Meta-analysis of neuroblastomas reveals a skewed ALK mutation spectrum in tumors with MYCN amplification

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Translational relevance

Our study yielded a number of important new insights with clinical implications. These findings are:

1. \textit{ALK} mutations are present in similar frequencies in all clinical stages of neuroblastoma (low as well as high stages).

2. F1174 hot-spot mutations are associated with \textit{MYCN} amplification and their combined occurrence leads to fatal disease outcome in all (except one) patients. A possible cooperation between the F1174 mutation and \textit{MYCN} amplification may have implications for targeted therapy.

3. F1174 mutations have a higher transforming capacity than R1275 mutations.

4. Chromosome 2 copy gain, including the \textit{ALK} locus, is associated with an increased \textit{ALK} expression which was found to be associated with a significantly worse outcome in the global population.

These findings shed a new and more detailed light on the distribution of \textit{ALK} mutations in neuroblastoma. This information may be of importance in the light of choice of risk-related therapy and development of future targeted therapies.
Abstract

Purpose: Activating mutations of the anaplastic lymphoma kinase (ALK) were recently described in neuroblastoma. We performed a meta-analysis of 709 neuroblastoma tumors to determine its frequency and mutation spectrum in relation to genomic and clinical parameters and studied the prognostic significance of ALK copy number and expression.

Experimental design: Frequency and type of ALK mutations, copy number gain and expression were analyzed in a new series of 254 neuroblastoma tumors. Data from 455 published cases were used for further in-depth analysis.

Results: ALK mutations were present in 6.9% of 709 investigated tumors and mutations were found in similar frequencies in favorable (INSS 1, 2 and 4S; 5.7%) and unfavorable (INSS 3 and 4; 7.5%) neuroblastomas (P=0.087). Two hotspot mutations, at positions R1275 and F1174, were observed (49% and 34.7% of the mutated cases respectively). Interestingly, the F1174 mutations occurred in a high proportion of MYCN amplified cases (P=0.001) and this combined occurrence was associated with a particular poor outcome, suggesting a positive cooperative effect between both aberrations. Furthermore, the F1174L mutant was characterized by a higher degree of auto-phosphorylation and a more potent transforming capacity as compared to the R1275Q mutant. Chromosome 2p gains, including the ALK locus (91.8%), were associated with a significantly increased ALK expression which was also correlated with poor survival.

Conclusions: ALK mutations occur in equal frequencies across all genomic subtypes but F1174L mutants are observed in a higher frequency of MYCN amplified tumors and show increased transforming capacity as compared to the R1275Q mutants.
Introduction

Neuroblastoma is the most common solid extracranial pediatric tumor with an annual incidence of 1 in 100,000 children below the age of 15 years (1). Despite intensive multimodal treatment, neuroblastoma remains fatal in almost half of the unfavorable patients. Insights into the molecular pathogenesis of this disease are required for the development of less toxic and more effective molecular targeted therapy. Detailed studies of patterns of DNA copy number alterations have been instrumental in our understanding of the clinical and biological heterogeneity of this tumor. Three major genomic subtypes represent more than 80% of all cases, i.e. hyperploid neuroblastoma with whole chromosome gains and losses, near diploid neuroblastoma with 11q deletions and 17q gain, and \textit{MYCN} amplified neuroblastoma with 1p deletions and 17q gain (2-5). The discovery of rare but recurrent high level amplification of the \textit{ALK} gene and a genetic study of familial neuroblastomas led to the discovery of activating \textit{ALK} mutations in neuroblastoma (6-10). The frequency of \textit{ALK} mutations in primary neuroblastoma varied between 6% and 11% in the different studies (6-10). The relatively low number of mutations described in each of the individual studies has precluded a thorough analysis of the frequency and distribution of recurrent \textit{ALK} mutations across the different genomic subtypes. Moreover, in the first published series, there was a significant bias towards analysis of high stage tumors, thus preventing a more general assessment of frequency and distribution of mutations across different stages (6-8). In our current study, we screened an additional 254 neuroblastoma cases including all clinical stages and genomic subtypes. In a meta-analysis, these findings were combined with those from 455 published cases (8-10) for which genomic subtype and clinical information were available. This strategy enabled us to analyze the \textit{ALK} mutation profile in relation to...
genomic and clinical data in 709 neuroblastomas which revealed a distinct mutation spectrum in relation to genomic subtype. Two hot-spot mutations, F1174L and R1275Q, were shown to induce a ALK auto-phosphorylation and were able to transform interleukin-3 (IL-3) dependent Ba/F3 cells into cytokine-independent growth. In addition, we also evaluated ALK gene expression levels and demonstrated that high ALK expression is correlated with poor survival.
Material and Methods

