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Human Cancer Biology

Clinical Cancer Research

Meta-analysis of Neuroblastomas Reveals a Skewed *ALK* Mutation Spectrum in Tumors with *MYCN* Amplification

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Abstract

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

Experimental Design: The 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 [International Neuroblastoma Staging System (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 autophosphorylation and a more potent transforming capacity as compared with 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 with the R1275Q mutants. *Clin Cancer Res; 16(17); 4353–62. ©2010 AACR.*

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 >80% of all cases, i.e., hyperploid neuroblastoma with whole chromosome gains and losses, near diploid neuroblastoma with 11q deletions and 17q gain, and *MYCN*-amplified neuroblastoma with 1p deletions and 17q gain (2–5). The discovery of rare but recurrent high-level amplification

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Translational Relevance

Our study yielded a number of important new insights with clinical implications. First, *ALK* mutations are present in similar frequencies in all clinical stages of neuroblastoma (low as well as high stages). Second, F1174 hotspot mutations are associated with *MYCN* amplification and their combined occurrence leads to fatal disease outcome in all (except one) patients. A possible cooperation between the F1174 mutation and *MYCN* amplification may have implications for targeted therapy. Third, F1174 mutations have a higher transforming capacity than R1275 mutations. Finally, chromosome 2 copy gain, including the *ALK* locus, is associated with an increased *ALK* expression that 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 *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.

of the *ALK* gene and a genetic study of familial neuroblastomas led to the discovery of activating *ALK* mutations in neuroblastoma (6–10). The frequency of *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 *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 *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 hotspot mutations, F1174L and R1275Q, were shown to induce ALK autophosphorylation and were able to transform interleukin-3 (IL-3)-dependent Ba/F3 cells into cytokine-independent growth. In addition, we evaluated *ALK* gene expression levels and showed that high *ALK* expression is correlated with poor survival.

Materials 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 International Neuroblastoma Staging System (INSS); ref. 11]. Patient information and genomic subtypes for 455 published tumors screened for *ALK* mutations were retrieved from the publications or were made available by the authors (Supplementary Table S1; refs. 8–10). In addition, 39 neuroblastoma cell lines were included. The cell lines were obtained from several sources (see Supplementary Table S2). 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 S3). 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).

Array comparative genomic hybridization copy number profiling

To determine DNA copy number alterations, array comparative genomic hybridization (arrayCGH) was done by using an in-house developed 1 Mb resolution bacterial artificial chromosome array (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, 150 ng of tumor and reference DNA were 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; ref. 12), including circular binary segmentation for scoring of DNA copy number alterations (13).

ALK gene expression data

Gene expression data were available for 440 tumors, comprising a published dataset of 251 tumors profiled on custom Agilent 44k arrays (ref. 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 RMAsketch), and an unpublished

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dataset of 88 tumors profiled on the Affymetrix HG-U133plus2.0 platform (normalized using MAS5 and downloaded from the R2 database).[15] Importantly, analyses were done 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 cutoff.

Tissue microarray and immunohistochemistry

For the establishment of a tissue microarray, 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 S4). 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 done using a monoclonal mouse anti-human CD246 ALK antibody (clone ALK1, Dako), and slides were scored for immunoreactive neuroblastoma cells where 0 meant no or weak staining intensity (<10% of the cells), 1 meant weak staining intensity (10-50% of the cells), 2 meant medium staining intensity (50-80% of the cells), and 3 meant 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 done when the neuroblastoma cell lines reached 70% confluency. 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% fetal bovine serum and 1 ng/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 done in triplicate. Transduced Ba/F3 cells were selected with G418 (500 μg/mL medium). The amount of viable cells was assayed on regular intervals. For proliferation curves, Ba/F3 cells were washed with PBS, and 5×10^5 cells were seeded in 5 mL medium without IL-3. Viable cells were counted using a Vi-CELL Cell Viability Analyzer (Beckman Coulter) on days 3, 5, 7, and 9.

Statistical analysis

Fisher's exact tests, Mann-Whitney tests, and correlation analyses were done using R (version 2.8.1). The R Survival package was used to generate Kaplan-Meier plots and to carry out log-rank analyses. Multivariate logistic regression analysis was done 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, nonfamilial primary neuroblastoma tumors, a total of 17 *ALK* mutations (6.7%) and 2 *ALK* amplifications (0.8%) were identified (Supplementary Fig. S1). 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 3.9% (10 of 254) and 2.0% (5 of 254) of the cases, respectively. 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; ref. 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 of 17 patients with an *ALK* mutation showed that these mutations were somatically acquired.

