High ALK receptor tyrosine kinase expression supersedes ALK mutation as a determining factor of an unfavorable phenotype in primary neuroblastoma

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Running title: ALK expression and mutation in neuroblastoma

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Statement of Translational Relevance

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 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 demonstrate that tumors with ALK mutations resemble neuroblastomas with high-level wild-type ALK expression in their global gene expression patterns, and that patients of these two 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 wild-type ALK, suggesting that ALK inhibitory drugs should be evaluated in second-line treatment strategies of all high-risk neuroblastoma patients with elevated ALK expression.
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 neuroblastoma.

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: Non-synonymous point mutations of ALK were detected in 21/263 neuroblastomas (8%). Tumors with ALK mutations exhibited about 2-fold elevated median ALK mRNA levels in comparison to tumors with wild-type ALK. Unexpectedly, the wild-type allele was preferentially expressed in 12/21 mutated tumors. Whereas survival of patients with ALK mutated tumors was significantly worse as compared to the entire cohort of wild-type ALK patients, it was similarly poor in patients with wild-type 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 wild-type 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 wild-type ALK expression levels in both their clinical and molecular phenotypes. These data suggest that high levels of mutated and wild-type ALK mediate similar molecular functions that may contribute to a malignant phenotype in primary neuroblastoma.
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. While fatal progression of the disease occurs frequently in children with disseminated tumors, 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 demonstrated 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 (PTN) and midkine (MDK) have been suggested to act as ligands for ALK in humans (10). Inappropriate ALK expression due to 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 1-4% and 6-8% 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 work, 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 1). All patients were enrolled in the German Neuroblastoma trials with informed consent. Patients’ age 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 fluorescence in situ hybridization and defined according to the guidelines of the European Neuroblastoma Quality Assessment Group (16). MYCN was amplified in 45 (17.1%) and non-amplified 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, while 194 and 192 tumors had a normal 1p (73.8%) and a normal 11q status (73.0%), respectively (non-informative 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 using the Puregene Blood Core Kit B (Qiagen, Hilden, Germany). Total RNA was isolated from 30 to 60 mg of the same snap-frozen tumors using the FastPrep FP120 cell disruptor (Qbiogene-Inc, Carlsbad, CA) and the TRlZol reagent (Invitrogen, Karlsruhe, Germany). RNA
integrity was assessed using the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) 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 using the primers generated by De Brouwer et al. for exons 21-22 (13) and by Chen et al. for all other exons (4) (Supplementary Table 2). PCR conditions were as follows: 95°C for 3 min, 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, and a final extension step at 72°C for 5 min. PCR reactions were performed in 20 µl with illustra Taq polymerase and PCR buffer according to the manufacturer's protocol (GE Healthcare, Munich, Germany). Purification and Sanger sequencing of the PCR products was performed by the Eurofins MWG Operon Sequencing Service (Eurofins MWG Operon, Ebersberg, Germany).

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

**DNA copy number quantification**

*ALK* copy number status was determined using real-time quantitative PCR with *TNFRSF17* and *SDC4* as normalizing reference genes and normal human genomic DNA (Roche Diagnostics, Mannheim, Germany) 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 RTprimerDB (http://medgen.ugent.be/rtprimerdb, *TNFRSF17*, ID 14; *SDC4*, ID 15; *ALK*, ID 8117) (19). Five µl amplification mixtures contained 2-12 ng of DNA, 1x SYBR green I mastermix (Eurogentec S.A., Seraing, Belgium) and 250 nM of each
primer. PCR reactions were performed in a 384-well plate on a LightCycler 480 (Roche). The cycling conditions comprised 10 min polymerase activation at 95°C and 45 cycles of 15 s at 95°C and 30 s at 60°C, followed by a dissociation curve analysis from 60 to 95°C in order to verify amplification specificity. The haploid ALK copy number for each sample was calculated using the real-time PCR data analysis software qbasePLUS (http://www.qbaseplus.com) (20). Haploid copy numbers >4 were considered as amplification.

