Mutation-Independent Anaplastic Lymphoma Kinase Overexpression in Poor Prognosis Neuroblastoma Patients

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Abstract
Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase predominantly expressed in the developing nervous system. Recently, mutated ALK has been identified as a major oncogene associated with familial and sporadic neuroblastomas (NBL). Yet, a direct correlation between endogenous expression level of the ALK protein, oncogenic potential, and clinical outcome has not been established. We investigated ALK genetic mutations, protein expression/phosphorylation, and functional inhibition both in NBL-derived cell lines and in 34 localized and 48 advanced/metastatic NBL patients. ALK constitutive phosphorylation/activation was observed in high-ALK expressing cells, harboring either a mutated or a wild-type receptor. No activation was found in cell lines with low expression of wild-type ALK. After 72 hours of treatments, small molecule ALK inhibitor CEP-14083 (60 nmol/L) induced growth arrest and cell death in NBL cells overexpressing wild-type (viability: ALKhigh 12.8%, ALKlow 73%, P < 0.0005; cell death: ALKhigh 56.4%, ALKlow 16.2%, P = 0.0001) or mutated ALK. ALK protein expression was significantly up-regulated in advanced/metastatic compared with localized NBLs (ALK overexpressing patients: stage 1-2, 23.5%; stage 3-4, 77%; P < 0.0001). Interestingly, protein levels did not always correlate with ALK genetic alterations and/or mRNA abundance. Both mutated and wild-type ALK receptor can exert oncogenic activity in NBL cells. However, wild-type ALK receptor requires a critical threshold of expression to achieve oncogenic activation. Overexpression of either mutated or wild-type ALK defines poor prognosis patients. Alternative mechanisms other than direct mutations and/or gene amplification regulate the ALK level of expression in NBL cells. Wild-type ALK is a potential therapeutic target for advanced/metastatic NBLs. [Cancer Res 2009;69(18):7338–46]

Introduction
Neuroblastoma (NBL) is a common and highly aggressive childhood tumor resulting from the malignant transformation of immature neural crest-derived cells (1, 2). Two thirds of the cases occur in children younger than 5 years. Despite current intensive multimodality treatments, children with metastatic NBL at diagnosis have a 5-year overall survival that does not exceed 40%. Approximately 10% of the patients with localized disease at diagnosis experience a relapse, and 20% of them become refractory to current therapies (3, 4). Additional therapeutic approaches are needed to improve the prognosis of NBL patients with advanced stage disease.

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase (TK) originally identified as the COOH terminal part of the transforming fusion protein nucleophosmin (NPM)-ALK in anaplastic large cell lymphomas (ALCL; refs. 5, 6). ALK is widely expressed in the embryonic nervous system, and its expression declines significantly after birth. It has been proposed that endogenous ALK expression promotes neurite outgrowth and motor neuron generation (7–9). The mechanisms whereby ALK is physiologically activated have not been completely elucidated. In humans, the heparin-binding growth factor pleiotrophin (PTN) has been shown to bind and activate ALK, although this issue is somewhat controversial (7–11). In a study aimed at comparing gene expression in human fetal neuroblast and NBL, ALK has been detected among the genes up-regulated in unfavorable NBLs compared with fetal neuroblasts, suggesting a possible oncogenic role for ALK (12). Recently, ALK was identified as a major predisposition gene in familial NBL, which accounts for 1% to 2% of NBL cases. In this study gene gain, amplification and point mutations were identified in germline and somatic mutations in familial and high-risk patients, respectively (13). Increasing evidence point to ALK as a crucial oncogene associated with NBL (14–17). These results open new avenues to future investigations aimed at developing possible therapeutic strategies based on the inhibition of ALK-mediated signaling.

In the present work, we directly tested the efficacy of small molecule ALK inhibitors on NBL cell growth and survival. Our results show that ALK inhibition, by small molecule inhibitors, specifically hampers cell proliferation and induces cell death of NBL cells expressing high levels of constitutively active ALK. ALK inhibition was effective on NBL cell lines carrying either a mutated or a wild-type gene. The immunohistochemical analysis of 82 sporadic NBL primary tumors showed a correlation between aberrant ALK level of expression and NBL progression and clinical
outcome. Our findings provide experimental basis for pharmacologic inhibition of ALK kinase activity as a possible therapeutic strategy for the treatment of ALK overexpressing NBL patients.

