High ALK Receptor Tyrosine Kinase Expression Supersedes ALK Mutation as a Determining Factor of an Unfavorable Phenotype in Primary Neuroblastoma

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Abstract

Purpose: Genomic alterations of the anaplastic lymphoma kinase (ALK) gene have been postulated to contribute to neuroblastoma pathogenesis. This study aimed to determine the interrelation of ALK mutations, ALK expression levels, and clinical phenotype in primary neuroblastomas.

Experimental Design: The genomic ALK status and global gene expression patterns were examined in 263 primary neuroblastomas. Allele-specific ALK expression was determined by cDNA cloning and sequencing. Associations of genomic ALK alterations and ALK expression levels with clinical phenotypes and transcriptomic profiles were compared.

Results: Nonsynonymous point mutations of ALK were detected in 21 of 263 neuroblastomas (8%). Tumors with ALK mutations exhibited about 2-fold elevated median ALK mRNA levels in comparison with tumors with wild-type (WT) ALK. Unexpectedly, the WT allele was preferentially expressed in 12 of 21 mutated tumors. Whereas survival of patients with ALK mutated tumors was significantly worse as compared with the entire cohort of WT ALK patients, it was similarly poor in patients with WT ALK tumors in which ALK expression was as high as in ALK mutated neuroblastomas. Global gene expression patterns of tumors with ALK mutations or with high-level WT ALK expression were highly similar, and suggested that ALK may be involved in cellular proliferation in primary neuroblastoma.

Conclusions: Primary neuroblastomas with mutated ALK exhibit high ALK expression levels and strongly resemble neuroblastomas with elevated WT ALK expression levels in both their clinical and molecular phenotypes. These data suggest that high levels of mutated and WT ALK mediate similar molecular functions that may contribute to a malignant phenotype in primary neuroblastoma. Clin Cancer Res; 17(15); 1–11. ©2011 AACR.

Introduction

Neuroblastoma is a pediatric tumor of the developing sympathetic nervous system, accounting for about 8% of childhood cancers (1). The biological and clinical behavior of neuroblastoma is remarkably heterogeneous. Fatal progression of the disease occurs frequently in children with disseminated tumors, whereas spontaneous regression or differentiation into benign ganglioneuroma is regularly observed in infants. The genetic etiology and molecular mechanisms of the different neuroblastoma subtypes have remained enigmatic. Yet, it has been shown in recent years that aggressive neuroblastomas and those with the capacity to regress spontaneously differ in a number of molecular characteristics (2), suggesting that they represent different subtypes of the disease (3).

In 2008, it has been reported that potentially activating mutations in the anaplastic lymphoma kinase (ALK) gene may account for most cases of familiar neuroblastoma and a fraction of sporadic neuroblastomas (4–7). ALK is a receptor tyrosine kinase involved in neuronal differentiation (8, 9), and pleiotrophin (PIN) and midkine (MDK) have been suggested to act as ligands for ALK in humans (10). Inappropriate ALK expression because of
Activating mutations of the anaplastic lymphoma kinase (ALK) gene have recently been identified in 8% of primary neuroblastoma. This finding has focused intense interest in the development of innovative treatment strategies for high-risk neuroblastoma patients by using inhibitors directed toward activated ALK, and first clinical trials with ALK inhibitory drugs have been initiated. In this study, we show that primary neuroblastomas with ALK mutations invariably exhibit elevated ALK expression levels. We furthermore show that tumors with ALK mutations resemble neuroblastomas with high-level wild-type (WT) ALK expression in their global gene expression patterns, and that patients of these 2 subtypes are characterized by similar prognostic marker profiles and unfavorable clinical courses. These data indicate that high ALK expression levels mediate similar molecular functions in primary neuroblastoma with mutated or WT ALK, suggesting that ALK inhibitory drugs should be evaluated in second-line treatment strategies of all high-risk neuroblastoma patients with elevated ALK expression.

Chromosomal translocations has been observed in several types of cancer, and constitutive ALK activity has been shown to induce malignant transformation both in vitro and in vivo (11), thus representing a potential molecular target for selective tyrosine kinase inhibitors (11, 12). In neuroblastoma, somatically acquired genomic amplification and mutation of ALK occur in 1% to 4% and 6% to 8% of primary tumors, respectively (4–7, 13). In addition, it was shown in cell line models that ALK mutations are likely oncogenic events that confer malignant properties to the cells. The association of ALK mutations with the clinical phenotype of the disease has remained contradictory. Some authors suggested an association of ALK mutations with an aggressive phenotype (4, 5), whereas others described ALK mutations in the entire spectrum of sporadic (6, 13) and familiar neuroblastoma (7). In addition to genomic alterations of ALK, elevated ALK expression levels have previously been reported for neuroblastoma (14, 15). However, the interrelation of ALK mutations, ALK expression levels, and clinical phenotype has remained elusive.