Gene expression analyses
Gene expression profiles were generated as dye-flipped dual-color duplicates using customized 11K oligonucleotide-microarrays as described (21). The ALK gene was represented by probe A_23_P324304. As a reference, pooled total RNA from 100 primary neuroblastomas was used. Data pre-processing, quality control analyses and normalization were performed 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 (RT-qPCR), single-stranded cDNA was generated from total RNA using the Superscript II First-Strand Synthesis System (Invitrogen). RT-qPCR was performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with SYBR Green chemistry using the standard curve method. To prevent amplification of contaminating genomic DNA, primer sequences were selected allowing intron-spanning amplification (Supplementary Table 2). 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 min in RIPA buffer (50 mM HEPES, pH 7.4, 150 mM 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 sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with 8% or 4-12% 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% BSA in 0.05% Tween-20/PBS before incubation with monoclonal primary antibodies (rabbit anti-human ALK, dilution 1:500; rabbit anti-human phospho-(y1604)-ALK, dilution 1:500; rabbit anti-human phospho-STAT3, dilution 1:2000, rabbit anti-human phospho-AKT, dilution 1:500, rabbit anti-human phospho-ERK1/2, dilution 1:1000; all Cell Signaling Technology, Danvers, MA) and horseradish peroxidase-labeled secondary goat anti-rabbit antibody (dilution 1:1000; Dako, Glostrup, Denmark). 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% FCS, L-glutamine and antibiotics. The cell line was authenticated by STR genotyping (DSMZ, Braunschweig, Germany). 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, USA), 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, Carlsbad, CA). 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 24h before being lysed on ice in RIPA buffer (50 mM HEPES,
ph7.4, 150 mM 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-square-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.
wild-type) and ALK expression (continuous) were fitted by stepwise-backward selection. The
likelihood ratio test p-value for inclusion was <0.05 and for exclusion >0.1.

Principal component analysis (PCA) and analysis of variance (ANOVA) of gene
expression data was performed using Rosetta Resolver Software. To test for global gene
expression differences between ALK-WT<sup>high</sup> tumors, ALK-WT<sup>low</sup> 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 to
centroid distances obtained from 1000 random permutations of the group labels. The number
of differentially expressed genes was calculated with either pair-wise t tests or by one-way
ANOVA over all three groups; for this, only genes (probes) in a list with a false discovery rate
<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 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 over-representation analysis of Gene Ontology (GO) categories by GOstats (25). Over-representation analysis of Gene Ontology categories was determined by Fisher's exact test ($p<0.05$ after Benjamini-Hochberg correction for multiple testing) 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 wild-type (WT) ALK was observed in 2/263 tumors. Point mutations were detected in 23/263 cases, 21 of which were heterozygous non-synonymous mutations corresponding to an overall prevalence of 8.0% (Table 1). Nine different nucleotide exchange mutations were observed, including three 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, while the other is currently in complete remission. Non-synonymous ALK mutation (ALK\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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 Figure 1).

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 using microarrays (21, 26), and
validated in 81 samples by RT-qPCR (Supplementary Figure 2). \textit{ALK} \textsuperscript{mutated} neuroblastomas exhibited significantly higher \textit{ALK} transcript levels than tumors with WT \textit{ALK} (p<0.001). The median expression in tumors with \textit{ALK} amplification and \textit{ALK} mutation was about 20-fold and 2-fold higher than in WT \textit{ALK} tumors, respectively (Fig. 2A, Supplementary Table 3). In contrast, \textit{ALK} mRNA levels did not differ between subgroups with different \textit{ALK} mutations (F1174L vs. R1275Q vs. other mutation, p=0.345, Fig. 2A). In addition, high \textit{ALK} mRNA expression correlated well with strong \textit{ALK} protein expression in most primary tumors as determined by Western blot analysis (Supplementary Fig. 3).

Unexpectedly, sequencing of cloned \textit{ALK} transcript fragments from \textit{ALK} \textsuperscript{mutated} neuroblastomas revealed a significant preponderance of WT allele expression (WT vs. mutated allele expression in the entire \textit{ALK} \textsuperscript{mutated} cohort, p=0.034). In particular, the WT allele was preferentially expressed (≥2-fold higher expression than the mutated allele) in all tumors with the F1174L mutation (Table 1). A more heterogeneous pattern of \textit{ALK} allele expression was 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 \textit{ALK} are sufficient to contribute to the tumor phenotype, or that \textit{ALK} expression levels rather than the mutation status represent a determining factor for neuroblastoma tumor behavior.

