Characterization of Rearrangements Involving the ALK Gene Reveals a Novel Truncated Form Associated with Tumor Aggressiveness in Neuroblastoma

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Abstract
Activating mutations of the ALK gene have been identified in sporadic and familial cases of neuroblastoma (NB), a cancer of the peripheral nervous system, and are thought to be the primary mechanism of oncogenic activation of this receptor in this pediatric neoplasm. To address the possibility that ALK activation may occur through genomic rearrangements as detected in other cancers, we first took advantage of high-resolution array-comparative genomic hybridization to search for ALK rearrangements in NB samples. Using complementary experiments by capture/paired-end sequencing and FISH experiments, various types of rearrangements were fully characterized, including partial gains or amplifications, in several NB cell lines and primary tumors. In the CLB-Bar cell line, we described a genomic rearrangement associated with an amplification of the ALK locus, leading to the expression of a 170 kDa protein lacking part of the extracellular domain encoded by exons 4 to 11, named ALK4-11. Analysis of genomic DNA from the tumor at diagnosis and relapse revealed that the ALK gene was amplified at diagnosis but that the rearranged ALK allele was observed at the relapse stage only, suggesting that it may be implicated in tumor aggressiveness. Consistently, oncogenic and tumorigenic properties of the ALK4-11 variant were shown after stable expression in NIH3T3 cells. Moreover, we documented an increased constitutive kinase activity of this variant, as well as an impaired maturation and retention into intracellular compartments. These results indicate that genomic rearrangements constitute an alternative mechanism to ALK point mutations resulting in receptor activation. Cancer Res; 73(1); 195–204. ©2012 AACR.
ALK signaling that has been shown to induce cell transformation in vitro and in vivo (4, 5). The ALK receptor is, therefore, an attractive therapeutic target in an expanding number of human malignancies, both in children and adults (16).

In the present work, we fully characterized various types of rearrangements involving the ALK gene in several NB cell lines and primary tumors. We documented a rearrangement leading to a novel truncated ALK variant, exhibiting oncogenic properties and associated with tumor aggressiveness.

Materials and Methods

NB cell lines and primary tumors

NB cell lines have been described previously (17–19). The CLB-Bar cell line was established from a stage 4 NB tumor at relapse. A subset of primary NB tumors previously characterized by whole-genome array-comparative genomic hybridization (CGH) was further analyzed in this study (15).

Array-CGH analysis

A custom NimbleGen ultrahigh density oligonucleotide array was designed for this study. We used a 4-plex format with 72,000 features starting from chromosome 2 position 29,249,000 to position 30,018,000 [all base pair positions are referring to human genome build 36 (hg18)], thereby covering the entire ALK gene and extending 20 kb upstream and downstream of the gene with 1 feature every 11 bases. The precise procedure is described in supplementary methods. Twenty-six cell lines were analyzed on this array: 106C, CHP212, CLB-Bar, CLB-Bar, CLB-Br, CLB-Car, CLB-Ga, CLB-Ge, CLB-Ma, CLB-Re, CLB-Tr, GI-M-EN, IMR32, LAN-1, N206/Kelly, SJNB-1, SJNB-6, SJNB-8, SJNB-12, SK-N-AS, SK-N-BE, SK-N-BE(2C), SK-N-BE(M17), SK-N-DZ, SK-N-SH, and TR14. We also studied three primary tumors showing amplification at the ALK locus, 1 tumor with amplification near the ALK locus and 9 tumors exhibiting 2p gain, with breakpoint in the vicinity of the ALK gene.

For a subset of samples, Affymetrix Genome-Wide Human SNP 6.0 Array was used to obtain a whole genomic profile according to Affymetrix recommendations (Supplementary Methods).

Capture and paired-end sequencing

The Sure Select method (Agilent Technologies) was used to capture regions of interest. The bait library was designed using the Agilent e-array software. The complete ALK genomic locus, together with 50 kb upstream and 30 kb downstream of the gene, was covered at ×5, excluding repeated regions. Boundaries of other amplified regions on the 2p chromosome of IMR32 and NB838 samples, defined by SNP 6.0 arrays, were also included in the captured regions (Supplementary Table S1). Libraries for paired-end sequencing were constructed for 10 samples (CLB-Bar, CLB-Br, CLB-Ga, CLB-Ge, CLB-Ma, CLB-Re, IMR32, N206, NB838, and SJNB8) from genomic DNA according to the manufacturer's protocol (Illumina). Capture was conducted on the bait library according to the protocol provided by Agilent Technologies. Paired-end sequencing (2 × 50 bp) was conducted on the Illumina Genome Analyzer II. Reads were analyzed as described in Supplementary Methods.