Mutation analysis was also done 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 S1).

Meta-analysis of *ALK* mutations reveals an increased occurrence of the F1174 mutation in *MYCN*-amplified tumors

We carried out 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 S1). The 709 samples had in total 49 *ALK* mutations (6.9%). The two most frequently occurring mutations were the F1174 (34.7%) and R1275 mutations (49%; Supplementary Fig. S1). When the entire group of different *ALK* mutations was taken into account in the evaluation of the overall frequency of *ALK* mutations, no significant difference was observed for the frequency of mutations in favorable (INSS 1, 2, and 4S; 14 of 245, 5.7%) versus unfavorable (INSS 3 and 4; 33 of 440, 7.5%) neuroblastomas (Fisher's exact test $P = 0.087$). 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*

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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

	Amplification or mutation vs wild-type	Mutation vs wild-type	Amplification vs wild-type	F1174 versus wild-type	R1275 versus wild-type	F1174 versus R1275
MYCN status	2.05E-04*	7.74E-02†	6.49E-07*	1.16E-03*	6.09E-01	5.31E-02†
1p-deletion‡	9.94E-02†	8.21E-01	8.03E-02†	3.57E-01	7.68E-01	2.91E-01
3p-deletion‡	3.45E-02†	5.54E-02†	1.00E-00	1.00E-00	2.36E-01	1.00E-00
11q-deletion‡	5.45E-02†	8.19E-02†	1.00E-00	1.00E-00	2.09E-01	5.00E-01
2p-gain‡	7.98E-01	1.00E-00	1.00E-00	6.02E-01	1.00E-00	5.26E-01
17q-gain‡	6.85E-01	6.72E-01	1.00E-00	7.03E-01	1.00E-00	1.00E-00
Age (<> 1 year)	4.07E-01	4.47E-01	7.73E-01	8.09E-02†	6.58E-01	9.33E-02†
Stage	9.94E-02†	1.66E-01	4.64E-01	6.70E-01	4.56E-01	8.13E-01
Genomic subtype	9.32E-04*	9.35E-02†	5.04E-06*	2.20E-02*	7.71E-01	3.92E-01

NOTE: The last column gives the results of the comparison of the cases with the F1174 mutation versus cases with the R1275 mutation.

*$P < 0.05$.

†$P < 0.1$.

‡Data are based on two of the four datasets for which detailed arrayCGH data were available.

status in the F1174 mutated cases versus the tumors with wild-type *ALK* ($P = 0.001$; Fig. 1A and Table 1). *ALK* F1174 mutations were found in 1.3% of the *MYCN*-single copy tumors, compared with 6.1% of *MYCN*-amplified tumors. Of the 17 tumors with the F1174 mutation, 58.8% had *MYCN* amplification, compared with a frequency of *MYCN* amplification of 21.6% in the tumors with wild-type *ALK* (Fig. 1A). In contrast, the frequency of *MYCN* amplification was similar for R1275 mutated versus wild-type cases. As most cell lines were *MYCN* amplified, we sequenced 39 neuroblastoma cell lines to verify whether the association of F1174 with *MYCN* amplification could also be detected. Indeed, F1174 mutations were present in 5 of 27 *MYCN*-amplified cell lines whereas only one R1275 mutation was present in this cohort (Supplementary Table S2).

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%), with 79 remaining unclassified (Supplementary Fig. S2).

A 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 of 49) were present in subtype 1 tumors (Fig. 1B).

Correlation of *ALK* mutation with survival

No significant survival differences were found in tumors with or without *ALK* mutations (or amplifications; log-rank $P = 0.317$; Fig. 2A). However, when the survival of patients with R1275 mutation or wild-type patients was compared with patients with the F1174 mutation type, Kaplan-Meier analysis showed significant survival differences ($P = 0.027$ and $P = 0.002$; Fig. 2B and C). This might largely be explained by the high frequency of *MYCN* amplification within the F1174 mutated tumors compared with the R1275 mutated tumors.