Materials and Methods

Patients. Eighty-two patients with NBL at diagnosis were studied. All patients were diagnosed and staged according to the International Neuroblastoma Staging System (18). MYCN locus amplification was assessed in all patients by fluorescence in situ hybridization analysis. Of these 82 patients, 11 had MYCN amplification (stage 1-2: one patient, stage 3-4: 10 patients). Response to therapy was evaluated according to the International Neuroblastoma Response Criteria (18).

Cell lines. IMR-32 cell lines were obtained from the American Type Culture Collection; UKF-NB3 and SK-N-SH cell lines were kindly provided by Dr. M. Rodolfo from our Institute, and the NB5 cell line by Dr. S. Morris (St. Jude Children’s Research Hospital). NB-INT1 were obtained by SK-N-SH subcloning. The t(2;5)-positive SU-DHL1 anaplastic lymphoma cell line was used as positive control.

Antibodies. Anti-ALK antibodies recognizing intracellular regions: mouse monoclonal antibody ALKc (provided by Dr. B. Falini, University of Perugia) and rabbit polyclonal antibody ALK11 (provided by Dr. S. Morris). Phosphorylated Tyr664 ALK and the rabbit h-actin were purchased from Cell Signaling Technology. Antibodies against phosphorylated Tyr (clone 4G10), phosphorylated Thr202/Tyr204 extracellular signal-regulated kinase 1/2 (ERK1/2), and phosphorylated Ser727 signal transducers and activators of transcription 3 (STAT3) were from Upstate Biotechnology.

CEP compounds. The small molecule ALK inhibitors, CEP-14083 and CEP-14513, were reported previously (19). Stock solutions of CEP-14083 and CEP-14513 at 4 mmol/L in DMSO (Sigma Chemical Co.) were subdivided in aliquots (50 μL) and stored at −20 °C.

Construction of Tet-inducible RNA interference–ALK. The Tet-inducible RNA interference (pTET)–ALKsiRNA construct carrying a doxycycline-inducible form of the RNA polymerase III (H1 promoter) was assembled to drive the expression of specific ALK small interfering RNA (siRNA) according to the procedure described by van de Wetering and colleagues (20). Briefly, phosphorylated ALK specific oligonucleotides (100 pmol of each) were annealed and cloned in BglII and HindIII cloning sites of the pTER vector (kindly provided by Dr. H. Clevers, Center for Biomedical Genetics). The ALK oligonucleotides used were:

5′-GATCCCGCGCCGCTACTGCCCCTGGAGTTCAAGAGACTCCAGGGGCAGTAGCGGCGG-3′.
5′-AGCTTTTCCAAAAAGCCGCTACTGCCCCTGGAGTCTCTTGAACTCCAGGGGCAGTAGCGGCGG-3′.

Generation of ALK doxycycline-inducible siRNA cell line. pcDNA6TR vector (Invitrogen) was stably transfected into IMR-32–derived cells using Lipofectamine 2000 transfection kit (Invitrogen) in accordance with the manufacturer’s instructions to generate IMR-32–derived cells expressing the Tet repressor. The IMR-32–pcDNA6TR vector cell line was then transfected with pTET-ALK2061. Double-stable Tet-cell line IMR-32-siALK2061 was selected for 3 wk with Zeocin (Invitrogen), and individual clones were obtained by limiting dilution. Expression of ALKsiRNA was induced by adding 1 μg/mL doxycycline in culture cell plates, and cell proliferation inhibition was tested by [3H]thymidine uptake.

Viability assay. NBL cells were seeded in flat-bottomed 96-well plates at 10,000 to 30,000 cells per well in a volume of 200 μL in supplemented...
medium, whereas lymphoma-derived cells were seeded in U-bottomed 96-well plates at 5,000 cells per well. For proliferation assay, 8 h before harvesting onto glass fiber filters, 1 μCi of [3H]thymidine was added to each well. Incorporation of [3H]thymidine was measured using a filter scintillation counter (1430 MicroBeta, Wallac). For viability assay, after 72 h of culture, 25 μL of 5 mg/L MTT (Sigma) were added directly to the cells followed by an additional 4 h incubation, and then 100 μL DMSO were added. The absorbance of individual wells was read at a wavelength of 550 nm.