\textit{High ALK expression correlates with an adverse neuroblastoma phenotype independent of the genomic ALK status}

Based on these findings, we hypothesized that high \textit{ALK} expression levels should correlate with an adverse phenotype of neuroblastoma irrespective of the genomic \textit{ALK} status. Indeed, \textit{ALK} transcript levels were significantly higher in subgroups characterized by adverse prognostic markers than in favorable subgroups in the WT \textit{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 \textit{MYCN}, p=0.011; 11q-loss vs. 11q normal, p=0.010). Moreover, \textit{ALK} expression was significantly associated with poor
patient survival in a univariate Cox regression model using ALK mRNA levels as a continuous variable (EFS, p=0.005, hazard ratio [HR] 3.27, 95% confidence interval [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<sub>low</sub>; >25<sup>th</sup> and <50<sup>th</sup> percentile, ALK-WT<sub>intermediate-low</sub>; >50<sup>th</sup> and <75<sup>th</sup> percentile, ALK-WT<sub>intermediate-high</sub>; >75<sup>th</sup> percentile, ALK-WT<sub>high</sub>; n=60 each; Fig. 2B and 3). To determine whether ALK expression and ALK mutation status are independent prognostic markers, the two 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 4). 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 obtained in the forward and backward models based on OS (probably due to 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<sub>high</sub> and ALK<sub>mutated</sub> neuroblastomas were compared, since 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), classification according to our gene expression-based classifier (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<sub>high</sub> and ALK<sub>mutated</sub> cases, respectively; p=0.008). In addition, clinical courses of patients with ALK<sub>mutated</sub> and ALK-WT<sub>high</sub> tumors were similar in both the entire cohort and after excluding MYCN amplified neuroblastomas (Fig. 3, Supplementary Fig. 4). Taken together, these data indicate that ALK<sub>mutated</sub> and ALK-WT<sub>high</sub> neuroblastomas exhibit highly similar clinical phenotypes.
ALK\textsuperscript{mutated} and ALK-WT\textsuperscript{high} neuroblastomas show similar molecular phenotypes

To investigate transcriptomic characteristics of tumors with and without ALK mutations, we performed principal component analyses (PCA) using global gene expression information of all samples. Here, ALK-WT\textsuperscript{low} and ALK-WT\textsuperscript{high} tumors formed separate subgroups, while ALK\textsuperscript{mutated} tumors showed a similar distribution to ALK-WT\textsuperscript{high} tumors in both the entire cohort and after excluding MYCN amplified neuroblastomas (Fig. 4, Supplementary Fig. 5). Of note, those four ALK\textsuperscript{mutated} neuroblastomas showing lower ALK expression levels than ALK-WT\textsuperscript{high} tumors were more closely associated with the ALK-WT\textsuperscript{low} subgroup (Supplementary Fig. 5).

To validate these observations, the ALK-WT\textsuperscript{low}, ALK-WT\textsuperscript{high} and ALK\textsuperscript{mutated} subgroups were compared in a pair-wise manner using analysis of centroid distances, ANOVA and t-test statistics (Table 2). Both ALK\textsuperscript{mutated} and ALK-WT\textsuperscript{high} tumors differed significantly from ALK-WT\textsuperscript{low} tumors (p<0.001) by analysis of centroid distances. In contrast, ALK-WT\textsuperscript{high} and ALK\textsuperscript{mutated} tumors appeared to be more similar (p=0.027). Accordingly, only few transcripts were differentially expressed between these two subgroups, whereas multiple genes were differentially expressed between ALK-WT\textsuperscript{low} and ALK-WT\textsuperscript{high} or ALK\textsuperscript{mutated} neuroblastomas.