Neuroblastoma patients and cell lines

In total, 254 primary untreated neuroblastoma tumors with a tumor percentage > 60% were investigated including 44 stage 1, 30 stage 2, 34 stage 3, 113 stage 4 and 33 stage 4S tumors (according to the INSS staging system (11)). Patient information and genomic subtypes for 455 published tumors screened for ALK mutations were retrieved from the publications or made available by the authors (supplementary Table 1) (8-10). In addition, 39 neuroblastoma cell lines were included. The cell lines were obtained from several sources (see supplementary Table 2). All of the cell lines were genotyped by DNA fingerprinting (PowerPlex, Promega).

Genomic DNA was isolated using the Qiagen DNA isolation kit (Qiagen) or a standard proteinase K/SDS procedure.

ALK DNA sequence analysis

For the first tumor cohort (146 cases) and the 39 cell lines, all 29 ALK coding exons were analyzed, whereas the remaining 108 tumors were screened for only the tyrosine kinase domain. Constitutional DNA from blood samples was available and analyzed for 12 of the 17 patients with a mutation in the primary tumor. Exons were amplified from genomic DNA (primer information in supplementary Table 3). PCR products were subjected to directional or bidirectional sequencing using BigDye Terminator V1.1/V3.1 Cycle Sequencing chemistry on an ABI3730XL sequencer (Applied Biosystems). Electropherograms were analyzed using Seqscape v2.5 software (Applied Biosystems).
ArrayCGH copy number profiling

In order to determine DNA copy number alterations, arrayCGH was performed by using an in-house developed 1 Mb resolution BAC array (37 samples) as previously described (3) or by using a custom-designed 44K array enriched for regions with recurrent imbalances in neuroblastoma (1p, 2p, 3p, 11q, 17) (217 samples) (Agilent Technologies). For the latter, a total of 150 ng of tumor and reference DNA was labeled with Cy3 and Cy5, respectively (BioPrime ArrayCGH Genomic Labeling System, Invitrogen). Further processing was done according to the manufacturer's guidelines. Features were extracted using the feature extraction v10.1.0.0.0 software program and processed with an in-house developed visualization software arrayCGHbase (http://medgen.ugent.be/arrayCGHbase) (12) including circular binary segmentation (CBS) for scoring of DNA copy number alterations (13).

ALK gene expression data

Gene expression data were available for 440 tumors, comprising of a published dataset of 251 tumors profiled on custom Agilent 44k arrays (14) (downloaded from the EBI ArrayExpress database (E-TABM-38)), an unpublished dataset of 101 tumors profiled on the Human Exon 1.0 ST Affymetrix arrays (normalized and summarized at the transcript level using RMA Sketch) and an unpublished dataset of 88 tumors profiled on the Affymetrix HG-U133plus2.0 platform (normalized using MAS5 and downloaded from the R2 database) (Koster et al., submitted). Importantly, analyses were performed on sets of tumors profiled on the same platform. For Kaplan-Meier and log-rank analysis, ALK expression levels were digitalized using the median expression value as cut-off.
**Tissue micro-array and immunohistochemistry**

For the establishment of a tissue micro-array, three representative areas from each tumor were selected on H&E stained slides from 70 formalin-fixed and paraffin-embedded primary untreated neuroblastoma tumors (supplementary Table 4). Neuroblastoma tumors were classified according to the International Neuroblastoma Pathology Classification (INPC) scoring system (15) which divides tumors into undifferentiated, poorly differentiated and differentiating. Of each tumor, three cores were punched into the recipient block. Immunohistochemistry was performed using a monoclonal mouse anti-human CD246 ALK antibody (clone ALK1, Dako) and slides were scored for immunoreactive neuroblastoma cells where 0 = no weak staining (<10% of the cells), 1 = weak staining intensity (10-50% of the cells), 2 = medium staining intensity (50-80% of the cells), 3 = high staining intensity (>80% of the cells). The medium score of the three punches was calculated for Kaplan-Meier and log-rank analysis.