**Cell cycle determination.** Ethanol-fixed cells were treated with DNase-free RNase (Boehringer Mannheim) and stained with 10 μg/mL propidium iodide (Sigma). DNA content and distribution of individual cells into different phases of the cell cycle was assessed by using FACScan flow cytometry and CellQuest software (Becton Dickinson). The ranges for G0-G1, S, G2-M, and sub-G1 phase cells were established based on the corresponding DNA content of histograms.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded tumor samples were classified according to the conventional International Neuroblastoma Pathology Classification system into undifferentiated, poorly differentiated, and differentiating NBs (21). Immunohistochemical detection of ALK was performed using the anti-ALK mouse antibody Ab-1 (Clone 5A4; NeoMarkers). Antibody retrieval was achieved by using the steamer heat method. ALK staining was visualized by polymer detection peroxidase assay (UltraVision LP, Lab Vision) and diaminobenzidine (Dako) as chromogen.

ALK staining was visualized by polymer detection peroxidase assay (UltraVision LP, Lab Vision) and diaminobenzidine (Dako) as chromogen. H&E sections were reviewed in conjunction with the immunohistochemical stains. The type of reactive cells and the pattern of reactivity (nuclear/cytoplasmic) were registered. Immunostaining was scored, taking into account the percentage of antibody-reactive neoplastic cells and the intensity of staining as follows: 1+, <10% reactive neoplastic cells; 2+, between 20% and 50% reactive neoplastic cells; 3+, >50% reactive neoplastic cells, heterogeneous with low/moderate-intensity staining; 3+, >50% reactive neoplastic cells, heterogeneous with moderate/high intensity of staining; 4+, >75% reactive neoplastic cells with strong intensity of staining.

**DNA sequencing.** A total of 30 ng of genomic DNA from 43 NB samples was PCR amplified for exons 21 to 27 of ALK gene (Supplementary Table S1), and products were purified with ExoSAP-IT (GE Healthcare). Bidirectional sequencing was performed by BigDye Terminator v1.1 kit (Applied Biosystems) on the ABI-Prism 3130 genetic analyzer (Applied Biosystems). Sequencing outputs were analyzed with Sequencher 4.8 software. All DNA sequence alterations were not present in the single-nucleotide polymorphism database.

**Multiplex ligation-dependent probe amplification.** Copy number variation of ALK locus was investigated by multiplex ligation-dependent probe amplification. A total of 20 ng of genomic DNA was amplified with primers specific for chromosome 2p23-24 (MRC Holland), including ALK and MYCN loci, according to the manufacturer’s instructions.

**Statistical analysis.** All values were calculated as means and 95% confidence intervals. The Student’s t test was used to test for differences in the means between two groups. Frequencies of patients with negative/low and high ALK immunoreactivity were compared across distinct classes of NBL stages using the Fisher exact test. Complete follow-up was available for all patients. Event-free survival time was calculated from the time of diagnosis to the time of progression or relapse. The life table curves were constructed using the Kaplan-Meier method, and the group comparisons were made using the log-rank test. Time to event for event-free survival was defined as the time from diagnosis until the time of first occurrence of relapse, progression, or death or until last contact if no event occurred. P values of <0.05 were considered statistically significant. P values of <0.01 were considered very statistically significant.

**Results**

**ALK kinase activation in human NBL cells.** We surveyed a panel of human NBL cell lines to identify cell lines with different levels of endogenous ALK protein (Fig. 1A and Supplementary S3B).

In UKF-NB3 and IMR-32 cell lines, the total amount of ALK receptor (200 kDa) was comparable with the level of NPM-ALK (80 kDa) present in the ALCL-derived cell line SU-DHL1 characterized by high level of expression (Fig. 1A). NB-INT1 cell line, characterized by low ALK expression, required a previous protein enrichment step through a selective ALK immunoprecipitation (Fig. 1B). No ALK was detectable in NB5 cells (Fig. 1A and B).

