Emergence of New ALK Mutations at Relapse of Neuroblastoma

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A B S T R A C T

Purpose
In neuroblastoma, the ALK receptor tyrosine kinase is activated by point mutations. We investigated the potential role of ALK mutations in neuroblastoma clonal evolution.

Methods
We analyzed ALK mutations in 54 paired diagnosis–relapse neuroblastoma samples using Sanger sequencing. When an ALK mutation was observed in one paired sample, a minor mutated component in the other sample was searched for by more than 100,000× deep sequencing of the relevant hotspot, with a sensitivity of 0.17%.

Results
All nine ALK-mutated cases at diagnosis demonstrated the same mutation at relapse, in one case in only one of several relapse nodules. In five additional cases, the mutation seemed to be relapse specific, four of which were investigated by deep sequencing. In two cases, no mutation evidence in only one of several relapse nodules. In five additional cases, the mutation seemed to be relapse specific, four of which were investigated by deep sequencing. In two cases, no mutation evidence was observed at diagnosis. In one case, the mutation was present at a subclonal level (0.798%) at diagnosis, whereas in another case, two different mutations resulting in identical amino acid changes were detected, one only at diagnosis and the other only at relapse. Further evidence of clonal evolution of ALK-mutated cells was provided by establishment of a fully ALK-mutated cell line from a primary sample with an ALK-mutated cell population at subclonal level (6.6%).

Conclusion
In neuroblastoma, subclonal ALK mutations can be present at diagnosis with subsequent clonal expansion at relapse. Given the potential of ALK-targeted therapy, the significant spatiotemporal variation of ALK mutations is of utmost importance, highlighting the potential of deep sequencing for detection of subclonal mutations with a sensitivity 100-fold that of Sanger sequencing and the importance of serial samplings for therapeutic decisions.

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INTRODUCTION

Current treatment approaches in cancer often lead to initial response followed by secondary progression that presents a therapeutic challenge because of resistance to conventional chemotherapy treatment. Thus, genetic characterization of cancer cells provides invaluable information for the identification of molecular therapeutic targets. Importantly, particular genetic alterations may be selected for or emerge during treatment. Subclonal driver mutations might play a role in tumor progression, and the presence of driver mutation—harboring subclones at diagnosis, which might expand at relapse, has been linked to adverse outcomes.

In neuroblastoma, the most frequent extracranial solid cancer of early childhood, tumor progression is often associated with limited therapeutic possibilities, underlining the need of molecular analyses. Genetic alterations in neuroblastoma at diagnosis mainly concern copy number alterations, with MYCN amplification in 20% to 25% of cases, and other copy number changes over extensive chromosome regions. Only a few recurrently altered genes, such as chromatin-remodeling or neurtogenesis genes, have been reported, targeted by either small interstitial structural alterations or mutations. Activating point mutations in the tyrosine kinase domain of ALK, the most frequent mutations in neuroblastoma, are detected at diagnosis in approximately 8% to 10% of patients and play an important role in neuroblastoma oncogenesis.

These alterations can be targeted using ALK inhibitors, and in vitro and in vivo models have
indicated their potential usefulness in the presence of an activating ALK mutation.18–20 A phase I/II study of crizotinib, a dual ALK/MET inhibitor, suggests possible efficacy in neuroblastoma harboring ALK mutations.21 Thus, it will be crucial to define treatment indications depending on the precise molecular characterization of ALK mutations in neuroblastoma.

Recently a relapse-specific ALK mutation has been described, correlating with unresponsiveness to therapy and indicating that the determination of the ALK status at tumor progression is critical.22 However, the actual frequency of ALK mutations at relapse has not yet been studied. We have studied 54 paired diagnosis–relapse neuroblastoma samples to analyze the frequency of ALK mutations at relapse and to define their potential role in clonal evolution.

### METHODS

**Patients**

Patients with neuroblastoma of all stages were included in this study if tumor samples collected in the participating laboratories both at diagnosis and at relapse were available (24 Swedish, 28 French, and two Belgian patients; Data Supplement). Patients were treated according to relevant national or international treatment protocols (Data Supplement). Ethics approval of protocols was obtained according to national guidelines, and written informed consent was obtained from parents according to national law. In France, this study was authorized by the ethics committee (Comité de Protection des Personnes Sud-Est IV), L07-95, and L12-171. In Belgium, the ethics committee EC/2006-124 approved this work. In Sweden, this study was authorized by the local ethical committees 09-1368, 09-473 (Gothenburg), and 07-069 (Uppsala). Diagnosis samples were obtained from the primary tumor site in the majority of patients (92%) according to clinical guidelines. Relapse samples were obtained from progressing/relapsed primary tumor in 68% of patients and from metastatic sites in 32% of patients (Data Supplement).

