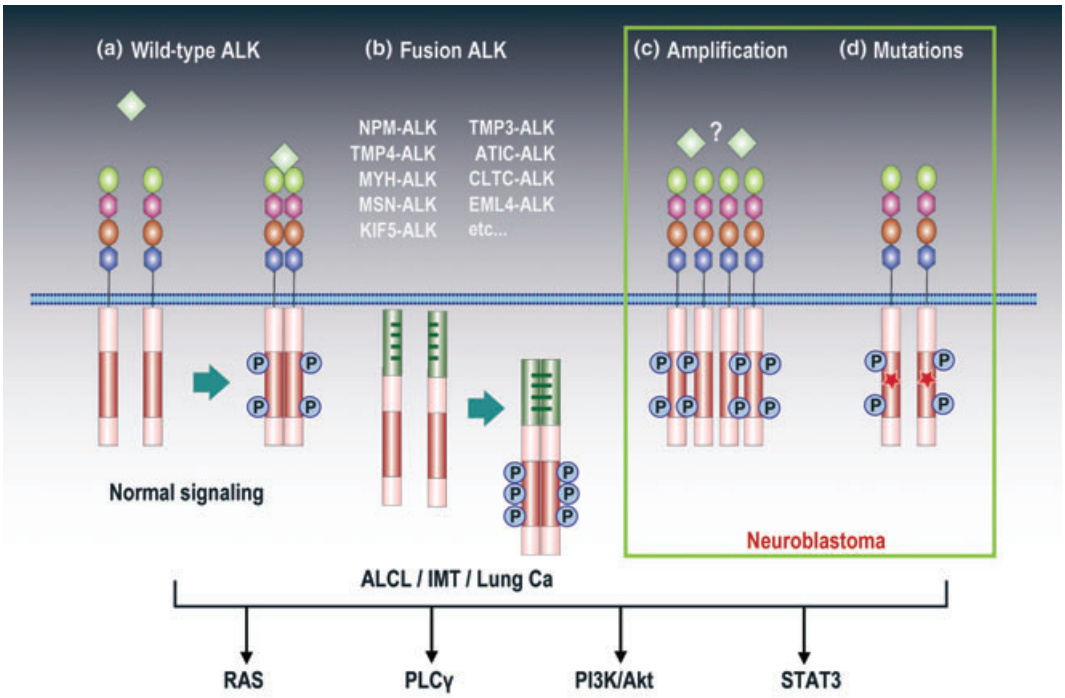

Fig. 2. Aberrant activation of ALK in human cancers. (a) Ligand-dependent physiological activation of wild-type ALK. (b) Fusion ALK kinases found in anaplastic large cell lymphoma (ALCL) and non-small-cell lung cancer (Lung Ca), such as NPM–ALK and EML4–ALK, self-dimerize through their N-terminal domains derived from fusion partners, leading to their transphosphorylation and constitutive activation of the kinase. In a subset of neuroblastoma, aberrant activation of ALK occurs by gene amplification (c) or somatic/germline mutations (d). Activated ALK transmits constitutive signals through downstream pathways, which is thought to be important for tumorigenesis. IMT indicates inflammatory myofibroblastic tumor.

using transgenic mouse models with *ALK* fusion genes; mice carrying *NPM–ALK* or *EML4–ALK* transgenes under *Vav* or *CD4,* or s*urfactant protein C* promoter develop aggressive lymphoma or adenocarcinoma of the lung, respectively.[59–61] The aberrant kinase activity of these ALK-fusion proteins is thought to be caused by transphosphorylation upon self-dimerization through their N-terminal domain derived from the fusion partners. Mutations or deletions of the dimerization domain of NPM–ALK and EML4–ALK result in loss of the transforming capacity of the fusion kinases.[55,57] The constitutive active fusion kinases transmit signals through activation of a variety of signal transducers, including PLCγ, PI3K/AKT, STAT3 and RAS.[62–67]

In neuroblastoma, on the other hand, aberrant activation of ALK kinase is caused by gene amplification[31] or mutations.[11–14] Thus, ALK represents a unique type of oncogenic kinase, in that it is deregulated either by gene fusions, or by gene amplification or mutations, depending on the tumor type.