**Western blotting**

Lysis of cells was performed when the neuroblastoma cell lines reached 70% confluence. Total cell lysates (50 µg of protein) were analyzed by standard procedures (16) using anti-phospho-ALK (Tyr1604), anti-ALK, (Cell Signaling) and anti-ERK2 (Santa Cruz) antibodies. The Aida Image Analyzer v.4.22 was used for quantification of western blots.

**Transformation assay**

Ba/F3 cells were cultured in RPMI-1640 supplemented with 10% FBS and 1ng/mL murine IL-3 (Peprotech). ALK F1174L and ALK R1275Q constructs were generated by PCR, cloned into the retroviral vector pMSCV-neo (Clontech) and transduced in
Ba/F3 cells. Experiments were performed in triplicate. Transduced Ba/F3 cells were selected with G418 (500µg/mL medium). The amount of viable cells was assayed on regular intervals. For proliferation curves, Ba/F3 cells were washed with PBS and 5 x 10^5 cells were seeded in 5mL medium without IL-3. Viable cells were counted using a Vi-CELL Cell Viability Analyzer (Beckman Coulter) on day 3, 5, 7 and 9.

Statistical analysis

Fisher’s exact tests, Mann-Whitney tests and correlation analyses were performed using R (version 2.8.1). The R Survival package was used to generate Kaplan-Meier plots and to perform log-rank analyses. Multivariate logistic regression analysis was performed using the glm function (R-base package).
Results

**ALK mutation analysis in 254 primary neuroblastoma tumors and 39 neuroblastoma cell lines**

In a series of 254 sporadic, non-familial primary neuroblastoma tumors, a total of 17 *ALK* mutations (6.7%) and 2 *ALK* amplifications (0.8%) were identified (supplementary Figure 1). In a first series of 146 cases, all 29 *ALK* coding exons were analyzed but no mutations were found outside the tyrosine kinase domain. Therefore, only the tyrosine kinase domain was analyzed for the remaining 108 tumors. The most frequent mutations were located at residues R1275 and F1174 and were detected in respectively 3.9% (10/254) and 2.0% (5/254) of the cases. A third recurrent but less frequent mutation affecting residue F1245 (6-9) was not detected in our series. One of the mutations previously reported in only one tumor was also detected as a single case in our series (Y1278S) (10), thus providing further evidence for its contribution to neuroblastoma pathogenesis. In addition to the previously reported mutations we observed one new missense mutation, R1231Q. Sequence analysis of the constitutional DNA of 12 out of 17 patients with an *ALK* mutation showed that these mutations were somatically acquired.

Mutation analysis was also performed in 39 neuroblastoma cell lines. All mutations, except for one (D1091N in LAN-6), were exclusively found within the tyrosine kinase domain. Recurrent mutations were found in two cell lines that were previously not analyzed, STA-NB-8 and NB-14. All results of previously reported cell lines were in concordance with published results except for one (see supplementary Table 1).
Meta-analysis of ALK mutations reveals an increased occurrence of the F1174 mutation in MYCN amplified tumors