We investigated gene amplification events and missense mutations as possible causes of ALK overexpression in UKF-NB3 and IMR-32. The degree of the ALK locus amplification was negligible in both cell lines (Supplementary Fig. S1A; Supplementary Table S1). The mutational analysis revealed that UKF-NB3 carries the R1275Q mutation previously described in familial and sporadic NBL cases (13), whereas IMR-32 cell line carries a wild-type ALK gene as for NB-INT1 and NB5 cells (Supplementary Table S1).

ALK activation status was evaluated both by Western blot analysis and in vitro kinase assay (Fig. 1C). UKF-NB3 and IMR-32 cell lines showed constitutive ALK phosphorylation as opposed to NB-INT1 and NB5 cells, which tested virtually negative. Consistently, autokinase activity was detected in UKF-NB3 and IMR-32 cell lines but not in NB-INT1 and NB5 cells. Intriguingly, the 140 kDa isoform of ALK receptor was tyrosine phosphorylated and maintained autokinase activity despite the lack of its NH2 terminal oligomerization domain (22).

ALK activation has been recently correlated to point mutations in its TK domain (13). Interestingly, our findings indicate that constitutive ALK phosphorylation can also occur in NBL cells harboring a wild-type allele (IMR-32).

As PTN-induced ALK activation has been described (23, 24), we investigated whether the active state of ALK in IMR-32 could be ascribed to its putative ligand. Two secreted PTN isoforms (PTN15 and PTN18) have been known. It has been proposed that PTN15 activates ALK through a direct interaction, whereas PTN18 acts through the PTN18/ALK receptor protein tyrosine phosphatase βζ (RPTPβζ) signaling pathway (25, 26). Reverse transcription–PCR analysis of RPTPβζ expression revealed that RPTPβζ transcripts were only present in the ALK-negative NB5 cells, suggesting that the PTN18/RPTPβζ pathway is not involved in ALK activation in the NBL cell lines analyzed (Supplementary Fig. S2A). Both PTN18 and PTN18 were detected in culture supernatants from high-ALK expressing cells (UKF-NB3, IMR-32), low-ALK expressing cells (NB-INT1), and ALK-negative cells (NB5). In particular, the conditioned medium from IMR-32 showed a very low amount of PTN15 compared with PTN18 (Supplementary Fig. S2B).

**Wild-type ALK native receptor displays oncogenic potential in NBL cells.** Our results show that ALK constitutive phosphorylation occurs in the high-ALK expressing NBL cell line IMR-32 harboring a wild-type receptor. To reveal native ALK receptor tumorigenic potential, we investigated the effect of the down-modulation of ALK expression on cell growth and survival. A doxycycline-inducible siRNA strategy was used to generate IMR-32-siALK1061 stable transfectants. In our experimental setting, ALK expression was strongly down-regulated after 2 weeks of doxycycline treatment (Fig. 2A). The peak of ALK depletion correlated with a complete inhibition of cell growth, indicating that wild-type ALK plays a pivotal role in the control of NBL cell proliferation (Fig. 2B).

**ALK down-regulation results in inhibition of cell growth and induction of cell death.** To further assess the relevance of ALK activation and ALK-mediated signaling for NBL tumor growth and survival, we tested the effects of direct ALK kinase inhibition by the small ALK kinase inhibitors CEP-14083 and CEP-14513. CEP
compounds have been shown to potently inhibit the kinase activity of NPM-ALK in lymphoma cells (19).

As shown in Fig. 3A, CEP-14083 and CEP-14513 inhibited the phosphorylation of both ALK and ALK-associated signaling molecules (i.e., STAT3, AKT, and ERK1/2) in a dose-dependent manner in IMR-32 cells. We, thus, concluded that, in addition to rearranged ALK, CEP compounds efficiently target also the native ALK receptor.