Biological consequences of ALK mutations

Most reported *ALK* mutations occurred within the kinase domain, in which three highly conserved amino acid positions, F1174, F1245 and R1275, were predominantly affected, suggesting their functional importance for the regulation of kinase activity[11–14] (Figs 3,4). The F1174 residue is located at the end of the Cα1 helix and corresponds to equivalent positions mutated in EGFR (V769) and ERBB2 (V769), while the F1245 lies in the catalytic domain and corresponds to the L833 residue of EGFR, a mutation of which is reported to be associated with gefitinib resistance in lung cancer (Fig. 5).[13] The R1275 position lies within the activation loop and is invariably changed to glutamine, and amino acid substitution at this position to a positively charged one would displace the loop to positions that permit autophosphorylation and autoactivation of the kinase (Fig. 5).[68,69] However, the distributions of these mutations were different between sporadic cases and familial cases; R1275 mutations are commonly found in both sporadic and familial cases, while no germline mutations involving the F1174 or F1245 position have been reported.[11–14] Because not all mutant *ALK* carriers develop neuroblastoma (i.e. incomplete penetrance), a germline *ALK* mutation is not fully oncogenic and additional genetic events are thought to be required for neuroblastoma development. *ALK* mutations tend to be associated with advanced diseases and also with *MYCN* amplification in sporadic neuroblastoma cases, although the trend was not clear for germline *ALK* mutations.[11–14]

When expressed in NIH3T3 cells, the predominant kinase domain mutant (F1174L) and a juxtamembrane mutant (K1062M) are shown to have transforming capacity; mutant-transduced cells display increased colony formation in soft agar and tumor generation in nude mice, whereas the mutant kinases show increased autophosphorylation and *in vitro* kinase activity compared with wild-type ALK.[14] In addition, when introduced into an IL-3-dependent cell line, BaF3, the two major kinase domain mutants (F1174L and R1275Q), render the cell line independent of IL-3.[13] Expression of the F1174L mutant in NIH3T3 and Ba/F3 cells leads to constitutive activation of the downstream signaling pathways of the ALK kinase, as demonstrated by increased levels of phosphorylated ERK1/2, STAT3 and AKT.[13,14] These functional and biochemical studies together indicate that these ALK mutants are actually oncogenic and could be responsible for the pathogenesis of neuroblastoma.

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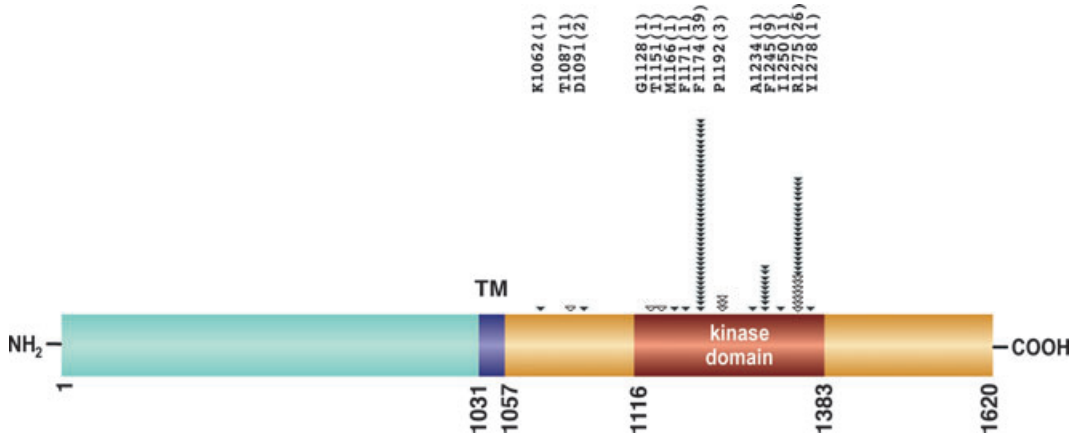

Fig. 3. Frequency and distribution of *ALK* mutations reported in familial and sporadic cases of neuroblastoma.[11–14,27] Locations of somatic and germline mutations of *ALK* in each case or family are depicted by filled and open arrows, respectively. The exact positions and amino acids involved are indicated on the top, where the number of reported mutations is indicated in parenthesis.

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                    1174
HUMAN ALK     ...ALIISKFNHQNIVR..
HUMAN LTK     ...ALIISKFRHQNIVR..
HUMAN INSR    ...ASVMKGFTCHHVVR..
HUMAN IGF1R   ...ASVMKEFNCHHVVR..