We performed a meta-analysis on the new cohort of 254 cases together with 455 previously analyzed and published cases to relate the ALK mutations to clinical and genomic data (8-10) (Table 1 and supplementary Table 1). The 709 samples have in total 49 ALK mutations (6.9%). The two most frequently occurring mutations are the F1174 (34.7%) and R1275 mutation (49%) (supplementary Figure 1). When taking into account the entire group of different ALK mutations when evaluating the overall frequency of ALK mutations, no significant difference was observed for the frequency of mutations in favorable (INSS 1, 2 and 4S) (14/245, 5.7%) versus unfavorable (INSS 3 and 4) (33/440, 7.5%) neuroblastomas (Fisher-exact test P=.087). Mutation frequencies were also compared within different stages, MYCN status or age but no significant differences were found (see Table 1 for P-values). However, when looking at the different types of mutations in relation to the clinical and genomic parameters, we noticed a skewed distribution for MYCN status in the F1174 mutated cases versus the tumors with wild-type ALK (P=.001) (Figure 1A and Table 1). ALK F1174 mutations were found in 1.3% of the MYCN-single copy tumors, compared to 6.1% of MYCN amplified tumors. Of the 17 tumors with the F1174 mutation, 58.8% had MYCN amplification, compared to a frequency of MYCN amplification of 21.6% in the tumors with wild-type ALK (Figure 1A). In contrast, the frequency of MYCN amplification was similar for R1275 mutated versus wild-type cases. As most cell lines are MYCN amplified, we then sequenced 39 neuroblastoma cell lines in order to verify whether the association of F1174 with MYCN amplification could also be detected. Indeed, F1174 mutations were present in 5 out of 27 MYCN amplified cell
lines whereas only one R1275 mutation was present in this cohort (supplementary Table 2).

**Frequency of ALK mutations according to genomic subtype**

To further explore the relationship between ALK mutation status and genomic alterations, we classified the tumors into genomic subclasses based on arrayCGH data (3). For 659 tumors, we could establish the genomic subtype: subtype 1 with numerical imbalances only (n=218); subtype 2A with 11q deletion and without MYCN amplification (n=126); subtype 2B with MYCN amplification (n=158); and subtype 3 without any detectable DNA copy number alterations (n=78). This classification covered most of the cases (88%) whereas 79 remained unclassified (supplementary Figure 2).

Comparison of the ALK mutation frequency in relation to genomic subtype revealed that ALK mutations were most frequently observed in MYCN amplified tumors (subtype 2B) (8.9% mutated), followed by subtype 1 tumors (7.3% mutated), subtype 2A tumors (4.0%) and subtype 3 tumors (1.3%). Interestingly, all infrequent mutations (6/49) were present in subtype 1 tumors (Figure 1B).

**Correlation of ALK mutation with survival**

No significant survival differences were found in tumors with or without ALK mutations (or amplifications) (log-rank $P=.317$) (Figure 2A). However, when comparing survival of patients with R1275 mutation or wild-type patients with patients with the F1174 mutation type, Kaplan-Meier analysis showed significant survival differences ($P=.027$ and $P=.002$) (Figure 2B and 2C). This might largely be explained by the high frequency of MYCN amplification within the F1174 mutated tumors compared to the R1275 mutated tumors.
Interestingly, although not statistically significant, we noticed that 9 out of 10 patients with a *MYCN* amplification and F1174 mutation died of disease, suggesting that within the *MYCN* amplified subgroup, patients with the F1174 mutation may have an particularly poor survival as compared to a 32% 5-year overall survival rate for *MYCN* amplified cases without the F1174 mutation.

**ALK amplification**

Overall, *ALK* amplifications could be detected in only 12 out of 709 tumors (1.7%) and none of these carried activating *ALK* mutations. All except one *ALK* amplified tumors were also found to be *MYCN* amplified (*P*<.001) which accounts for 6.7% of the total of *MYCN* amplified tumors. Like *ALK* mutation, amplification is not a statistically significant independent marker for survival when analyzed in a model with *MYCN*, stage and age in a logistic regression analysis (data not shown).

**ALK low copy number gain, gene expression and survival**

Low copy number gain of chromosome 2p material or whole chromosome 2 gain was detected in 19.3% (49/254) and 17.7% (45/254) of the cases, respectively, in keeping with the high occurrence reported in previous studies (6, 8-10, 17). No focal low copy number gains were detected for *ALK*. In cases with partial 2p gain, the extra chromosomal segments varied in size from 15-87 Mb and included the *MYCN* gene except for one case. In the latter, *MYCN* amplification was present together with a more distal 15 Mb gain with a telomeric breakpoint immediately proximal to the *ALK* locus (supplementary Figure 3). The *ALK* gene was included in all except four cases of partial 2p gain (91.8%). An interesting observation, particularly in view of the high frequency of *ALK* copy number gain, was that 2p gains encompassing the *ALK* locus
were present in only 2 out of 17 mutated tumors (11.8%) indicating that 2p gain is not a common mechanism for increasing mutated ALK copy number.