At effective concentrations, CEP inhibitors did not affect the survival of NB5 cells that do not express ALK. Remarkably, NBL cells expressing low levels of native ALK (NB-INT1, ACN, SK-N-AS) evidenced a similar behavior to NB5 (Fig. 3B and Supplementary Fig. S3). CEP-14083 and CEP-14513 efficiently inhibited proliferation of NBL cell lines expressing high levels of ALK either with a mutated TK domain (i.e., UKF-NB3 and SK-N-SH) or carrying a wild-type TK domain (IMR-32, IMR-5, GILIN, HTLA, SK-N-BE2). Within 72 hours, ~50% reduction of proliferation rate was observed in the presence of 15 to 30 nmol/L CEP-14083 and 45 nmol/L CEP-14513. Notably, CEP-14083 inhibited more effectively the proliferation of NBL than ALCL cells included as positive control (Fig. 3B and Supplementary Fig. S3), whereas the inhibitory activity of CEP-14513 was equivalent in the NBL and lymphoma cell lines. Inhibition of ALK kinase activity induced by CEP inhibitors corresponded to a significant increase of cell death in UKF-NB3 and IMR-32 cells compared with NB-INT1 (Fig. 3C), suggesting a link between the level of expression of endogenous ALK protein and its oncogenic activity.

**ALK overexpression in poor prognosis NBL patients.** The observation that high level of ALK expression correlates with
constitutive kinase activation in NBL cell lines prompted to question the in vivo ALK expression in NBL patients. To address ALK potential clinical relevance, we investigated ALK expression in paraffin-embedded primary tumors from 82 NBL patients by immunohistochemistry. The patient cohort included 34 cases with localized tumors (stage 1-2), 19 cases with stage 3 NBLs, and 29 cases with metastatic disease at diagnosis (stage 4).

ALK immunoreactivity was detected in 75 cases (91.5%) and in each and every group of histologic differentiation (Fig. 4). All the seven tumor samples found to be negative for ALK were from stage 1-2 patients. In all positive samples, ALK localization displayed a membrane/cytoplasmic pattern. Staining was observed exclusively in tumor cells, whereas Schwannian stromal cells and lymphoid infiltrates tested consistently negative.

![Figure 4.](image)
ALK gene copy number alterations that could lead to the overexpression of ALK observed in poor prognosis NBLs, indicating a possible association between protein level of expression and tumor aggressiveness (P < 0.0001; Fig. 4A; Table 1). Indeed, the 3-year EFS probability for patients with absent/low (0, 1+) and high (3+, 4+) ALK positivity was 0.87 ± 9 and 0.32 ± 7, respectively (P < 0.001; Fig. 4B). ALK overexpression was equally observed in cases with and without MYCN amplification (data not shown).

Yet, we identified eight stage 1-2 patients showing high ALK expression and five stage 3-4 patients with low ALK expression. It is important to note that six of these stage 1-2 patients experienced a clinical progression and two died within 9 months from the relapse. Conversely, stage 3-4 patients with low ALK expression are long-term survivors: three had a continuous complete remission after 49, 51, and 54 months from diagnosis, and two had a very good partial remission after 38 and 40 months from diagnosis (Table 1).

ALK overexpression in NBL tumor samples did not associate with activating mutations. Recent data point to the involvement of mutations in the TK domain of ALK in NBL aggressiveness (13–17). To establish a link between missense mutations and the overexpression of ALK observed in poor prognosis NBLs, we sequenced the entire TK domain of 21 stage 1-2 and 22 stage 3-4 patients described in Fig. 4.

As expected, activating mutations were observed only in tumor samples displaying high immunoreactivity for ALK: one sample from stage 1-2 and three samples from stage 3-4 NBLs. However, the majority (18 of 22, 81.8%) of the tumor samples showing high ALK immunoreactivity was characterized by wild-type TK domain both in stage 1-2, as well as in stage 3-4 NBLs (Table 2). Interestingly, the patient with localized tumor displaying an elevated amount of mutated ALK (sample ID 2875) did not experience any disease progression, as opposed to relapsing stage 1-2 patients (sample ID 1227, 1363, 1458; 1600, 1668) characterized by high ALK immunoreactivity and a wild-type ALK TK domain.

As amplification of the genomic ALK locus has been proposed as mechanism of ALK up-regulation (27), we assayed the occurrence of ALK gene copy number alterations that could lead to the overexpression of the ALK protein observed in stage 1-2 tumors. No high-level focal amplification was observed (Table 2). One case showed unbalanced gain of a large genomic region at 2p including the ALK locus, and one additional case showed a chromosome 2 trisomy. The remaining tumor samples had a normal 2n copy of ALK, including stage 1-2 patients who died of the progressive disease (sample ID 1458, 1668).