PCR validation of identified rearrangements

Information about primers used to confirm rearrangements predicted by capture and paired-end sequencing is listed in Supplementary Table S2.

FISH experiments

For FISH experiments, we used either the LSI ALK dual color, Break Apart Rearrangement probe (Vysis) or bacterial artificial chromosome (BAC) probes or five-kb PCR products synthesized using TaKaRa ExTaq (Lonza) labeled with various fluorochromes by a nick-translation kit (Vysis). Primers used to generate the PCR fragment probes as well as BACs used for FISH experiments are listed in Supplementary Table S3. FISH experiments were conducted as previously described (19). Images were captured with Metamorph software and analyzed with ImageJ software.

ImmunobLOTS

Total ALK was detected using Zymed 18-0266 rabbit polyclonal antibody (Invitrogen). An antibody (number 3341, Cell Signaling Technologies) directed against the phosphorylated Y1604 residue was used to reveal phosphorylated ALK. Western blot analyses were conducted using anti-ERK1/2 (9102), antiphospho-ERK1/2 (4377), anti-AKT (9272), antiphospho-AKT (4056), anti-STAT3 (9132), and antiphospho-STAT3 (9145) antibodies from Cell Signalling Technologies.

ALK immunoprecipitation and proteomic analysis

Cell extracts were prepared as previously described (10). 10 mg of proteins were incubated with a mix of rabbit polyclonal and mouse monoclonal anti-ALK antibodies (20) for 2 hours at 4°C then with protein G Sepharose beads (Sigma) for 1 hour at 4°C. The immunoprecipitates were washed twice with cold PBS supplemented with 0.1% Triton then resuspended in gel loading buffer (50 mmol/L Tris pH = 8.2, Urea 8M, Thiouraea 2M) and resolved by SDS-PAGE. For proteomic analysis, label-free quantitative analysis of immunopurified proteins was conducted as described in Supplementary Methods.

Transforming potential of ALK

NIH3T3 cells were cultured as previously described (21). The cDNA encoding the ALK variant was generated from total RNA of the CLB-Bar cell line using high-fidelity PCR. Its sequence was checked by Sanger sequencing and cloned into the pcDNA3 expression vector. Stably transfected cells were obtained after transfection using Lipofectamine LTX reagent and selected by 400 μg/ml geneticin (Gibco).

For proliferation assays 20,000 stably transfected cells were plated in duplicates in 24-well plates. The number of living cells was counted 3 and 6 days after using a Vi-cell XR counter (Beckman Coulter). For soft agar assays, 250,000 stably transfected cells were mixed in 0.3% agar with complete medium and plated on 0.3% agarose-coated 6-well plates (in triplicate). After 17 days, colonies were manually counted. Tumor formation was evaluated 21 days after subcutaneous injections of 1 million transfected cells in Nude mice.
In vitro kinase assay

In vitro kinase assay was conducted as previously described (10) using 500 μg of NIH3T3 cell lysates.

Deglycosylation of ALK with F and H-endoglycosidases and cell surface protein biotinylation

Deglycosylation and cell surface protein biotinylation experiments were conducted on NIH3T3 cells stably expressing WT ALK or the ALK

Results

Copy number changes within the ALK gene in NB samples identified by high-resolution array-CGH