In this article, we determined the contribution of genomic ALK alterations and ALK expression to the clinical and molecular phenotypes of primary neuroblastomas. We assessed the frequency of genomic ALK alterations in a large and representative neuroblastoma cohort, evaluated the relationship of ALK mutations and ALK expression levels, and investigated the association of genomic and transcriptomic ALK status with global gene expression patterns of the tumors as well as prognostic markers and clinical outcome of the patients.

**Material and Methods**

**Patients**

The study comprised primary neuroblastoma samples from 263 patients (Supplementary Table S1). All patients were enrolled in the German Neuroblastoma trials with informed consent. The age of patients at diagnosis ranged from 0 to 295 months (median age, 15 months). Median follow-up for patients without fatal events was 84 months. Five-year event-free survival (EFS) of the total cohort was 0.69 ± 0.03 and 5-year overall survival (OS) 0.80 ± 0.03. Stage was classified according to the International Neuroblastoma Staging System (INSS): stage 1, n = 68; stage 2, n = 43; stage 3, n = 41; stage 4, n = 80; stage 4S, n = 31. The distribution of age and stage in this cohort was representative of the German NB97 trial. Chromosomal alterations were determined by FISH and defined according to the guidelines of the European Neuroblastoma Quality Assessment Group (16). MYCN was amplified in 45 (17.1%) and nonamplified in 215 cases (81.7%; missing MYCN status, n = 3). Loss of chromosome 1p or 11q was observed in 61 (23.2%) and 59 tumors (22.4%), respectively, whereas 194 and 192 tumors had a normal 1p (73.8%) and a normal 11q status (73.0%), respectively (noninformative cases for 1p and 11q, n = 8 and n = 12, respectively). A favorable and an unfavorable histology according to the Shimada system (17) were diagnosed in 148 (56.3%) and 88 tumors (33.5%), respectively (missing information, n = 27). Response to treatment was defined according to the revised criteria of the International Neuroblastoma Response Criteria (INRC).

**Sample preparation**

Tumor samples were checked by a pathologist for at least 60% tumor content. DNA was isolated from approximately 20 mg of snap-frozen tissue obtained before cytotoxic treatment by using the Puregene Blood Core Kit B (Qiagen). Total RNA was isolated from 30 to 60 mg of the same snap-frozen tumors by using the FastPrep FP120 Cell Disruptor (Qbiogene-Inc.) and the TRIZol reagent (Invitrogen). RNA integrity was assessed by using the 2100 Bioanalyzer (Agilent Technologies) considering only samples with an RNA Integrity Number of at least 7.5.

**Sequencing of DNA and cDNA**

For sequencing of the ALK gene, exons encoding the kinase domain (i.e., exons 20, 21–22, 23, 24, and 25) were PCR amplified by using the primers generated by De Brouwer and colleagues for exons 21–22 (13) and by Chen and colleagues for all other exons (ref. 4; Supplementary Table S2). PCR conditions were as follows: 95°C for 3 minutes, 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. PCR reactions were conducted in 20 μl with illustra Taq polymerase and PCR buffer according to the manufacturer’s protocol (GE Healthcare). Purification and Sanger sequencing of the PCR products...
was done by the Eurofins MWG Operon Sequencing Service (Eurofins MWG Operon).

Allele-specific expression was determined by amplification of transcript fragments encompassing the respective point mutations by using reverse transcriptase PCR (RT-PCR), followed by cloning and sequencing. PCR fragments were cloned into plasmid vectors by using the TOPO TA Cloning Kit (Invitrogen), and sequenced by using the BigDye Terminator Sequencing Kit (Applied Biosystems). Primer sequences for amplification are available from the authors upon request.