Absence of germline promoter mutations affecting ALK expression. As the underlying genetic causes determining high levels of ALK expression remained to be determined in a considerable proportion of patients, we investigated whether missense mutations in the promoter sequence could account for ALK overexpression (28, 29). Genomic DNA from eight cell lines and 29 patients (ALK negative/low, 9 patients; ALK moderate, 6 patients; ALK high, 14 patients) expressing wild-type ALK was subjected to sequence analysis in the 2,564-bp region upstream of the translation start codon containing the putative promoter of ALK. No sequence variants were found.

Finally, the relation between ALK protein and transcript expression was further investigated within additional 18 tumor samples classified for their protein expression level quantified by immunohistochemistry. mRNA levels were assayed by Taqman real-time PCR. As shown in Fig. 4C, the abundance of ALK transcripts did not always correlate with the level of ALK immunoreactivity detected in primary NBLs. ALK mRNA expression was comparable in tumors showing low and high ALK immunoreactivity (P = 0.7323), whereas it was lower in samples with moderate ALK immunoreactivity (P = 0.0315 and P = 0.04, statistically significant).

### Discussion

Recent evidence indicates a pivotal role for the ALK TK receptor in both familial and sporadic NBL pathogenesis (13–17). A significant correlation between activating mutations in the ALK TK domain and poor clinical outcome has been shown in sporadic NBLs. Yet, mutations at the ALK locus have only been reported for a limited number of cases. Missense mutations have been found in 8.4%, whereas focal amplifications have been detected in 3.5% of the surveyed high-risk patients (13). The limited incidence of ALK mutations in sporadic NBLs suggests that ALK-independent oncogenic mechanisms are common in NBL pathogenesis. However, this observation does not exclude the possible involvement of the wild-type ALK protein in the pathogenic process. To gain more insight into the role of the wild-type receptor, we investigated ALK expression in both NBL cell lines and NBL patients. Data presented in this study indicate that the native ALK receptor can exert oncogenic activity in NBL cells, in addition to its mutated isoforms.

Previous studies indicated that NBL-derived cell lines harboring mutated ALK alleles exhibit constitutive ALK phosphorylation (13, 16). Interestingly, we observed aberrant phosphorylation in both UFK-NB3 cell line carrying the R1275Q mutation and IMR-32 cell line that carries a wild-type ALK. This finding indicates that mechanisms other than genetic alterations can lead to constitutive ALK activation. We did not observe ALK phosphorylation in NB-INT1 cells, which showed very low ALK expression compared with IMR-32 and UFK-NB3, suggesting a positive correlation between ALK abundance and its constitutive activation.

IMR-32 cells displayed a higher activity of the 200-kDa band compared with the UKF-NB3 cells in the kinase assay. These differences are in agreement with similar findings by Miyake and colleagues (30) showing differences in the extent of kinase activity of ALK in NBL cell lines with high level of expression of ALK. Interestingly, also the 140-kDa isoform results phosphorylated exclusively in high-ALK expressing cells. It is intriguing to

### Table 1. Overall expression of ALK in NBL patients

<table>
<thead>
<tr>
<th>INSS stage</th>
<th>ALK immunoreactivity</th>
<th>Negative/low*</th>
<th>Moderate</th>
<th>High*</th>
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<tr>
<td>1-2</td>
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<td>19</td>
<td>7</td>
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<td></td>
<td>5</td>
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<td>37</td>
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*P < 0.0001 at Fisher exact test comparing the frequency of patients with negative/low and high ALK immunoreactivity across distinct classes of NBL stages.

Six of eight patients had a clinical progression of the disease; two of six relapsed patients died of the disease.

Three complete remissions and two stable disease.
hypothesize that ALK isoform-140 could contribute to determining the ultimate oncogenic potential of ALK in addition to the ALK native receptor isoform-200. The presence of the ALK isoform-140 suggests distinctive biological traits associated with ALK receptor activation and/or molecular processing that differentiate NBLs from other ALK expressing tumors, such as lymphoma.