To identify rearrangements targeting the ALK locus, we searched for intragenic transitions in copy number using an ultrahigh density array covering the entire ALK gene in 13 primary tumors selected for ALK amplification or 2p gain and in 26 NB cell lines (see Material and Methods). The whole genomic profile of cases presenting such rearrangements associated with ALK amplification was further explored using SNP 6.0 arrays. Fig. 1A shows amplification of the ALK locus as well as several other amplified regions on the 2p arm in the CLB-Bar, IMR32, and NB838 samples. Analysis of the ALK locus with our ultrahigh density array indicated that these 3 samples had amplified and rearranged ALK alleles. In the CLB-Bar cell line, most of the locus was amplified; however, a 39-kb region from 29,668,100 to 29,707,200 positions in intron 3 showed an absence of amplification (Fig. 1B). In the IMR32 cell line, array-CGH identified a partial amplification of the ALK locus, between positions 29,550,200 and 29,777,700, the corresponding breakpoints being located in introns 4 and 2, respectively. For the NB838 tumor, harboring a whole ALK amplification, we observed a breakpoint in intron 2 close to position 29,790,000. In five samples, copy number changes were observed within the ALK locus in the absence of amplification (Fig. 1B).
Various ALK rearrangements characterized by capture and paired-end sequencing

To further explore ALK rearrangements observed by array-CGH and document the nature of the involved partner, we conducted paired-end sequencing after DNA capture with a specific bait library. Files were processed to identify genomic rearrangements that matched with the breakpoints detected by array-CGH. For the breakpoints identified by array-CGH for which this analysis did not identify any rearrangement, a further exploration of the capture data files was conducted with less stringent filters in the vicinity of the expected regions of breakpoint.

The capture and paired-end sequencing strategy identified an inverted duplication in CLB-Br and a large duplication in SJNB8, which were experimentally validated (Supplementary Table S2). In CLB-Br, the rearrangement was shown to be somatic, as it was not detected in the lymphocytes of the patient (data not shown). This rearrangement targeted the intron 11 of the ALK gene, however the exact position of the breakpoint could not be determined because of homology between regions of the 2p chromosome involved in this duplication, i.e. sequences extending from positions 29,335,931 to 29,336,086 and from positions 29,336,256 to 29,336,425. No fusion gene is expected from this rearrangement. In SJNB8, the ALK gene broken in exon 23 is fused to intron 11 of the CLIP4 gene. These 2 genes are fused in the opposite transcriptional orientation and are therefore not expected to lead to fusion transcripts and proteins.

For the CLB-Re cell line, at least seven copy number translocations were detected within the ALK locus (Fig. 1B) and the capture approach allowed the identification of several intra and interchromosomal rearrangements that were experimentally validated. In particular three deletions were confirmed, as well as 3 interchromosomal rearrangements with chromosome 3 (Supplementary Table S2). FISH analysis revealed the presence of 2 abnormal derivatives in all examined metaphases of this tetraploid cell line (18), which is consistent with a highly rearranged ALK gene in a homogeneous cell population (Supplementary Fig. S1).

In the CLB-Ga cell line, the capture approach identified a rearrangement between the intron 1 of the ALK locus and a telomeric sequence (Supplementary Table S2). The repeated structure of the telomeric sequence precluded the design of primers for PCR confirmation. However, sequencing of larger fragments using a whole-genome mate-pair strategy (unpublished results) identified the same rearrangement and allowed PCR primers design and validation on genomic DNA. The precise nature of the telomere involved in this rearrangement remains unknown at that time.

For the NB176 primary tumor, inverse PCR (Supplementary Methods) allowed the characterization of an unbalanced...
translocation between the \textit{ALK} gene and a region of chromosome 1 that does not contain any gene (Supplementary Table S2). The positions of the breakpoints are fully consistent with the array-CGH profile showing 1p deletion and 2p gain in this sample.

The case of the N206/Kelly cell line is different. Indeed, a translocation involving the \textit{ALK} locus, detected by FISH analysis using the Break Apart Vysis probe, has been reported in this cell line (22). Interestingly, we did not observe any transition in copy number within the \textit{ALK} locus, neither with our specific array, nor with the SNP 6.0 array. Nevertheless, the capture and paired-end sequencing approach allowed the identification of an interchromosomal rearrangement between the \textit{ALK} gene and the \textit{CLPTM1L} gene located on chromosome 5 (Supplementary Table S2). Reverse-transcriptase PCR (RT-PCR) confirmed the expression of a fusion transcript between exon 4 of the \textit{ALK} gene and exon 8 of the \textit{CLPTM1L} gene (data not shown). As the \textit{CLPTM1L} sequence is not in frame, a fusion protein of 405 amino acids is predicted containing the first 385 amino acids of the \textit{ALK} receptor rapidly followed by a STOP codon. FISH analysis with several BACs scattered over the \textit{ALK} gene clearly showed that this cell line harbors 2 normal copies of the \textit{ALK} gene and 1 copy disrupted between positions 29,351,163 and 29,513,137 (Supplementary Fig. 2 and Supplementary Table S2). This observation is, therefore, consistent with the absence of any transition in copy number within the \textit{ALK} locus and suggests that one part of the disrupted \textit{ALK} copy may be translocated to chromosome 5.