In order to evaluate the possible impact of ALK copy number gain on ALK expression, we compared Affymetrix exon array expression data and arrayCGH data for 101 neuroblastomas. This showed a strong correlation between copy numbers of the ALK gene and expression levels (Spearman correlation coefficient: 0.308, \(P=0.002\), one ALK amplified sample was omitted from this analysis) (supplementary Figure 4). This was confirmed by Mann-Whitney analysis comparing the expression in tumors with normal ALK copy number versus tumors with ALK gain (CGH result >0.3) (\(P=0.001\)).

Next, we evaluated the relation between ALK gene expression and survival. We therefore analyzed expression levels in three independent datasets including a total of 440 tumor samples (14) (Koster et al., submitted). Each analysis was performed on samples profiled on one particular platform. These showed a correlation of ALK gene expression with survival in the global patient population (log-rank p-values for overall and progression-free survival <.05) (Figure 3A-C). Multivariate logistic regression analysis (in a model testing MYCN status, age, stage and ALK expression) could demonstrate an independent prognostic value for ALK mRNA expression in the larger dataset (14) (Odd’s ratio: 2.94 (95%CI: 1.29-6.69), \(p=1.02E-2\)), but this could not be confirmed in the other two smaller datasets.

**ALK immunoreactivity**

In addition to the relation between ALK transcript expression and survival, we also investigated the ALK protein expression status and patient survival using a tissue micro-array (TMA) containing 70 primary tumors. Kaplan-Meier and log-rank analysis
show a significant correlation between ALK protein expression and overall survival (OS; \( P = .014 \)) and progression free survival (PFS; \( P = .002 \)) (Figure 4). The three cases with \( ALK \) mutation (R1275Q) present on the TMA have a median expression value (score 2) whereas in the remaining \( ALK \) wild-type tumors, ALK reactivity varied from low (score 0) to high (score 3).

For the evaluation of the ALK activity, the level of activated/phosphorylated ALK (p-ALK) was investigated. However, as the specificity of p-ALK antibodies remains to be determined, we performed Western blot analysis rather than immunohistochemistry experiments on a panel of 22 neuroblastoma cell lines. This allowed for comparison of \( ALK \) mRNA expression levels (\( P = .011 \)) and native ALK protein levels (\( P = 1.55E-07 \)) versus p-ALK protein level. We clearly demonstrate a significant correlation between both \( ALK \) mRNA expression levels and native ALK protein levels with p-ALK protein levels (supplementary Figure 5). Interestingly, we could also show that cell lines harboring the F1174 mutation (except for the SK-N-SH cell line with very low ALK expression levels) or ALK amplification have relatively more phosphorylated ALK than cell lines with the other hotspot mutation. Moreover, cell lines with wild type ALK have very low levels of phosphorylated ALK (supplementary Figure 6 and supplementary Figure 7).

Transforming capacity of \( ALK \) hot-spot mutations

Given the observed concordance between mutation type and ALK activity, we also compared the transforming capacity of both \( ALK \) hotspot mutations in IL-3 dependent Ba/F3 cell lines.
Although both mutants were able to transform Ba/F3 cells to IL-3 independent growth, cells expressing \textit{ALK} F1174L transformed the cells significantly faster than Ba/F3 cells expressing \textit{ALK} R1275Q (Figure 5).
Discussion

The present meta-analysis of ALK mutations of 709 neuroblastomas in relation to genomic profiles and clinical parameters resulted in a number of new important observations. First, substitutions at residue F1174, one of the two hotspot mutations, were significantly overrepresented in MYCN amplified tumors. Moreover, patients with the F1174 mutation present with a particularly poor outcome. Second, our results indicate that, although both hotspot mutations have constitutive ALK phosphorylation, the F1174L mutation has stronger auto-phosphorylation and transformation capacity of Ba/F3 cells than the R1275Q mutation. Third, in contrast to some previous reports, we show that ALK mutations also occur in a significant proportion of tumors with favorable stages 1, 2 and 4s (5.7% versus 7.5% in stage 3 and 4 tumors). Fourth, we show that copy number gain of the chromosome 2 region encompassing ALK is associated with an increased ALK expression. Fifth, increased ALK expression is associated with a worse outcome in the global population. Finally, we show that chromosome 2p is not frequently gained in tumors with ALK mutations, indicating that mutated ALK alleles are not selected for high expression by copy number gain.