                    1245
HUMAN ALK     ...EENHFIHRDIAARN..
HUMAN LTK     ...EENHFIHRDIAARN..
HUMAN INSR    ...NAKKFVHRDLAARN..
HUMAN IGF1R   ...NANKFVHRDLAARN..

                    1275
HUMAN ALK     ...GDFGMARDIYRASY..
HUMAN LTK     ...GDFGMARDIYRASY..
HUMAN INSR    ...GDFGMTRDIYETDY..
HUMAN IGF1R   ...GDFGMTRDIYETDY..
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Fig. 4. Alignment of amino acids of ALK among different species. Conserved amino-acids among different insulin receptor family kinases are shown by gray boxes and the mutated positions are shown in red.

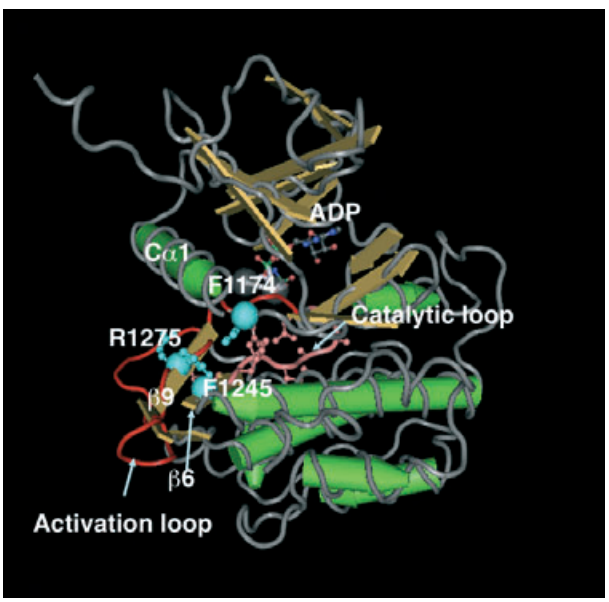

Fig. 5. A 3-D structure of the kinase domain of ALK kinase predicted from that solved for IGF-1R, where the positions of three major mutations are indicated by light blue spheres. Activation and catalytic loops are depicted by red and pink wires.

Effects of ALK inhibition on ALK fusion kinases

The critical role of *ALK* mutations in neuroblastoma development is further supported by the experiments using inhibition of mutant ALK. Tumor suppressive effects of ALK inhibition have been well documented in *NPM–ALK*-positive ALCL and *EML4–ALK*-positive NSCLC. NVP–TAE684 is a highly potent and selective small molecule ALK inhibitor, which blocks the growth of ALCL-derived cell lines with very low IC_{50} values between 2 and 10 nM.[70] NVP–TAE684 treatment of ALCL-derived cell lines induces rapid and sustained inhibition of phosphorylation of NPM–ALK and its downstream signaling, leading to cell cycle arrest and apoptosis.[70] NVP–TAE684 also induces varying degrees of growth suppression in *EML4–ALK*-bearing lung cancer cell lines, including NCI-H3112, NCI-H2228 and DFCI032.[67,71] PF-2341066 was another compound, which was initially identified as an orally available c-Met inhibitor in biochemical enzymatic screens, but was subsequently found to show selective inhibition of ALK.[72,73] It is highly selective for both ALK and c-Met kinases, being almost 20-fold selective for ALK and c-Met compared with 120 other kinases.[73] PF-2341066 inhibited cell growth of NPM–ALK-positive ALCL-derived cell lines, as well as EML4–ALK-positive NSCLC-derived cell lines with decreased downstream signaling pathways, although their IC_{50} values were significantly higher than those of NVP–TAE684.[71,72] Recently, Soda *et al.* generated transgenic mice, in which the *EML4–ALK*-transgene was selectively expressed in the developing lung under the *surfactant protein C* promoter.[61] All mice developed multiple lung adenocarcinomas soon after birth, which were successfully treated with a 2,4-pyrimidinediamine derivative that specifically inhibits ALK kinase.[61] These observations strongly support that aberrant ALK activity of ALK-fusion proteins is central to the development of ALCL and NSCLC.