**Sanger Sequencing of the ALK Receptor Tyrosine Kinase Domain**

Paired diagnosis–relapse tumor samples were included if the samples contained more than 50% tumor cells by pathologic examination. After DNA extraction using standard procedures, mutations of the kinase domain were searched for by Sanger sequencing (Data Supplement). We have studied 54 paired diagnosis–relapse neuroblastoma samples to analyze the frequency of ALK mutations at relapse and to define their potential role in clonal evolution.

**RESULTS**

To determine the frequency of ALK mutations at relapse, we performed Sanger sequencing of the ALK receptor tyrosine kinase domain in a series of 54 paired diagnosis–relapse neuroblastoma samples. ALK mutations were observed in nine of 54 diagnostic samples (Tables 1 and 2). For all nine cases, the same mutation was also detected by Sanger sequencing in a sample at relapse. In one case (NB1224), an ALK mutation, detected at diagnosis, was seen at relapse in only one of several samples. Indeed, the ALK mutation was found in only one of the stroma-poor tumor nodules and not in the stromarich tissue surrounding the nodules (Fig 1). A germline ALK mutation was observed in only one case (NB0073).

At relapse, new ALK mutations were also detected by Sanger sequencing in five additional cases (Table 1). These were a F1174L mutation (exon 23) in two cases, a F1174S mutation (exon 23) described previously in one case,22 and a Y1278S mutation and R1275Q mutation (exon 25) in the other two cases.

In cases where ALK mutations were detected by Sanger sequencing in only one of all available tumor samples, we determined whether the ALK mutation might have gone undetected in the other samples as a result of a limit in sensitivity of the Sanger technique. We used the IonTorrent PGM technique to resequence the relevant hotspots in all available tumor samples of case NB1224 and of four of five cases with an ALK mutation seen only at relapse (NBG12, NBG17, NB3038, and NB1382). For the fifth case, no sufficient material was available for the PGM analysis. Using the 318 chip (Life Technologies), a high coverage was achieved for all cases at the relapsed regions, with more than 100,000 reads per position after application of bioinformatics filters (Data Supplement).