\textbf{Complex patterns of 2p coamplification involving the \textit{ALK} gene}

For IMR32, the capture and paired-end sequencing strategy indicated that the 2 breakpoints located on each side of the \textit{ALK} partial amplicon were pasted to a part of chromosome bands 2p14 and 2p16.2, also amplified in this cell line. Additional links between the other 2p amplicons were detected as indicated in Supplementary Table S2 and confirmed by PCR. Altogether, it is possible to draw a scheme representing the structure of a complex amplicon containing various parts of chromosome 2, including a piece of the \textit{ALK} gene (Fig. 2A). FISH experiments confirmed that the different parts of this structure were indeed present in a common homogeneous
staining region (hsr), previously identified on a derivative chromosome der1 t(1qter-1p32:17q21-17qter(inshsr2)) (ref. 18; Fig. 2B).

We applied the same approach for the primary tumor NB838 and the breakpoint revealed by array-CGH was precisely mapped at position 29,778,881 in the second intron of the ALK gene (Supplementary Table S2). The ALK gene was fused to repeated sequences precluding the identification of the exact partner involved in the rearrangement. A closer examination of the capture and paired-end data revealed other rearrangements within the ALK gene with various 2p amplicons, indicating a highly rearranged ALK locus in this tumor (data not shown).

Finally, for the CLB-Bar cell line, the same strategy revealed a more complex pattern of rearrangements targeting the ALK gene than first expected from the ultrahigh density array. It indeed confirmed the 2 breakpoints initially detected by array-CGH but also revealed additional breakpoints within the ALK locus (Supplementary Table S2). A more detailed examination of this array was consistent with these results, and other 2p amplified regions detected by SNP 6.0 array appeared to be involved in some rearrangements with the ALK locus (Supplementary Table S2 and Supplementary Fig. S3). Importantly, the rearrangement between positions 29,328,032 (intron 11) and 29,707,160 (intron 3) juxtaposes the region encoding exons 1 to 3 to the one encoding exons 12 to 29 of the ALK gene (Fig. 3A).

This rearrangement was validated by PCR and complementary experiments revealed that a variant ALK protein is produced from this genomic rearrangement (vide infra). FISH experiments confirmed that the 2 ALK pieces involved in the rearrangement, A1 and A4, were coamplified in a common population of dmin (Fig. 3B). These dmin also contained the 2p ampiclons located at around 9, 14, 20, and 22 millions, as well as the A2 and A3 regions of the ALK gene. The MYCN locus was amplified in a distinct population of dmin (Supplementary Fig. S3).
The rearranged ALK locus is only observed at relapse

As the CLB-Bar cell line was derived from a NB tumor at relapse, we sought to determine when ALK amplification and rearrangement occurred. First, FISH analysis with MYCN and ALK probes on tumor appositions revealed a great heterogeneity in the amplification of both loci in the tumor at diagnosis (Fig. 3C, left). At relapse, heterogeneity was significantly reduced and most of the nuclei exhibited amplification of MYCN and ALK (Fig. 3C, right). Second, PCR analysis indicated that the rearranged ALK locus was present in the tumor at relapse, and is therefore not an event acquired during the culture process. However, this rearrangement was not detected in DNA extracted from the tumor at diagnosis (Fig. 4A). SNP 6.0 array analysis revealed that the pattern of amplicons on chromosome 2p in the CLB-Bar cell line is similar to the profile of the tumor at relapse but different from the profile of the tumor at diagnosis (Fig. 4B). Strikingly, the ALK locus was already amplified in the tumor at diagnosis (Fig. 4B and C) but, in agreement with data obtained by PCR, it did not appear rearranged in the tumor at diagnosis (Fig. 4C). Altogether, these results indicate that the ALK gene was amplified at diagnosis in a subset of tumor cells and that the rearranged allele was observed at the relapse stage only, suggesting that it may be implicated in the aggressiveness of the tumor.