Interestingly, although not statistically significant, we noticed that 9 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 a particularly poor survival as compared with 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 of 709 tumors (1.7%), and none of these carried activating *ALK* mutations. All *ALK*-amplified tumors, except for one, were also found to be *MYCN* amplified ($P < 0.001$), 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

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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 of

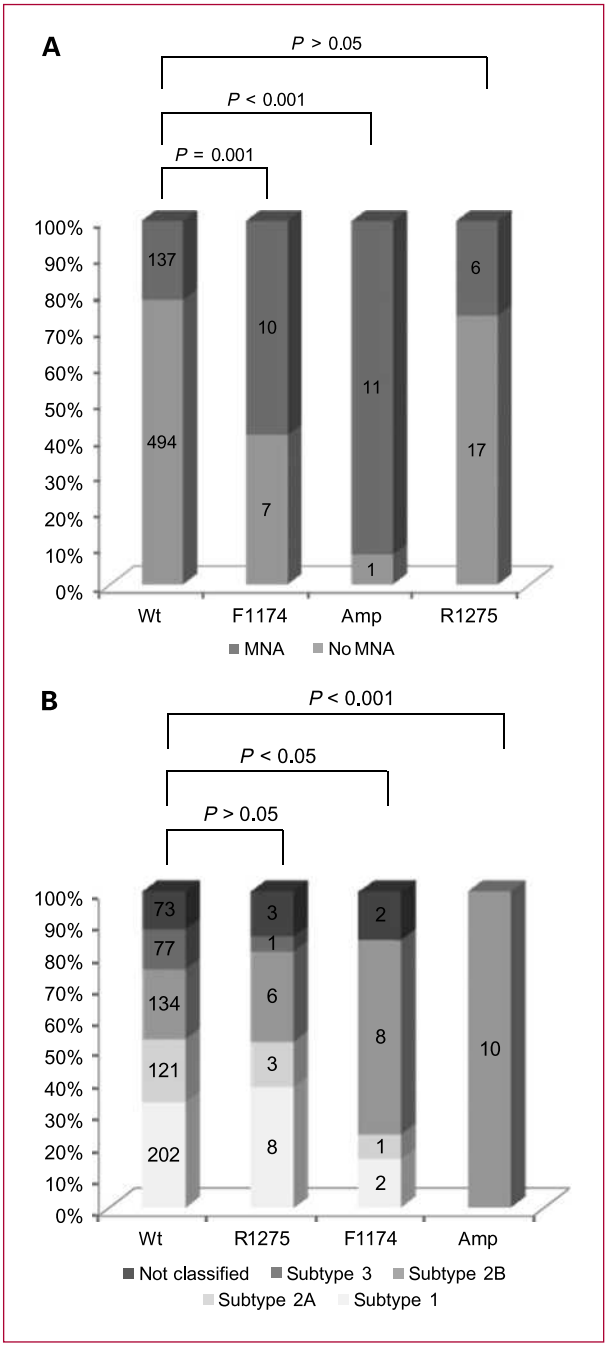

Fig. 1. Comparison of the distribution of *MYCN* status (A) and genomic subtype (B) in tumors with one of the two frequent *ALK* mutations or *ALK* amplification versus wild-type tumors. Wt, wild-type *ALK*; Amp, *ALK* amplification; MNA, *MYCN* amplification.