The functional relevance of the high proportion of F1174 mutations in the subset of MYCN amplified ALK mutated neuroblastomas remains undetermined but their co-occurrence may suggest a cooperative effect between both aberrations in these tumors. The F1174 mutation might contribute to an additional growth and survival benefit in MYCN amplified neuroblastoma cells which may explain the particularly poor survival of these patients. The fact that neuroblastoma cell lines with MYCN amplifications have a relatively high frequency of F1174 mutations might also point at...
a particular growth advantage that may have facilitated in vitro growth of these cells.

Of further interest in this context is the observation that F1174 mutations in the germline have not been reported up to now, which could suggest embryonic lethality. Of particular interest was the observation that only one of the 10 patients with MYCN amplification together with a F1174 mutation survived, in contrast to a 32% 5-year overall survival rate in patients with MYCN amplified tumors without the F1174 mutation.

The different distribution across the genomic subtypes and the adverse impact of the F1174 mutations on survival raise the question whether the F1174 and R1275 mutations may execute distinct effects on tumor biology. George and colleagues have previously also shown that both F1174L and R1275Q mutants could transform Ba/F3 cells, but their analysis did not reveal major differences in oncogenic potential between these two mutants, in part because the proliferation data were not reported in detail. In our hands, the F1174L mutant transformed the Ba/F3 cells more efficiently than the cells expressing the R1275Q mutant. This correlated with higher auto-phosphorylation levels of ALK FL, which was not observed for the RQ mutant.

Occurrence of mutations of particular genes in relation to genomic subgroups have been reported in certain tumor entities such as PIK3CA mutations in head and neck squamous carcinomas without EGFR amplification and beta-catenin mutations in medulloblastomas with loss of chromosome 6 (18-20). However, to the best of our knowledge, a different distribution for mutations within the same functional domain of one specific gene, as observed here for the F1174 mutation in ALK, has not been reported.

Our study also showed, in contrast to some of the initial studies, that ALK mutations occur in fairly equal frequency in both low and high stage tumors. Therefore, mutation
analysis should also be performed in patients with low stage tumor and clinical characteristics and behavior of such tumors should be carefully monitored in further studies.

In addition to mutations, ALK activation can also result from high level gene amplification as demonstrated by previous studies (8-10). Meta-analysis showed that this is a recurrent but rare mechanism, detected in only 1.7% of the cases. In keeping with previous studies, amplification of ALK almost exclusively occurs in MYCN amplified tumors. Apart from such rare ALK high level amplification, high stage neuroblastoma tumors often exhibit gain of a large part of 2p that mostly encompasses the ALK locus. In our cohort, mutation analysis showed that only a minority of tumors with 2p gain carried ALK mutations, although 2p gains are present in as much as 19.3% of all neuroblastomas with segmental imbalances. This observation indicates that 2p gain does not act as a mechanism for increased copy number of mutated ALK, in contrast to what has been described for other oncogenes in other tumor entities (21-22). In view of the variability of centromeric breakpoints for 2p gains but almost consistent presence of ALK in these segments, one could assume that low copy number gain of ALK could also infer a growth or survival advantage for neuroblastoma cells. To test this hypothesis we analyzed the relation between ALK copy number and expression and the impact of increased ALK gene expression on survival. ALK gene expression was indeed shown to be copy number sensitive and increased ALK expression correlated with poor survival. Using immunostaining, Passoni et al. (2009), recently demonstrated that ALK overexpression correlated with patient survival although no correlation was found between mRNA and protein expression in their tumor cohort (23), which is in contrast with our observations in cell lines. Our findings, together with those of Passoni et al...
(2009) indicate that increased ALK expression might be functionally relevant. Therefore, patients with increased ALK expression might benefit from future clinical trials with ALK inhibitors.