To gain insight into the molecular mechanisms by which ALK may exert its effects on neuroblastoma pathogenesis, the phosphorylation status of ALK targets was examined in neuroblastoma cell lines carrying either WT or mutated ALK transgenes and in primary tumors. In the cell line model, strong phosphorylation of ALK itself was observed in F1174L mutants upon ALK induction, while weak to modest ALK phosphorylation occurred in WT clones and R1275Q mutants, respectively (Supplementary Fig. 6). In addition, ALK induction resulted in a discrete to moderate increase of p-STAT3 and p-ERK/1/2 in clones FL8 and FL1 harboring the F1174L mutation, while 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. 3). 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<sub>high</sub> tumors (Supplementary Fig. 7). 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, 1733 genes were positively or negatively correlated with ALK expression in ALK<sub>mutated</sub> tumors (Supplementary Table 5A), while 823 genes were correlated with ALK expression in WT ALK tumors (Supplementary Table 5B). Of note, the overlap of genes correlated with ALK expression in WT ALK and ALK<sub>mutated</sub> tumors was exceptionally high (57%), and the direction of correlation was concordant (i.e., either positive or negative) for all these genes. Over-representation 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 6). 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<sub>mutated</sub> tumors, 21 and 14 significantly enriched GO categories were found among the genes positively and negatively correlated with ALK expression, respectively. Notably, 18/21 (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 in 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 21/263 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 demonstrate that mutations in the ALK tyrosine kinase domain are invariably associated with elevated ALK transcript levels in primary neuroblastoma. While 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 over-expression (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 ALKmutated 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<sup>high</sup> tumors in which ALK expression was as high as in ALK<sup>mutated</sup> 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<sup>low</sup> subgroup and patients with spontaneously regressing tumors (n=4) in the ALK-WT<sup>high</sup> 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<sup>mutated</sup> and ALK-WT<sup>high</sup> neuroblastomas revealed highly similar prognostic marker profiles and clinical outcomes in these patients. In line with this observation, global gene expression patterns of these two 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<sup>mutated</sup> 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 due to contaminating ALK transcripts from stromal cells, since only samples with a tumor content of >60% were analyzed, and non-malignant 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 report on preferential WT allele expression of an oncogene harboring putatively activating mutations. While the underlying processes of allele-specific expression are largely unexplored, a \textit{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 demonstrates 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 concordantly point to a pathogenetic role of ALK in malignant progression of both WT and mutant ALK primary neuroblastoma. These findings are in line with \textit{in vitro} studies demonstrating that knock-down or inhibition of ALK using siRNA or inhibitory small molecules, respectively, resulted in anti-tumorigenic 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 knock-down (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 \textit{in vivo} and \textit{in vitro} studies 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.

**Acknowledgements**

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References

22. Fischer M, Skowron M, Berthold F. Reliable transcript quantification by real-time reverse transcriptase-polymerase chain reaction in primary neuroblastoma using normalization to averaged expression levels of the control genes HPRT1 and SDHA. J Mol Diagn 2005;7: 89-96.


Legends to tables

**Table 1**: Summary of patient and tumor characteristics of neuroblastomas with genomic alterations of *ALK*. Allele expression indicates the numbers of wild-type (WT) and mutated (mut.) clones. Age at diagnosis is given in days. Amp, amplification; del, deletion; imb, imbalance; n.d., not done; DOD, death of disease; CR, complete remission; vgPR, very good partial remission; (*) homozygous mutation. Tumors with F1174L and R1275Q mutations are highlighted in gray.

**Table 2**: Summary of pair-wise comparisons of *ALK*-WT\textsubscript{low}, *ALK*-WT\textsubscript{high} and *ALK*\textsuperscript{mutated} neuroblastomas using analysis of centroid distances, ANOVA and t-test statistics. For t-test analysis and ANOVA, genes (probes) with a false discovery rate <0.05 were considered after correction for multiple testing by the Benjamini-Hochberg method.

**Supplementary Table 1**: Patient and tumor characteristics of the 263 neuroblastomas in this study. Indicated are the sample ID, gender (F, female; M, male), tumor stage, age (days), histological classification according to Shimada (F, favorable; UF, unfavorable; n.d., not determined), *MYCN* copy number status (1, *MYCN* not amplified as determined by FISH; amp., ≥5fold increase in *MYCN* signal numbers in relation to the numbers of chromosome 2; n.d., not determined), *ALK* mRNA expression values as log ratios according to the microarray data, and patient subgroup based on *ALK* genomic status and expression level (1, *ALK*-WT\textsubscript{low}; 2, *ALK*-WT\textsubscript{intermediate-low}; 3, *ALK*-WT\textsubscript{intermediate-high}; 4, *ALK*-WT\textsubscript{high}; 5, *ALK*\textsuperscript{mutated}; 6, *ALK* amplification).