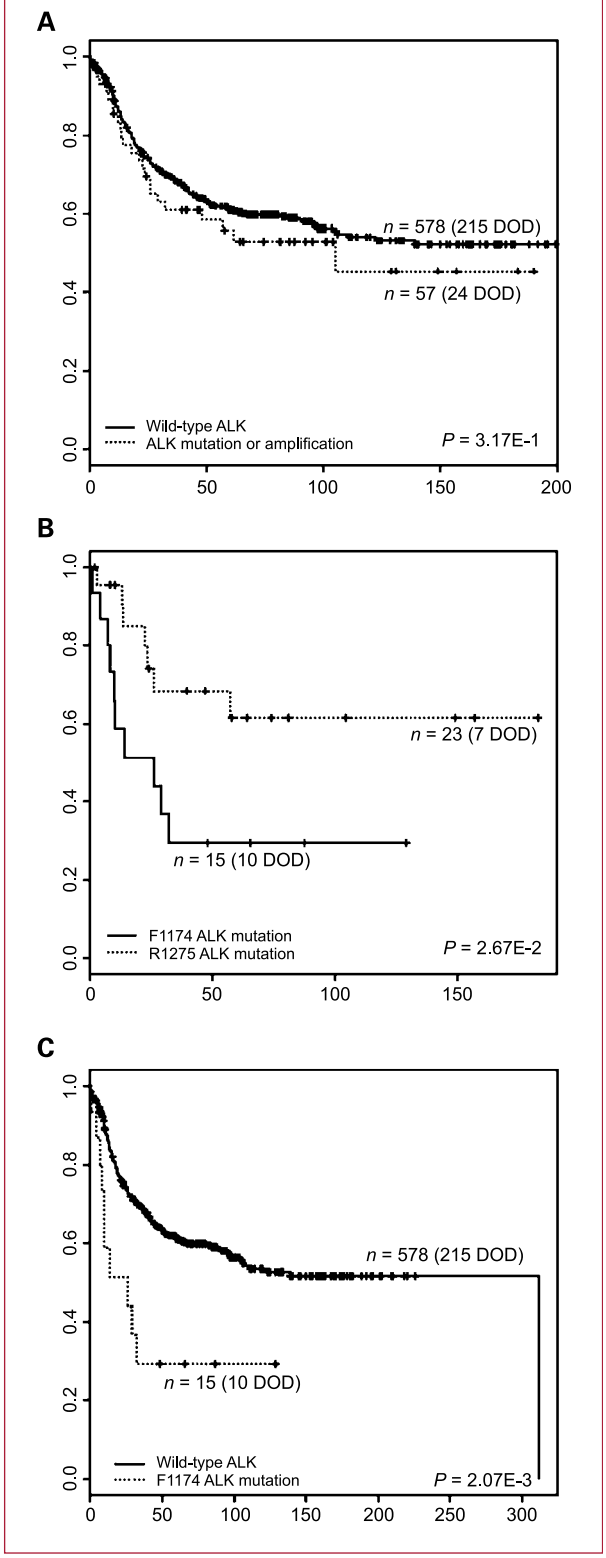

Fig. 2. Kaplan-Meier and log-rank analysis of *ALK*-mutated and *ALK*-amplified tumors versus *ALK* wild-type cases (A), F1174 mutated versus R1275 mutated cases (B), and F1174 mutated versus wild-type cases (C). DOD, dead of disease.

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254) and 17.7% (45 of 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 to 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 Fig. S3). 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 of 17 mutated tumors (11.8%), indicating that 2p gain is not a common mechanism for increasing mutated *ALK* copy number.

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 Fig. S4). 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 analyzed expression levels in three independent datasets including a total of 440 tumor samples (14).[16] Each analysis was done 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 <0.05; Fig. 3A-C). Multivariate logistic regression analysis (in a model testing *MYCN* status, age, stage, and *ALK* expression) could show an independent prognostic value for *ALK* mRNA expression in the larger dataset (ref. 14; odds ratio, 2.94; 95% confidence interval, 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 microarray containing 70 primary tumors. Kaplan-Meier and log-rank analysis show a significant correlation between ALK protein expression and overall survival (OS; $P = 0.014$) and progression-free survival (PFS; $P = 0.002$; Fig. 4). The three cases with *ALK* mutation (R1275Q) present on the tissue microarray had 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 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 carried out Western blot analysis rather than immunohistochemistry experiments on a panel of 22 neuroblastoma cell lines. This allowed for comparison of *ALK* mRNA expression levels ($P = 0.011$) and native ALK protein levels ($P = 1.55E-07$) versus p-ALK protein level. We clearly show a significant correlation between both *ALK* mRNA expression levels and native ALK protein levels with p-ALK protein levels (Supplementary Fig. S5). 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 do cell lines with the other hotspot mutation. Moreover, cell lines with wild-type ALK have very low levels of phosphorylated ALK (Supplementary Figs. S6 and S7).