To investigate the functional relevance of the activation of the wild-type ALK receptor in IMR-32 cells, we down-regulated both its expression using an inducible RNA interference approach and its kinase activity by small kinase ALK inhibitors CEP-14083 and CEP-14513. Inhibition of ALK in IMR-32 cells specifically hampered cellular proliferation and induced cell death, strongly supporting a functional relevant oncogenic activity mediated by the wild-type ALK receptor. Noteworthy, the inhibitory effect of CEP compounds was observed in NBL cell lines showing ALK overexpression and activation, harboring either a mutated (UKF-NB3, SK-N-SH) or a wild-type (IMR-32, HTLA, GILIN, IMR-5, SK-N-BE2) receptor but not in NB-INT1, ACN, and SK-N-AS cells expressing low amounts of ALK, underlining the requirement of a critical threshold of protein expression to achieve oncogenic activity.

The molecular mechanisms responsible for ALK overexpression in NBL cells need further elucidations. We detected negligible ALK amplification and no missense mutations in TK domain and/or in the promoter region that could justify the aberrant level of ALK protein expression. In addition, our results point to limited, if any, involvement of PTN in determining ALK constitutive oncogenic activation. Indeed, high-ALK expressing IMR-32 cells displayed ALK constitutive activation combined with low level of secreted PTN15. The analysis of transcript abundance in a restricted number of patients indicates that the accumulation of the ALK protein can occur independently from its transcriptional up-regulation. This observation outlines the involvement of posttranscriptional modifications, possibly interfering with protein stability. Indeed, a prolonged persistence of activated ALK on the cell surface due to reduced rate of internalization and the association of ALK with heat shock proteins have been described (31, 32).

The tenet that protein overexpression actively contributes to ALK-mediated oncogenic activity is further supported by the data obtained in NBL patients. Our immunohistochemical analyses conducted on a large cohort of patients showed major differences in ALK expression between stage 1-2 and stage 3-4 NBL patients. In general, localized and poorly aggressive tumors (stage 1-2) had a low level of ALK expression. In contrast, more aggressive and metastatic tumors (stage 3-4) exhibited ALK overexpression. However, exceptions to this trend were observed. Six of eight stage 1-2 patients whose tumors showed ALK overexpression had an unfavorable clinical outcome, whereas all five stage 3-4 patients with low level ALK expressing tumors had a favorable outcome. These observations point to ALK oncogenic relevance in NBL cells and corroborate ALK overexpression as an indicator of poor clinical outcome.

Previous studies showed no significant link between ALK expression and prognosis (27, 33). This discrepancy may be ascribed to differences in the size of the patient cohort (82 versus 16 and 22 patients), in the criterion of patient stratification (disease stage), and in the methodology used to assess ALK protein expression. In this study, we used an immunohistochemical approach to evaluate ALK expression in a large cohort of patients with NBL, and we found a strong association between ALK overexpression and clinical outcome. The results are in line with previous studies showing that ALK overexpression is a poor prognostic factor for NBL patients. These findings further support the potential role of ALK in the pathogenesis and progression of NBL and suggest that targeting ALK may be a promising therapeutic strategy for NBL patients.
stage versus MYCN amplification), and in the experimental protocol (immunohistochemistry versus Western blot analysis).

In agreement with the results showing that only 8% to 10% of tumors from high-risk patients had single-base substitutions consistent with activating mutations (13), we observed low frequency of ALK missense mutations in the NBL patients with high levels of ALK expression. The majority of advanced/metastatic patients (stage 3–4) were characterized by the expression of a wild-type ALK. Intriguingly, also the stage 1-2 patients who died of disease progression had wild-type ALK, further emphasizing both the oncogenic potential and the clinical implications of the ALK native receptor.

Despite progress in treatment modalities, children with metastatic NBL (stage 4) at diagnosis still have a 5-year overall survival of <40% (34–36). The possibility to develop alternative therapeutic strategies based on ALK molecular targeting for advanced/metastatic NBLs is an attractive opportunity. CEP-14083 and CEP-14513 exert potent cytotoxic effect on high-ALK expressing wild-type IMR-32 cells, an attractive opportunity. CEP-14083 and CEP-14513 exert potent cytotoxic effect in a broad cohort of high-risk NBL patients expressing high protein levels of either mutated or wild-type ALK receptor. This group of patients could be represented by relapsed stage 1-2 patients with progressive disease and the majority of advanced/metastatic stage 3-4 patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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