DNA copy number quantification

ALK copy number status was determined by using real-time quantitative PCR, with TNFRSF17 and SDC4 as normalizing reference genes and normal human genomic DNA (Roche Diagnostics) as calibrator sample (18). DNA from NB1, a neuroblastoma cell line with known ALK amplification, was used as a positive control. Primer sequences can be found in RprimerDB (http://medgen.ugent.be/rprimerdb; ref. 19). Five microliters of amplification mixtures contained 2 to 12 ng of DNA, 1 μM SYBR Green I Mastermix (Eurogentec S.A.) and 250 nmol/L of each primer. PCR reactions were conducted in a 384-well plate on a LightCycler 480 (Roche). The cycling conditions comprised 10 minutes polymerase activation at 95°C and 45 cycles of 15 seconds at 95°C and 30 seconds at 60°C, followed by a dissociation curve analysis from 60°C to 95°C to verify amplification specificity. The haploid ALK copy number for each sample was calculated by using the real-time PCR data analysis software qbasePLOD (http://www.qbaseplus.com; ref. 20). Haploid copy numbers more than 4 were considered as amplification.

Gene expression analyses

Gene expression profiles were generated as dye-flipped dual-color duplicates by using customized 11K oligonucleotide microarrays as described (21). The ALK gene was represented by probe A2_23_P324304. As a reference, pooled total RNA from 100 primary neuroblastomas was used. Data preprocessing, quality control analyses, and normalization were done as described. All raw and normalized microarray data are available at the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress; Accession: E-TABM-38, E-MTAB-161).

For real-time quantitative RT-PCR (qRT-PCR), single-stranded cDNA was generated from total RNA by using the Superscript II First-Strand Synthesis System (Invitrogen). qRT-PCR was done on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with SYBR Green chemistry by using the standard curve method. To prevent amplification of contaminating genomic DNA, primer sequences were selected allowing intron-spanning amplification (Supplementary Table S2). PCR reactions were run in duplicates for each sample and as triplicates for determination of standard curves. For normalization, the expression level of the target gene was divided by the geometric mean of expression levels of the control genes HPRT1 and SDHA as described (22).

Western blot analyses

To analyze expression of proteins, tumor tissue or cell lines were lysed on ice for 30 minutes in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.1% SDS, 1% Triton X-100, and 1% NP-40) supplemented with complete Protease Inhibitor Cocktail (Roche) and Phos-Stop (Roche). After centrifugation of lysates, 20 μg of protein were separated by SDS-PAGE with 8% or 4% to 12% Tris-glycine gels and transferred to nitrocellulose membranes by tank blotting or semi-dry blotting. The membranes were blocked with either 5% dry milk powder or 5% bovine serum albumin in 0.05% Tween-20/PBS before incubation with monoclonal primary antibodies (rabbit anti-human ALK, dilution 1:500; rabbit anti-human phospho-(γ1604)-ALK, dilution 1:500; rabbit anti-human phospho-STAT3, dilution 1:2,000, rabbit anti-human phospho-AKT, dilution 1:500, rabbit anti-human phospho-ERK1/2, dilution 1:1,000; all Cell Signaling Technology) and horseradish peroxidase-labeled secondary goat anti-rabbit antibody (dilution 1:1,000; Dako). The antigen–antibody complex was detected with the ECL Prime Western Blotting Detection Kit (GE Healthcare).

Cell lines and cell culture

The human neuroblastoma cell line SK-N-AS, which has been described to express low levels of wild-type (WT) ALK (7), was grown as monolayer in RPMI 1640 supplemented with 10% fetal calf serum, l-glutamine, and antibiotics. The cell line was authenticated by short tandem repeat genotyping (DSMZ). SK-N-AS cells were transfected by electroporation with pcDNA6/TR (Invitrogen) harboring the gene coding for the tetracyclin repressor, and single cell clones were raised by limited dilution and antibiotic selection (blasticidine). The cDNA encoding ALK (F1174L) was synthesized (Genescript), with HindIII and NotI restriction sites flanking the kinase domain without altering the protein sequence. In addition, the cDNA was flanked by attL sites for subsequent Gateway Cloning (Invitrogen). Alternative kinase cassettes, representing the WT sequence or a kinase domain harboring the R1275Q mutation, were synthesized and introduced by cloning via the HindIII and NotI restriction sites. WT ALK as well as ALK(F1174L) or ALK(R1275Q) cDNA were subcloned into pT-REx-DEST30 (Invitrogen), a vector for Tet-conditional expression, by a Gateway Cloning Reaction (Invitrogen). SK-N-AS-TR were transfected by electroporation with pT-REx-DEST30-wtALK, pT-REx-DEST30-ALK(F1174L), pT-REx-DEST30-ALK(R1275Q), or pT-REx-DEST30-GFP. Single cell clones were raised by selection with antibiotics G418 and blasticidine and by limited dilution. For conditional ALK or GFP expression, cells were treated with tetracyclin (1 μg/mL) for 24 hours before being lysed on ice in RIPA buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.1% SDS, 1% Triton X-100, and 1% NP-40) supplemented with complete Protease Inhibitor Cocktail (Roche) and Phos-Stop (Roche).
Statistical analyses