To determine the sensitivity of our technique, in a first step, the background variability resulting from the PGM sequencing was calculated for the control cell lines in the studied region. The mean overall coverage for the control cell lines was more than 175,000X. The mean overall background variability was 0.034% ± 0.035% for each base, except the reference base, with no significant outlier (Data Supplement). To determine the expected sensitivity, we then calculated which number of reads would be considered statistically different
**Table 1. Clinical and Tumor Genetic Data of Patients With ALK Mutations**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age at Diagnosis (months)</th>
<th>Stage (INSS)</th>
<th>Interval From Diagnosis to Relapse (months)</th>
<th>Relapse Type</th>
<th>Follow-Up (months from diagnosis)</th>
<th>Outcome</th>
<th>Genomic Profile at Diagnosis</th>
<th>ALK Change</th>
<th>Position and Mutation</th>
<th>ALK Detection by Sanger Sequencing</th>
<th>ALK Detection by PGM (% of total reads)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBG03</td>
<td>50</td>
<td>4</td>
<td>23</td>
<td>loc</td>
<td>29</td>
<td>DOD</td>
<td>MNA</td>
<td>F1174L</td>
<td>29443695 (TTC&gt;TATA)</td>
<td>Neg</td>
<td>Pos</td>
</tr>
<tr>
<td>NBG14</td>
<td>90</td>
<td>4</td>
<td>10</td>
<td>loc</td>
<td>55</td>
<td>NED</td>
<td>S</td>
<td>F1174L</td>
<td>29443695 (TTC&gt;TATA)</td>
<td>Neg</td>
<td>Pos</td>
</tr>
<tr>
<td>NBG21</td>
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<td>2b</td>
<td>11</td>
<td>loc</td>
<td>17</td>
<td>DOD</td>
<td>MNA</td>
<td>F1174L</td>
<td>29443695 (TTC&gt;TATA)</td>
<td>Neg</td>
<td>Pos</td>
</tr>
<tr>
<td>NB0175</td>
<td>101</td>
<td>2b</td>
<td>90</td>
<td>loc + meta</td>
<td>150</td>
<td>DOD</td>
<td>S</td>
<td>Y1278S</td>
<td>29432655 (TAC&gt;TCC)</td>
<td>Neg</td>
<td>Pos</td>
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<tr>
<td>NB0899</td>
<td>0.2</td>
<td>4</td>
<td>6</td>
<td>meta</td>
<td>136</td>
<td>DOD</td>
<td>N</td>
<td>R1275Q</td>
<td>29432664 (IGA&gt;CAA)</td>
<td>Neg</td>
<td>Pos</td>
</tr>
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<td>NB0824</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>meta</td>
<td>16</td>
<td>DOD</td>
<td>N</td>
<td>F1174L</td>
<td>29443695 (TTC&gt;TATA)</td>
<td>Neg</td>
<td>Pos</td>
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<td>NB1269</td>
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<td>4</td>
<td>10</td>
<td>loc</td>
<td>11</td>
<td>DOD</td>
<td>S</td>
<td>L1198M</td>
<td>29443631 ICTG&gt;ATG</td>
<td>Neg</td>
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<td>NB1224</td>
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<td>2b</td>
<td>4</td>
<td>loc</td>
<td>14</td>
<td>NED</td>
<td>S</td>
<td>R1275Q</td>
<td>29432664 (IGA&gt;CAA)</td>
<td>Neg</td>
<td>Pos</td>
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<td>NB0073</td>
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<td>4s</td>
<td>7</td>
<td>meta</td>
<td>272</td>
<td>NED</td>
<td>N</td>
<td>T1151R</td>
<td>29445273 (ACG&gt;AGG)</td>
<td>Pos</td>
<td>Pos</td>
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<tr>
<td>NB05</td>
<td>50</td>
<td>4</td>
<td>45</td>
<td>loc + meta</td>
<td>50</td>
<td>DOD</td>
<td>MNA</td>
<td>R1275Q</td>
<td>29432664 (IGA&gt;CAA)</td>
<td>Neg</td>
<td>Neg</td>
</tr>
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<td>NBG12</td>
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<td>4</td>
<td>9</td>
<td>meta</td>
<td>9</td>
<td>DOD</td>
<td>S</td>
<td>F1174S</td>
<td>29443696 (TTC&gt;TCC)</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>NBG17</td>
<td>29</td>
<td>4</td>
<td>13</td>
<td>meta</td>
<td>24</td>
<td>DOD</td>
<td>MNA</td>
<td>F1174L</td>
<td>29443695 (TTC&gt;TATA)</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>NB1382</td>
<td>4</td>
<td>4</td>
<td>51</td>
<td>loc + meta</td>
<td>63</td>
<td>DOD</td>
<td>S</td>
<td>Y1278S</td>
<td>29432655 (TAC&gt;TCC)</td>
<td>Neg</td>
<td>Neg</td>
</tr>
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<td>NB0308</td>
<td>3</td>
<td>2b</td>
<td>21</td>
<td>loc</td>
<td>93</td>
<td>NED</td>
<td>N</td>
<td>F1174L</td>
<td>29443695 (TTC&gt;TATA)</td>
<td>Neg</td>
<td>Neg</td>
</tr>
</tbody>
</table>

**NOTE.** For all patients with ALK mutations detected at diagnosis by Sanger sequencing, mutations were also detected by Sanger at relapse. In five cases, mutations were detected by Sanger at relapse only, two of which (NB0308 and NBG17) could be shown to harbor ALK mutations in a smaller fraction of cells or subclones at diagnosis as evidenced by PGM deep sequencing. Furthermore, for a cell line harboring an ALK mutation, the mutation was observed in a subclone of cells in the primary sample (CLB_Ba).

**Abbreviations:** DOD, dead of disease; INSS, International Neuroblastoma Staging System; loc, local relapse; meta, metastatic relapse; MNA, MYCN amplification; N, numerical chromosome alterations; ND, not done; NED, no evidence of disease; Neg, negative; PGM, Personal Genome Machine; Pos, positive; S, segmental chromosome alterations.