Characterization of the ALK$^{\text{D4–11}}$ protein variant in the CLB-Bar cell line

We further showed that the amplified and rearranged ALK allele in the CLB-Bar cell line is expressed at the protein level. Indeed, Western blot analysis revealed that a variant protein of 170 kDa was the main ALK form in this cell line (Fig. 5A). Mass spectrometry analysis determined that a part of the extracellular domain was missing in this 170 kDa form (Fig. 5B). A peptide overlapping the junction of the ALK variant between amino acids 317 and 681 was detected in the proteomic analysis confirming that the variant lacks the amino acids encoded by exons 3 and 681 (V, first amino acid encoded by exon 12).

Oncogetic properties and impaired maturation of ALK$^{\text{D4–11}}$

To further characterize the ALK$^{\text{D4–11}}$ variant, it was stably expressed in NIH3T3 cells (Fig. 6A). As compared with the WT receptor, this variant exhibited a much stronger in vitro kinase activity (Fig. 6B). Moreover, expression of the ALK$^{\text{D4–11}}$ variant favored NIH3T3 cell proliferation compared with the WT ALK receptor (Fig. 6C) and led to a higher number of colonies in a soft agar assay (Fig. 6D and E). Finally, subcutaneous injection in nude mice showed that cells expressing this variant induced tumors with a higher frequency (5/5) compared with cells expressing the WT receptor (2/5).

We also analyzed the glycosylation status and the subcellular localization of the ALK$^{\text{D4–11}}$ receptor. Figure 7A shows that the
ALK^{Δ4–11} variant was present as a major band sensitive to endoglycosidase H treatment, as it is the case for the lower band of the 220 kDa doublet of WT ALK. The various forms of WT ALK and the ALK^{Δ4–11} variant were sensitive to endoglycosidase F. These data provide evidence that the ALK^{Δ4–11} variant corresponds to a partially glycosylated form of ALK likely retained in the endoplasmic reticulum. Biotinylation experiments confirmed that the ALK^{Δ4–11} variant was indeed intracellular, as it was revealed by ALK antibodies but not by biotin, as the lower band of the 220 kDa doublet of WT ALK (Fig. 7B). The analysis of a subset of potential downstream targets of the ALK receptor did not reveal any significant differences between the ALK^{Δ4–11} form and the WT receptor (Fig. 7C).

Discussion

The present study reports that the ALK locus is targeted by rearrangements at a high frequency (23%) in NB cell lines and that rearrangements are also observed in a subset of NB tumors. ALK amplification and some intragenic rearrangements have been previously reported in NB but not characterized in details (6, 10, 11, 23, 24). Our results show that, in contrast to ALC, IMT, or NSCLC, neither the breakpoints observed within the ALK locus nor the involved partners were recurrent in the studied NB samples. It is noteworthy to mention that the ALK gene is highly expressed in most NB, which may render the locus especially prone to rearrangements. Such a link between transcriptional regulation and genesis of genomic aberrations has recently been reported in prostate tumors presenting with a TMPRSS2-ERG fusion, in which rearrangements breakpoints were enriched near open chromatin (25).

This work identified 9 samples, including 7 cell lines and 2 primary tumors, in which the ALK gene is targeted by rearrangements. In 3 cases, we could document that abnormal transcripts are expressed from these rearrangements. In the N206 sample, we identified a chimeric transcript between ALK and CLPTM1L that was not in frame and the biologic function of which remains to be defined. We observed a highly rearranged ALK locus in the CLB-Re sample. Recently, a whole-genome mate-pair analysis of this sample revealed an extensive number of rearrangements between 2 regions of chromosomes 2p and 3p (Boeva, Jouannet and colleagues, submitted for publication). This pattern of rearrangements potentially corresponds to the phenomenon of chromothripsis (26) that may provide an explanation for the multiple breakpoints observed in the ALK gene. RNA-Seq analysis using a paired-end strategy confirmed the chimera predicted from our capture data between exon 11 of ALK and exon 2 of FHIT (data not shown). Subsequent RT-PCR indicated that the fusion was not in frame. It remains to be determined whether the truncated ALK formed from this rearrangement harbors biologic activity.