**Supplementary Table 2**: Nucleotide sequences of the oligonucleotides used as primers in this study.

**Supplementary Table 3**: Relative expression levels of *ALK* in tumors with and without genomic alterations of *ALK*. The mean and median ratios as well as log ratios indicate *ALK*
expression levels in tumor subgroups in relation to the reference RNA used in the microarray experiments. In addition, the fold-change of mean and median ALK expression levels of the respective subgroups in comparison to tumors with WT ALK (top table) and in comparison to WT_{low} ALK neuroblastomas (bottom table) is indicated.

**Supplementary Table 4:** Multivariate Cox regression models based on EFS and OS considering ALK expression (continuous) and ALK mutation status (non-synonymous mutations vs. wild-type). Bold-face indicates statistical significance (p<0.05).

**Supplementary Table 5:** Correlation of each gene represented on the microarray with ALK expression levels in the subgroup harboring non-synonymous ALK mutations (A) and in the subgroup with wild-type ALK (B). Indicated are the microarray probe ID, the correlation coefficient, the ENSEMBL IDs of the respective genes and the gene symbols.

**Supplementary Table 6:** Results of the over-representations analysis of GO-Biological process categories by Fisher’s exact test, which has been calculated for genes positively (A) and negatively (B) correlated with ALK expression in neuroblastomas with ALK mutations, or positively (C) and negatively (D) correlated with ALK expression in wild-type ALK neuroblastoma. GOBPID, Gene Ontology biological process ID; adj.PvalueBH, adjusted p-value according to the Benjamini-Hochberg method.
Legends to figures

Figure 1: Kaplan-Meier estimates for event-free and overall survival of neuroblastoma patients without (blue, n=240) and with (red, n=21) ALK missense mutations (EFS at 5 years, 0.71±0.03 vs. 0.52±0.11, p=0.040; and OS at 5 years, 0.82±0.03 vs. 0.62±0.11, p=0.015). The two patients harboring ALK amplifications were excluded from this analysis.

Figure 2: Expression levels of ALK in neuroblastoma subgroups defined by the genomic status of ALK as determined by microarray analysis (A). Expression levels of ALK in neuroblastoma subgroups defined by the genomic status of ALK and ALK mRNA levels as determined by microarray analysis (B). Expression values are given as log-ratios. Boxes, median expression values (horizontal line) and 25th and 75th percentiles; whiskers, distances from the end of the box to the largest and smallest observed values that are less than 1.5 box lengths from either end of the box; open circles, outlying values.

Figure 3: Kaplan-Meier estimates for event-free and overall survival of neuroblastoma patients classified according to their ALK expression level and the ALK mutation status. EFS at 5 years of ALK-WTlow (dark blue) vs. ALK-WTintermediate-low (light blue) vs. ALK-WTintermediate-high (black) vs. ALK-WThigh (green) vs. ALKmutated (red): 0.83±0.05 vs. 0.73±0.06 vs. 0.69±0.06 vs. 0.59±0.07 vs. 0.52±0.11, respectively; OS at 5 years: 0.89±0.04 vs. 0.89±0.04 vs. 0.82±0.05 vs. 0.66±0.07 vs. 0.62±0.11, respectively. Comparison of EFS and OS of ALK-WThigh vs. ALKmutated, p=0.546 and p=0.652, respectively.

Figure 4: Principle component analysis (PCA) of gene expression profiles from ALK-WTlow (light blue), ALK-WThigh (dark blue) and ALKmutated (green) neuroblastomas as well as tumors with ALK amplification (pink).

Supplementary figure 1: Kaplan-Meier estimates for event-free and overall survival of neuroblastoma patients with a normal genomic ALK status (blue), F1174L mutation (red),
R1275Q mutation (green), or other ALK mutations (black). Pair-wise comparisons revealed no significant differences in the clinical courses between subgroups harboring different types of ALK mutations.

**Supplementary figure 2:** Correlation of ALK log expression values in 81 samples as determined by microarray and RT-qPCR (Pearson correlation coefficient, r=0.85).