Transforming capacity of *ALK* hotspot 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 *ALK* F1174L transformed the cells significantly faster than did Ba/F3 cells expressing *ALK* R1275Q (Fig. 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 autophosphorylation and transformation capacity of Ba/F3 cells than does 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

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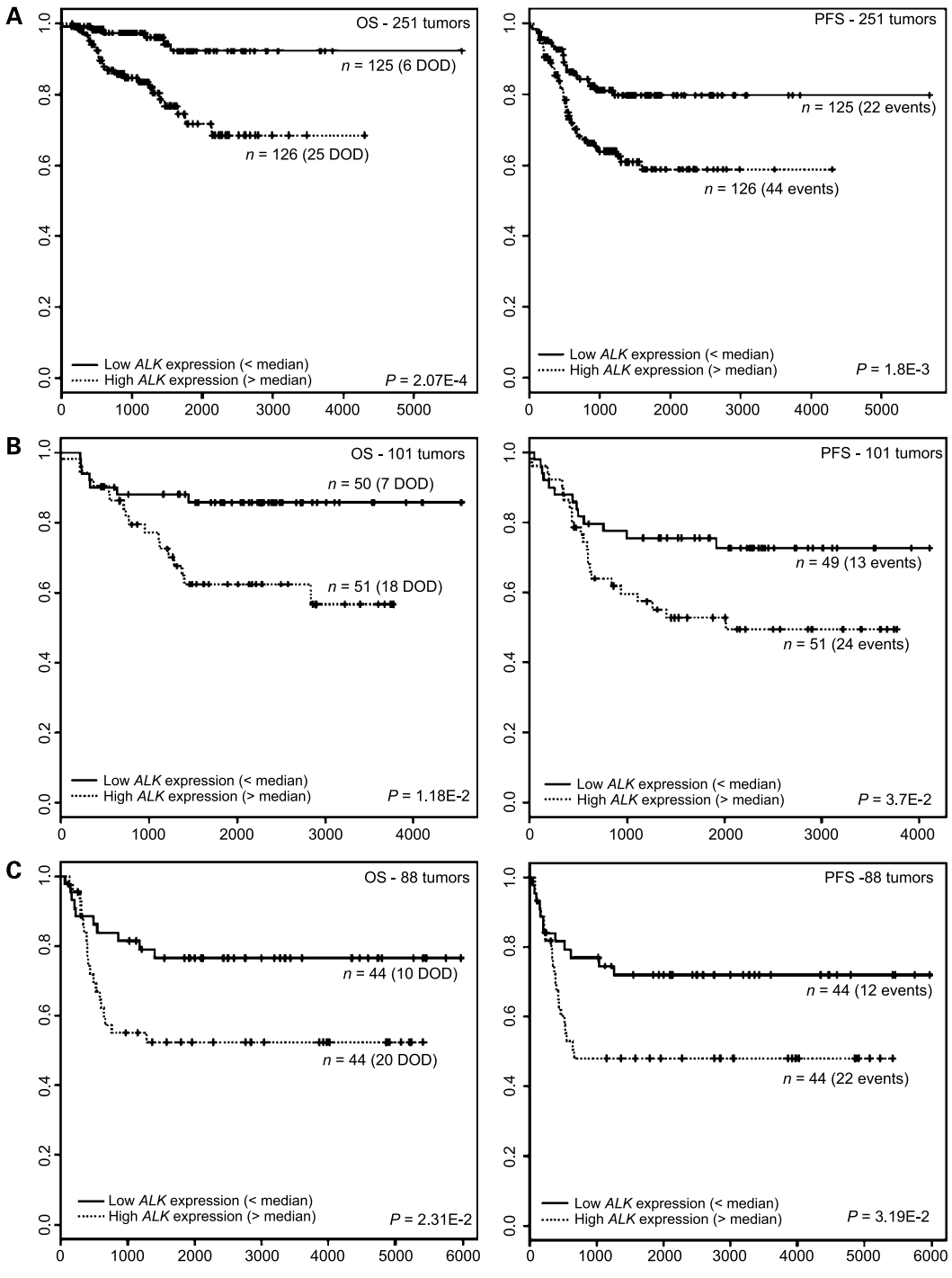

Fig. 3. Kaplan-Meier and log-rank analysis for overall (OS) and progression free survival (PFS) comparing tumors with high and low *ALK* mRNA expression in a published set of 251 neuroblastoma tumors (A; ref. 14), and in unpublished sets of 101 (B) and 88 (C) neuroblastoma tumors.

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.