In conclusion, this meta-analysis for the first time demonstrates that the recurrent F1174 mutation predominantly occurs in MYCN amplified tumors and clearly shows differences in the frequency and distribution of ALK mutations across the different genomic subtypes in neuroblastoma. The F1174 mutation might infer a poor prognosis in patients with MYCN amplification but further studies are needed to substantiate this hypothesis. Furthermore we could demonstrate that the F1174L mutant displayed a higher degree of ALK phosphorylation and tumorigenicity than the R1275Q mutant. No significant difference was observed in the frequency of ALK mutations between low and high stage tumors.
References


Table legend

Table 1: Results of Fisher-exact analysis comparing the distribution of genomic alterations, age, stage and genomic subgroup in cases with one of the frequent ALK mutations or amplification versus wild-type cases. The last column gives the results of the comparison of the cases with the F1174 mutation versus cases with the R1275 mutation (* = p<.05, . = p<.1, ** = data are based on 2 of the 4 datasets for which detailed arrayCGH data were available)
Figure legends

Figure 1: Comparison of the distribution of MYCN status (A) and genomic subtype (B) in tumors with one of the two frequent ALK mutations or ALK amplification versus wild-type tumors (wt = wild-type ALK, amp = ALK amplification, MNA = MYCN amplification)

Figure 2: Kaplan-Meier and log-rank analysis of ALK mutated and amplified tumors versus ALK wild-type cases (A), F1174 mutated versus R1275 mutated cases (B) and F1174 mutated versus wild-type cases (C) (DOD = dead of disease)

Figure 3: Kaplan-Meier and log-rank analysis for overall (OS) and progression free survival (PFS): comparing tumors with high and low ALK mRNA expression in (A) a published set of 251 neuroblastoma tumors (14), and in unpublished sets of (B) 101 and (C) 88 neuroblastoma tumors.

Figure 4: Kaplan-Meier and log-rank analysis for overall (OS) and progression free survival (PFS) comparing tumors with high (expression ≥ 2) and low (expression < 2) ALK protein expression (X-axis = number of days ; DOD = dead of disease)

Figure 5: Proliferation curve of Ba/F3 cells stably expressing the F1174L or R1275Q ALK mutants. Ba/F3 cells expressing mutant ALK as well as parental Ba/F3 cells were grown in the absence of IL-3 for a period of 9 days. A day 9 value for ALK F1174L was not included in the figure since the culture had reached maximal density before this time point.
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<td>7.98E-01</td>
<td>1.00E+00</td>
<td>1.00E+00</td>
<td>6.02E-01</td>
<td>1.00E+00</td>
<td>5.26E-01</td>
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<td>17q-gain**</td>
<td>6.85E-01</td>
<td>6.72E-01</td>
<td>1.00E+00</td>
<td>7.03E-01</td>
<td>1.00E+00</td>
<td>1.00E+00</td>
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<td>age (&lt;= 1 year)</td>
<td>4.07E-01</td>
<td>4.47E-01</td>
<td>7.73E-01</td>
<td>8.09E-02</td>
<td>6.58E-01</td>
<td>9.33E-02 .</td>
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<td>stage</td>
<td>9.94E-02 .</td>
<td>1.66E-01</td>
<td>4.64E-01</td>
<td>6.70E-01</td>
<td>4.56E-01</td>
<td>8.13E-01</td>
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<td>genomic subtype</td>
<td>9.32E-04 *</td>
<td>9.35E-02 .</td>
<td>5.04E-06 *</td>
<td>2.20E-02 *</td>
<td>7.71E-01</td>
<td>3.92E-01</td>
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Figure 1
Figure 2
Figure 3
Meta-analysis of Neuroblastomas Reveals a Skewed ALK Mutation Spectrum in Tumors with MYCN Amplification

Sara De Brouwer, Katleen De Preter, Candy Kumps, et al.

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