Associations of ALK mutations or ALK expression levels with prognostic markers were determined by U test or \( \chi^2 \) test were appropriate. Allele-specific expression in tumors with mutated ALK was assessed by Wilcoxon test. Kaplan–Meier estimates for EFS and OS were calculated and compared by log-rank test. Recurrence, progression, and death from disease were considered as events. For multivariate analysis, Cox proportional hazards regression model based on EFS and OS was applied. The factors ALK mutation (mutated vs. WT) and ALK expression (continuous) were fitted by stepwise-backward selection. The likelihood ratio test \( P \) value for inclusion was less than 0.05 and for exclusion more than 0.1.

Principal component analysis (PCA) and ANOVA of gene expression data was done by using Rosetta Resolver Software. To test for global gene expression differences between ALK-WT\(^{hgh}\) tumors, ALK-WT\(^{low}\) tumors, and tumors with ALK mutation, differences between group centroids were calculated as described (23, 24). In brief, normalized intensity values from all probes were averaged in each group to yield group centroids. The Euclidean distance between these vectors was calculated and compared with centroid distances obtained from 1,000 random permutations of the group labels. The number of differentially expressed genes was calculated with either pairwise \( t \) tests or by 1-way ANOVA over all 3 groups; for this, only genes (probes) in a list with a false discovery rate of less than 0.05 were considered, after correction for multiple testing by the Benjamini–Hochberg method. All calculations except ANOVA were carried out in R version 2.9.0 (http://www.r-project.org) with extension package limma (version 2.18.0).

To identify gene expression patterns associated with either mutated ALK or WT ALK in primary neuroblastoma, the correlation of ALK expression levels with all genes represented on the microarray was examined. Thresholds for high and low correlation were defined by using quantile–quantile plots of all correlation coefficients against a theoretical normal distribution. According to this procedure, thresholds for genes positively or negatively correlated with ALK expression in ALK-mutated tumors and in WT ALK tumors were defined as \( r \geq 0.5 \) or \( r \leq -0.5 \), and \( r \geq 0.25 \) or \( r \leq -0.25 \), respectively. Lists of genes positively or negatively correlated with ALK were subjected to overrepresentation analysis of Gene Ontology (GO) categories by GOstats (25). Overrepresentation analysis of Gene Ontology categories was determined by Fisher’s exact test \( (P < 0.05 \) after Benjamini–Hochberg correction for multiple testing) by using R version 2.11.0 with extension packages GOstats (v. 2.14.0) and GO.db (version 2.4.1). Correction for multiple testing was done by package multtest (v. 2.4.0).

Results

Association of genomic alterations of ALK with clinical variables in primary neuroblastoma

The prevalence of genomic ALK mutations was determined in a representative cohort of 263 primary neuroblastomas. Genomic amplification of WT ALK was observed in 2 of 263 tumors. Point mutations were detected in 23 of 263 cases, 21 of which were heterozygous nonsynonymous mutations corresponding to an overall prevalence of 8.0% (Table 1). Nine different nucleotide exchange mutations were observed, including 3 which had not been described previously. The most common mutations were F1174L \( (n = 5) \) and R1275Q \( (n = 8) \) as reported previously (4–7, 13).

Next, the association of genomic ALK alterations with patient clinical courses and prognostic markers was assessed. Both tumors with ALK amplification showed MYCN amplification and loss of chromosome 1p (Table 1), in line with previous observations (4–7). One of these patients succumbed to disease, whereas the other is currently in complete remission. Nonsynonymous ALK mutation \( (ALK_{\text{mutated}}) \) was not associated with stage 4 disease \( (P = 0.805) \), the genomic status of chromosome 1p \( (P = 0.283) \), or the Shimada classification \( (P = 0.195) \). Patients having ALK\(^{\text{mutated}}\) tumors tended to be older at diagnosis than those without mutations \( (P = 0.064) \). Although not statistically significant \( (P = 0.132) \), the prevalence of ALK mutations was twice as high in MYCN amplified tumors \( (n = 6, 14\%) \), as in tumors without MYCN amplification \( (n = 15, 7\%) \). Only a single ALK\(^{\text{mutated}}\) tumor showed loss of 11q, which is a significant inverse correlation of these genetic variables \( (P = 0.032) \). Whereas EFS and OS were significantly worse in ALK\(^{\text{mutated}}\) patients than in patients with WT ALK (Fig. 1), there was no significant difference in the clinical courses of patients with different types of ALK mutations (Supplementary Fig. S1).