Figure 6. Oncogenic properties of ALK^{Δ4–11}. A, Western blot analysis of NIH3T3 cells stably expressing ALK WT, ALK^{Δ4–11}, or ALK R1275Q. B, increased kinase activity of the ALK^{Δ4–11} variant. 32P incorporated in ALK immunoprecipitates is visualized by autoradiography (top) before immunoblotting with an anti-ALK antibody (bottom). C, NIH3T3 cells expressing ALK^{Δ4–11} showed increased proliferation compared with those expressing ALK WT. The number of living cells is reported at various time points. D, increased colony formation in soft agar assay of NIH3T3 cells expressing ALK^{Δ4–11} as compared with cells expressing ALK WT. E, colony number quantification of soft agar experiments conducted in triplicates.
We can, therefore, speculate that the ALK activation in a subset of NB samples through deletions of part of the extracellular domain of the receptor. These observations are reminiscent of the case of 2 others RTK, the PDGFRα and the delta epidermal growth factor receptor (ΔEGFR)/EGFRvIII for which in-frame deletion mutants of the extracellular domain have been described as potent oncogenes in various human cancers (28–31). These variant receptors have notably been shown to be activated in a ligand-independent manner, which could be the case for the ALKΔ4–11 as it lacks the putative ligand-binding site and allows proliferation of NIH3T3 cells under low serum conditions.

In conclusion, we document a novel ALK truncated form bearing oncogenic properties and associated with tumor aggressiveness. Our data suggest that it may be useful to investigate ALK amplification and rearrangement at the time of relapse in NB patients with refractory tumors using high-resolution SNP profiling or RT-PCR experiments. Patients presenting with tumors exhibiting ALK amplification and/or deletion of the extracellular domain may potentially benefit from ALK-targeted therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Figure 7. Impaired maturation of ALKΔ4–11 and analysis of downstream effectors. A, cell lysates from NIH3T3 cells expressing ALK WT or ALKΔ4–11 were untreated (C), treated with endoglycosidase F (F) or H (H), then analyzed by Western blot analysis using polyclonal anti-ALK. ALKΔ4–11 is endoglycosidase H sensitive, indicating an impaired protein maturation. B, biotinylation experiment showing total ALK protein detected with polyclonal anti-ALK in red and biotinylated proteins detected with streptavidin in green. The ALKΔ4–11 variant is not stained by biotin, showing its intracellular retention. C, cell extracts from NIH3T3 cells expressing ALK WT or ALKΔ4–11 were analyzed for pERK, pAKT, and pSTAT3.

In the CLB-Bar cell line, the genomic rearrangement targeting the ALK gene leads to the expression of a novel variant form of the ALK receptor. We have previously shown that this variant was highly phosphorylated as revealed by an antiphosphothreonine Y1586 ALK antibody (10). Using NIH3T3 cells, we have now documented an increased kinase activity of this variant compared with the WT receptor as well as its impaired maturation and intracellular retention. We also showed that the ALKΔ4–11 form exhibits oncogenic and tumorigenic properties. Such properties are similar to the ones reported for another short ALK form, ALKΔ1–3, also truncated in its extracellular domain (27). Whereas Okubo and colleagues observed an increased phosphorylation of STAT3 in cells expressing the ALKΔ1–3 form, expression of the ALKΔ4–11 variant was not associated with increased phospho-STAT3. This suggests that further characterization of signaling pathways triggered by the ALKΔ4–11 variant will therefore be required. Our results indicate that although rare, genomic rearrangements associated with amplification may lead to ALK activation in a subset of NB samples through deletions of part of the extracellular domain of the receptor. These observations are reminiscent of the case of 2 others RTK, the PDGFRα (PDGR receptor α) and the delta epidermal growth factor receptor (ΔEGFR)/EGFRvIII for which in-frame deletion mutants of the extracellular domain have been described as potent oncogenes in various human cancers (28–31). These variant receptors have notably been shown to be activated in a ligand-independent manner, which could be the case for the ALKΔ4–11 as it lacks the putative ligand-binding site and allows proliferation of NIH3T3 cells under low serum conditions.
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