**Supplementary figure 3:** Western blot analysis of ALK and of the phosphorylated ALK targets p-STAT3, p-AKT and p-ERK1/2 in primary neuroblastomas. The sample ID, the genomic status of ALK and ALK mRNA expression levels according to the classification into percentiles (see text) are indicated above the blot results: +++, very high ALK mRNA expression according to amplified ALK; ++, high ALK expression according to ALK-WT\textsuperscript{high}; +, intermediate-high ALK expression levels according to ALK-WT\textsuperscript{intermediate-high}, (+), intermediate-low ALK expression levels according to ALK-WT\textsuperscript{intermediate-low}, - - low ALK expression levels according to ALK-WT\textsuperscript{low}.

**Supplementary figure 4:** Kaplan-Meier estimates for event-free and overall survival of neuroblastoma patients classified according to their ALK expression level and the ALK mutation status after exclusion of patients with MYCN amplified tumors. EFS at 5 years of ALK-WT\textsuperscript{low} (dark blue) vs. ALK-WT\textsuperscript{intermediate-low} (light blue) vs. ALK-WT\textsuperscript{intermediate-high} (black) vs. ALK-WT\textsuperscript{high} (green) vs. ALK\textsuperscript{mutated} (red): 0.89±0.04 vs. 0.81±0.06 vs. 0.69±0.06 vs. 0.65±0.08 vs. 0.67±0.12, respectively; OS at 5 years: 0.96±0.03 vs. 0.96±0.03 vs. 0.84±0.05 vs. 0.74±0.07 vs. 0.80±0.10, respectively. Comparison of EFS and OS of ALK-WT\textsuperscript{high} vs. ALK\textsuperscript{mutated}, p=0.88 and p=0.65, respectively.

**Supplementary figure 5:** Principle component analysis (PCA) of gene expression profiles from ALK-WT\textsuperscript{low} (light blue), ALK-WT\textsuperscript{high} (dark blue) and ALK\textsuperscript{mutated} neuroblastomas (green and red) after exclusion of MYCN amplified tumors. Tumors with ALK mutations and high
ALK expression are indicated in green, whereas the four ALK mutated tumors having intermediate or low ALK expression are indicated in red.

**Supplementary figure 6:** Western blot analysis of ALK, phosphorylated ALK and the phosphorylated ALK targets p-STAT3, p-AKT and p-ERK1/2 in transgenic SK-N-AS cells harboring either WT ALK (WT), F1174L mutant ALK (FL), R1275Q mutant ALK (RQ) or GFP as a control. Results are shown for two clones per transgenic cell line before and after induction of the transgene by addition of tetracycline.

**Supplementary figure 7:** Expression of PTN and MDK in neuroblastoma subgroups defined by the genomic status of ALK and ALK mRNA levels as determined by microarray analysis (A). Correlation of PTN and MDK expression with ALK mRNA levels in WT ALK neuroblastomas (B). Expression values are given as log-ratios. Boxes, median expression values (horizontal line) and 25th and 75th percentiles; whiskers, distances from the end of the box to the largest and smallest observed values that are less than 1.5 box lengths from either end of the box; open circles, outlying values.
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Fig. 1

EFS vs. years

OS vs. years
**Fig. 2A**

**ALK log-ratio**

**ALK genomic status**
- Wild-type
- F1174L
- R1275Q
- Other mutation
- Amplification
Fig. 2B

![Box plot showing ALK log-ratio for different WT and ALK mutation statuses with p=0.755](image)

- WT<sub>low</sub>
- WT<sub>intermediate-high</sub>
- ALK<sub>mutated</sub>
- WT<sub>intermediate-high</sub>
- WT<sub>high</sub>
- amplification
Supplementary Fig. 1
Supplementary Fig. 2
Supplementary Fig. 3
Supplementary Fig. 4
Supplementary Fig. 5
**Supplementary Fig. 6**

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Supplementary Fig. 7A

![Box plots of PTN and MDK log-ratios for different genotypes and amplifications.](image)
High ALK receptor tyrosine kinase expression supersedes ALK mutation as a determining factor of an unfavorable phenotype in primary neuroblastoma

Johannes H. Schulte, Hagen S. Bachmann, Bent Brockmeyer, et al.

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