Genomic ALK alterations are associated with elevated ALK expression levels

To examine the influence of genomic ALK alterations on ALK transcript levels, relative ALK mRNA expression levels were determined in all 263 tumors by using microarrays (21, 26), and validated in 81 samples by qRT-PCR (Supplementary Fig. S2). ALK\(^{\text{mutated}}\) neuroblastomas exhibited significantly higher ALK transcript levels than tumors with WT ALK \( (P < 0.001) \). The median expression in tumors with ALK amplification and ALK mutation was about 20-fold and 2-fold higher than in WT ALK tumors, respectively (Fig. 2A, Supplementary Table S3). In contrast, ALK mRNA levels did not differ between subgroups with different ALK mutations (F1174L vs. R1275Q vs. other mutation, \( P = 0.345 \); Fig. 2A). In addition, high ALK mRNA expression correlated well with strong ALK protein expression in most primary tumors as determined by Western blot analysis (Supplementary Fig. S3).

Unexpectedly, sequencing of cloned ALK transcript fragments from ALK\(^{\text{mutated}}\) neuroblastomas revealed a significant preponderance of WT allele expression (WT vs. mutated allele expression in the entire ALK\(^{\text{mutated}}\) cohort, \( P = 0.034) \). In particular, the WT allele was preferentially expressed \( (\geq 2\text{-fold higher expression than the mutated allele}) \) in all tumors with the F1174L mutation (Table 1). A more heterogeneous pattern of ALK allele expression was
## Table 1. Summary of patient and tumor characteristics of neuroblastomas with genomic alterations of ALK

<table>
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<th>Allele expression (WT/mut.)</th>
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<th>Germline</th>
<th>Stage</th>
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<td>25</td>
<td>3824 G&gt;A</td>
<td>5/9</td>
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<td>4</td>
<td>1,841</td>
<td>no</td>
<td>del1p</td>
<td>no</td>
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<td></td>
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<td>710</td>
<td>amp</td>
<td>imb1p</td>
<td>n.d.</td>
<td>CR</td>
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</tbody>
</table>

NOTE: Allele expression indicates the numbers of WT and mutated (mut.) clones. Age at diagnosis is given in days. Abbreviations: amp, amplification; CR, complete remission; del, deletion; DOD, death of disease; imb, imbalance; n.d., not done; vgPR, very good partial remission. *Homozygous mutation.
detected in the subgroup of neuroblastomas with R1275Q or other mutations. Allele-specific expression was not associated with any particular clinical parameter. Together, these results suggest that either low levels of mutated ALK are sufficient to contribute to the tumor phenotype, or that ALK expression levels, rather than the mutation status, represent a determining factor for neuroblastoma tumor behavior.

High ALK expression correlates with an adverse neuroblastoma phenotype independent of the genomic ALK status