Effects of ALK inhibition on *ALK*-mutated neuroblastoma cell lines

In neuroblastoma, the predominant mechanism of ALK activation should be some conformational change caused by a point mutation typically involving the kinase domain, which potentially affects the kinetics of ALK inhibitors on the mutated kinase. However, as long as major ALK mutants are concerned, their kinase activity seems to be successfully inhibited by the currently available ALK inhibitors. Ba/F3 cells transduced with the F1174L or R1275Q ALK mutant were effectively killed by NVP–TAE684 or PF-2341066, whereas the cells transduced with a constitutive active FLT3 mutant or wild-type ALK were not.[13] Thus, both compounds specifically inhibit the kinase activity of these ALK mutants, although the inhibition is more efficient for F1174F than for R1275Q. In fact, many, if not all, neuroblastoma cell lines carrying mutated or amplified *ALK* alleles are shown to be sensitive to these ALK inhibitors.[12,13,71] Interestingly, the sensitivity of some neuroblastoma cell lines to small molecule ALK inhibitors was recognized prior to the discovery of *ALK* mutations in neuroblastoma. McDermott *et al.* tested more than 600 cancer cell lines for their sensitivity to NVP-TAE684 and/or PF-2341066 and found that neuroblastoma cell lines, as well as cell lines derived from ALCL and lung cancer, frequently show sensitivity to these inhibitors.[71] The dependence of *ALK*-mutated neuroblastoma to ALK inhibition is further confirmed by ALK knockdown experiments; shRNA-mediated knockdown of ALK in *ALK*-mutated neuroblastoma cell lines results in the suppression of cell growth, indicating that the major effect of ALK inhibitors on *ALK*-mutated neuroblastoma cell lines are mediated by their activity on ALK rather than off-target effects on other kinases.

As mentioned above, the sensitivity of *ALK*-mutated neuroblastoma cell lines to ALK inhibitors seems to substantially differ among cell lines, depending on the type of *ALK* mutations. The F1174L mutant seems to be more sensitive to NVP–TAE684 than the R1275Q mutant.[13] Some *ALK*-mutated cell lines were resistant to ALK inhibition; SMS–KCNR harbors the R1275Q mutation, but was not killed by NVP–TAE684 or shRNA, indicating that this cell line acquired some additional mutations, escaping from its dependence on ALK signaling.

Concluding remarks

Genetic analyses of neuroblastoma have revealed that aberrant activation of ALK kinase in human cancer is not only caused by gene fusions but also by gene amplification or germline/somatic mutations. However, probably the most significant impact of the discovery of *ALK* mutations in neuroblastoma would be the possibility of successful treatment of *ALK*-mutated neuroblastoma with small molecule ALK-inhibitors, which are now under development in several pharmaceutical companies. Because ALK expression is restricted to developing neural tissues and ALK-deficient mice develop normally,[37] mutated ALK is likely to be a plausible therapeutic target. Although the enthusiasm for *ALK*-targeted therapy for advanced neuroblastoma seems to be too early at this moment, an encouraging result was reported from a clinical trial of crizotinib (PF-2341066) for NSCLC carrying the *EML4–ALK* fusion gene. A total of 50 patients were evaluable for response, where 64% of the overall response rate and 90% of the disease control rate were obtained[74] with minimum adverse reactions. Nevertheless, the result in NSCLC is not easily translated into neuroblastoma cases. For example, while some ALK mutants are shown to be inhibited by the available ALK inhibitors *in vitro*, the impact of different mutation types on the action of inhibitors should be further evaluated. The effect of frequent co-existence of *MYCN* amplification with *ALK* mutations on sensitivity to ALK inhibitors is still elusive, although a cell line, KELLY, which carries both the F1174L mutation and *MYCN* amplification, was reported to be sensitive to NVP–TAE684.[13,71] Finally, the role of ALK inhibitors in *ALK*-non-mutated neuroblastoma is another interest. Some neuroblastoma cell lines (NBEBC1 and NB1771) were shown to be sensitive to shRNA-mediated ALK knockdown, even though they were reported to have no mutated *ALK* alleles.[11] Interestingly, ALK is phosphorylated in these cell lines at lower levels. Considering the frequent expression of ALK in neuroblastoma cells, it may be postulated that regardless of its mutation status, ALK play a positive role during the initiation and promotion of neuroblastoma, even though established tumors may or may not depend on the ALK activity. Clearly, much more work is required before the clinical role of ALK inhibitors in the treatment of advanced neuroblastoma is established.

Disclosure Statement

The authors have no conflict of interest.

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