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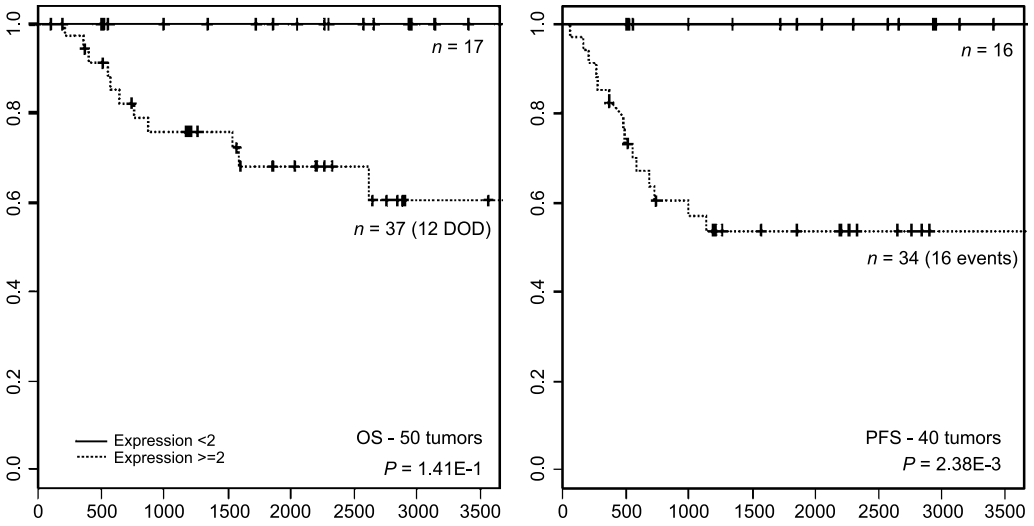

Fig. 4. Kaplan-Meier and log-rank analysis for overall (OS) and progression free survival (PFS) comparing tumors with high (expression ≥2) and low (expression <2) ALK protein expression. *X*-axis, number of days.

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 1 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 of 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 (7). In our hands, the F1174L mutant transformed the Ba/F3 cells more efficiently than did the cells expressing the R1275Q mutant. This correlated with higher autophosphorylation levels of ALK F1174L, which was not observed for the R1275Q mutant.

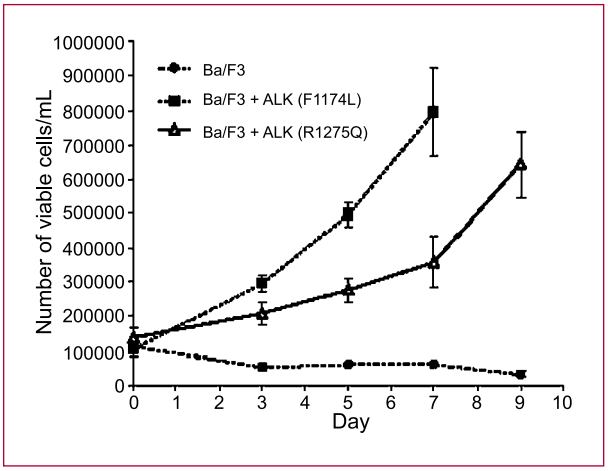

Fig. 5. Proliferation curve of Ba/F3 cells stably expressing the F1174L or R1275Q ALK mutants. Ba/F3 cells expressing mutant *ALK* as well as parental Ba/F3 cells were grown in the absence of IL-3 for a period of 9 days. A day 9 value for ALK F1174L was not included in the figure because the culture had reached maximal density before this time point.

The occurrence of mutations of particular genes in relation to genomic subgroups has been reported in certain tumor entities, such as *PIK3CA* mutations in head and neck squamous carcinomas without *EGFR* amplification and β-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 done in patients with low-stage tumors, and the 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 shown 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

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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 break points for 2p gains but almost consistent presence of *ALK* in these segments, one could assume that low copy number gain of *ALK* could also imply 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 showed 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 shows for the first time 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 suggest a poor prognosis in patients with *MYCN* amplification, but further studies are needed to substantiate this hypothesis. Furthermore we could show that the F1174L mutant displayed a higher degree of ALK phosphorylation and tumorigenicity than did the R1275Q mutant. No significant difference was observed in the frequency of *ALK* mutations between low- and high-stage tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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