On the basis of these findings, we hypothesized that high ALK expression levels should correlate with an adverse phenotype of neuroblastoma, irrespective of the genomic ALK status. Indeed, ALK transcript levels were significantly higher in subgroups characterized by adverse prognostic markers than in favorable subgroups in the WT ALK cohort (stage 4 vs. stages 1–3 and 4S, age >18 months vs. <18 months, 1p-loss vs. 1p normal, unfavorable vs. favorable Shimada classification, P < 0.001 each; amplified vs. non-amplified MYCN, P = 0.011; 11q-loss vs. 11q normal, P = 0.010). Moreover, ALK expression was significantly associated with poor patient survival in a univariate Cox regression model by using ALK mRNA levels as a continuous variable (EFS, P = 0.005, HR = 3.27, 95% CI: 1.43–7.47; OS, P = 0.003, HR = 5.13, CI: 1.72–15.30). Accordingly, WT ALK expression correlated inversely with patient survival in subgroups created by subdivision according to percentiles of ALK transcript levels (<25th percentile, ALK-WT<25>; 25th and <50th percentile, ALK-WT<50>; 50th and <75th percentile, ALK-WT<75>; 75th percentile, ALK-WT75); n = 60 each; Fig. 2B and 3). To determine whether ALK expression and ALK mutation status are independent prognostic markers, the 2 variables ALK expression (continuous) and genomic ALK status (mutated vs. WT) were analyzed in multivariate Cox regression models. Here, ALK expression, but not ALK mutation, was independently associated with patient outcome (Supplementary Table S4). In addition, the prognostic value of ALK mutation status and ALK expression levels was evaluated in the context of the current German risk estimation system, which utilizes the variables stage, age, MYCN status, and 1p status. In these multivariate analyses, stage, age, and 1p status were independent prognostic variables in the models based on EFS, whereas inhomogeneous results were observed in the forward and backward models on the basis of the OS (probably because of too few events to assess 6 prognostic markers in this cohort; data not shown).

Moreover, the distributions of prognostic markers and clinical courses of patients with ALK-WT<sup>high</sup> and ALK<sup>mutated</sup> neuroblastomas were compared, because these cohorts showed similar ALK expression levels (Fig. 2B). The subgroups did not differ in age (P = 0.624), stage (P = 0.565), MYCN status (P = 1.0), and classification according to our gene expression-based classifier (ref. 21; P = 0.519) or 1p-status (P = 0.604). A significant difference was observed only in the prevalence of 11q-loss (35.1% and 4.8% of the ALK-WT<sup>high</sup> and ALK<sup>mutated</sup> cases, respectively; P = 0.008). In addition, clinical courses of patients with ALK<sup>mutated</sup> and ALK-WT<sup>high</sup> tumors were similar in both the entire cohort and after excluding MYCN amplified neuroblastomas (Fig. 3, Supplementary Fig. S4). Taken together, these data indicate that ALK<sup>mutated</sup> and ALK-WT<sup>high</sup> neuroblastomas exhibit highly similar clinical phenotypes.

ALK<sup>mutated</sup> and ALK-WT<sup>high</sup> neuroblastomas show similar molecular phenotypes

To investigate transcriptomic characteristics of tumors with and without ALK mutations, we carried out PCA by using global gene expression information of all samples. Here, ALK-WT<sup>low</sup> and ALK-WT<sup>high</sup> tumors formed separate subgroups, whereas ALK<sup>mutated</sup> tumors showed a similar distribution to ALK-WT<sup>high</sup> tumors in both the entire cohort and after excluding MYCN amplified neuroblastomas (Fig. 4, Supplementary Fig. S5). Of note, those 4 ALK<sup>mutated</sup> neuroblastomas showing lower ALK expression levels than ALK-WT<sup>high</sup> tumors were more closely associated with the ALK-WT<sup>low</sup> subgroup (Supplementary Fig. S5). To validate these observations, the ALK-WT<sup>low</sup>, ALK-WT<sup>high</sup>, and ALK<sup>mutated</sup> subgroups were compared in a pairwise manner by using analysis of centroid distances, ANOVA, and t-test statistics (Table 2). Both ALK<sup>mutated</sup> and ALK-WT<sup>high</sup> tumors differed significantly from ALK-WT<sup>low</sup> tumors.
in F1174L mutants upon ALK induction, whereas weak to modest ALK phosphorylation occurred in WT clones and R1275Q mutants, respectively (Supplementary Fig. S6). In addition, ALK induction resulted in a discrete to moderate increase of p-STAT3 and p-ERK1/2 in clones FL8 and FL1 harboring the F1174L mutation, whereas it had no effect on target phosphorylation in all other clones. In primary tumors, the levels of phosphorylated ALK targets were highly heterogeneous, and neither correlated with genomic alterations of ALK nor ALK expression (Supplementary Fig. S3). Together, these data suggest that activation of known ALK targets may be limited and significantly modulated by other factors in neuroblastoma in vivo. In addition, analysis of transcript levels of the putative ALK ligands PTN and MDK revealed a discrete correlation of MDK with ALK expression and slightly elevated MDK expression levels in ALK-WT<sup>high</sup> tumors (Supplementary Fig. S7). The significance of this finding for neuroblastoma pathogenesis, however, remains uncertain.

To identify gene expression patterns associated with either mutated or WT ALK in primary neuroblastoma, the correlation of ALK expression with all genes represented on the microarray was examined in these subgroups. In total, 1,733 genes were positively or negatively correlated with ALK expression in ALK<sup>mutated</sup> tumors (Supplementary Table S5A), whereas 823 genes were correlated with ALK expression in WT ALK tumors (Supplementary Table S5B). Of note, the overlap of genes correlated with ALK expression in WT ALK and ALK<sup>mutated</sup> tumors was exceptionally high (57%), and the direction of correlation was concordant (i.e., either positive or negative) for all these genes. Overrepresentation analysis of Gene Ontology categories revealed 59 GO categories significantly enriched among the genes positively correlated with ALK expression in tumors with WT ALK (Supplementary Table S6). These categories were related to cell-cycle regulation, DNA replication, cell division, DNA repair, and protein ubiquitinylation. In contrast, no GO category was significantly enriched for genes negatively correlated with ALK expression. In ALK<sup>mutated</sup> tumors, 21 and 14 significantly enriched GO categories were found among the genes positively and negatively correlated with ALK expression, respectively. Notably, 18 of 21 (86%) of the categories enriched for positively correlated genes were also found among the 59 categories identified in WT ALK tumors. These findings support the hypothesis that high expression levels of WT ALK and high expression levels of mutated ALK have a similar impact on the molecular phenotype of primary neuroblastoma which may be related to cellular proliferation.

### Discussion

Activating mutations and amplification of the ALK gene have been described to contribute to neuroblastoma pathogenesis (4–7). The interrelation of ALK mutations, ALK expression, and clinical phenotype, however, has remained ambiguous so far. In this study, heterozygous missense mutations were detected in 21 of 263 tumors (8%), which
is in line with frequencies reported previously (4–7, 13). In contrast to results of a recent meta-analysis (13), ALK mutations were associated with a worse EFS and OS in this study (Fig. 1). This discrepancy might be attributed to a different composition of the cohort in the study of De Brouwer, which contained substantially more high-risk patients. It must be stressed, though, that ALK mutations did not show a clear correlation with established prognostic markers in both studies, and that mutations were detected in patients throughout the whole spectrum of the disease in the present survey, including spontaneously regressing stage 4S patients (NB068 and NB052).

The relationship of ALK mutation and ALK expression has been investigated in neuroblastoma cell lines (5, 7) and in small patient cohorts (27, 28) so far. In this study, we show that mutations in the ALK tyrosine kinase domain are invariably associated with elevated ALK transcript levels in primary neuroblastoma. Although the molecular mechanism of this finding remains unclear, it appears reasonable to assume that mutated ALK promotes its own expression via a feed-forward regulatory loop. A similar mechanism has been described for the ErbB2 receptor tyrosine kinase in breast cancer, which actively induces its own overexpression (29). Yet, the observation of elevated ALK expression levels in a substantial fraction of WT ALK tumors may suggest additional mechanisms promoting ALK expression. Alternatively, somatic mutations may be preferentially acquired in ALK loci showing high transcriptional activity.
Together, results from our study and others substantiate that ALK expression is regularly elevated in ALK\textsuperscript{mutated} primary neuroblastoma (27, 28), however, the molecular mechanisms underlying this phenomenon are still to be elucidated.

The association of WT ALK expression levels with the clinical phenotypes of neuroblastoma has remained uncertain to date (13–15, 28, 30). Here, we show that elevated ALK mRNA levels are associated with an unfavorable neuroblastoma phenotype independent of the genomic ALK status, indicating a role of elevated ALK expression in the development of aggressive neuroblastoma. Patients with ALK-WT\textsuperscript{high} tumors in which ALK expression was as high as in ALK\textsuperscript{mutated} tumors had a similar poor outcome as those with ALK mutations, indicating that both patient subgroups may benefit from ALK-targeted therapies. However, analogous to the broad range of clinical phenotypes of patients with mutated ALK, we observed both patients with fatal outcome in the ALK-WT\textsuperscript{low} subgroup and patients with spontaneously regressing tumors (n = 4) in the ALK-WT\textsuperscript{high} subgroup. Accordingly, neither ALK mutation status nor ALK expression turned out to be independent prognostic markers in multivariate analyses considering variables of the current German risk estimation system. In light of the results of this study and others (13) as well as the high prognostic accuracy of recently published complex DNA- or RNA-based prognostic classifiers (21, 26, 31–33), it remains questionable whether ALK mutation status or expression level will be useful for risk estimation of neuroblastoma patients in the future.

Comparison of ALK\textsuperscript{mutated} and ALK-WT\textsuperscript{high} neuroblastomas revealed highly similar prognostic marker profiles and clinical outcomes in these patients. In line with this observation, global gene expression patterns of these 2 subgroups were also alike. In addition, more than half the genes that correlated with ALK expression in WT ALK tumors were also associated with ALK expression in mutated tumors. Gene Ontology categories of transcripts positively correlated with ALK expression were enriched for functions related to cellular proliferation in both ALK\textsuperscript{mutated} and WT ALK tumors. Together, these data strongly suggest that high expression levels of WT ALK and mutated ALK have similar effects on the neuroblastoma biological phenotype that may be related to tumor growth.

Unexpectedly, we observed that levels of phosphorylated ALK targets were highly heterogeneous in primary tumors and did neither correlate with ALK expression nor with the ALK mutation status of the tumor, indicating substantial influences of other pathways on the activation of these proteins in primary neuroblastomas. Moreover, the WT ALK allele was found to be preferentially expressed in many primary ALK\textsuperscript{mutated} neuroblastomas. It appears unlikely that this finding was because of contaminating ALK transcripts from stromal cells, because only samples with a tumor content of more than 60% were analyzed, and nonmalignant cells in neuroblastomas have been shown to be ALK-negative (28). Allelic variations in gene expression have been shown to contribute to human variability and disease including cancer (34–37). To our knowledge, however, this is the first article on preferential WT allele expression of an oncogene harboring putatively activating mutations. Although the underlying processes of allele-specific expression are largely unexplored, a cis effect of the ALK mutation such as reduced RNA stability of the affected ALK transcript might contribute to this unexpected observation (37). Besides the molecular mechanisms of allele-specific expression, it remains to be determined whether the mutated ALK protein, the enhanced ALK expression, or both confer the functions of this tyrosine kinase in ALK\textsuperscript{mutated} neuroblastoma.

Taken together, this study shows that primary neuroblastomas with mutated ALK invariably exhibit high ALK expression levels with preferential expression of the WT allele in some cases. ALK\textsuperscript{mutated} tumors strongly resemble ALK-WT\textsuperscript{high} neuroblastomas in both their clinical phenotypes and their transcriptomic profiles. The unfavorable patient outcome of these subgroups and the ALK-associated gene expression patterns concordant point to a pathogenetic role of ALK in malignant progression of both WT and mutant ALK primary neuroblastoma. These findings are in line with in vitro studies showing that knockdown or inhibition of ALK by using siRNA or inhibitory small molecules, respectively, resulted in antitumorigenic effects in neuroblastoma cell lines with high ALK expression levels irrespective of the presence of activating mutations (6, 7, 28). In contrast, neuroblastoma cell lines with low-level ALK expression were not susceptible to ALK knockdown (e.g., cell lines SK-N-DZ, NGP), ALK inhibition (e.g., NB5, NB-INT1), or both (SK-N-AS) in these studies. The consistency of the results from both in vivo and in vitro studies

### Table 2.

Summary of pairwise comparisons of ALK-WT\textsuperscript{low}, ALK-WT\textsuperscript{high}, and ALK\textsuperscript{mutated} neuroblastomas by using analysis of centroid distances, ANOVA and t-test statistics. For t-test analysis and ANOVA, genes (probes) with a FDR < 0.05 were considered after correction for multiple testing by the Benjamini–Hochberg method.

<table>
<thead>
<tr>
<th></th>
<th>ALK mutated vs. ALK WT\textsuperscript{low}</th>
<th>ALK mutated vs. ALK WT\textsuperscript{high}</th>
<th>ALK WT\textsuperscript{low} vs. ALK WT\textsuperscript{high}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centroid distance (P value)</td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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<tr>
<td>ANOVA (genes)</td>
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<tr>
<td>t-test (genes)</td>
<td>1,567</td>
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suggest the level of expression rather than the activating mutation as the primary mediator of the molecular functions of ALK in established neuroblastoma. These data, however, do not rule out the possibility that activating ALK mutations may play a critical role in neuroblastoma initiation and early development, which remains to be addressed in future studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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High *ALK* Receptor Tyrosine Kinase Expression Supersedes *ALK* Mutation as a Determining Factor of an Unfavorable Phenotype in Primary Neuroblastoma

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