*Signifies a base frequency as detected by PGM with a statistically significant difference from the controls.
from the background. Bonferroni’s correction was applied, because multiple tests were performed for each base at each position. Considering a mean coverage of 175,000×, a variation supported by 296
reads, or observed with a frequency of 0.17%, would result in a statistically significant difference from the controls (two-sided Fisher’s exact test). For the studied tumor samples, the background variability was not different from that of the controls, except the mutation hotspots, and no mutations outside the mutation hotspots were detected.

In a next step, to analyze more precisely the mutation hotspots, the percentage of bases at coordinates 29443696, 29443695, 29432664, and 29432655, corresponding to the mutations F1174S(T>C) in NBG12, F1174L(C>A) in NBG17 and NB0308, R1275Q(G>A) in NB1224, and Y1278S(A>C) in NB1382, respectively, were studied in detail in the respective samples (Table 3). For each case, the frequency of bases at a given position was compared with frequencies observed in the controls (two-sided Fisher’s exact test; Tables 1, 2, and 3). As expected, the PGM analysis detected all mutations seen by Sanger sequencing and enabled further precision regarding their allele frequency. In case NBG12, PGM analyses of the previously described F1174L mutation observed by Sanger sequencing, was compared with the corresponding primary sample. In case CLB-Ma, PGM analysis of the primary tumor tissue Ma(PT) did not reveal a higher frequency of the mutation base compared with the controls, indicating that in the studied sample, no ALK-mutated subclone could be detected (Tables 2 and 3). However, the cell line was established from invaded bone marrow. Because no sample of this bone marrow was available for PGM analysis, it cannot be excluded that an ALK-mutated subclone might have been present in the metastatic site. For CLB-Ba, PGM analysis of the primary sample [invaded bone marrow, Ba(P_T_BM)] from which the cell line was directly established revealed the presence of the mutation in 6.6% of the studied DNA fragments, indicating the presence of an ALK-mutated subclone in the primary sample, which then expanded during establishment of the cell line harboring the known ALK mutation in all cells (Tables 2 and 3; Fig 2).

With the increasing importance of targeted therapies, full characterization of molecular genetic events in cancer cells becomes crucial. The new, high-resolution next-generation sequencing techniques now enable the evaluation of biomarkers for more precise studies of clonal evolution in sequential samples from the same patient.

In neuroblastoma, only few studies have analyzed the genetic alterations at relapse, with no alterations specific for relapse described to date. One recent report has described a relapse-specific ALK mutation. We now report on the search for ALK mutations in 54 paired diagnosis–relapse neuroblastoma samples. In this study of samples from patients who experienced relapse, we observed ALK mutations at diagnosis in 17% of the patients, suggesting that at diagnosis, in patients who experience relapse, the incidence of ALK mutations might be higher than that reported in the overall population. Furthermore, we demonstrate an emergence of ALK mutations was not seen at relapse. At relapse, a different mutation was observed in 19.125% (Fisher’s exact test, P < 10−16; Data Supplement). These mutations led to the same AA change, indicating a mutation switch between diagnosis and relapse with an expected identical functional consequence.

Finally, to search for further evidence of clonal selection of ALK-mutant cells, two established cell lines (CLB-Ma and CLB-Ba), with a previously described F1174L mutation observed by Sanger sequencing, were compared with the corresponding primary sample. In case CLB-Ma, PGM analysis of the primary tumor tissue Ma(PT) did not reveal a higher frequency of the mutation base compared with the controls, indicating that in the studied sample, no ALK-mutated subclone could be detected (Tables 2 and 3). However, the cell line was established from invaded bone marrow. Because no sample of this bone marrow was available for PGM analysis, it cannot be excluded that an ALK-mutated subclone might have been present in the metastatic site. For CLB-Ba, PGM analysis of the primary sample [invaded bone marrow, Ba(PT_BM)] from which the cell line was directly established revealed the presence of the mutation in 6.6% of the studied DNA fragments, indicating the presence of an ALK-mutated subclone in the primary sample, which then expanded during establishment of the cell line harboring the known ALK mutation in all cells (Tables 2 and 3; Fig 2).

**DISCUSSION**

Table 2. Patient Data According to Cell Line

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Age at Diagnosis (months)</th>
<th>Stage (INSS)</th>
<th>Primary (%)</th>
<th>Sample Cells</th>
<th>Tissue From Which Cell Line Was Established</th>
<th>Follow-Up From Diagnosis (months)</th>
<th>Outcome</th>
<th>Genomic Profile</th>
<th>AA Change</th>
<th>Position and Mutation</th>
<th>ALK Detection by Sanger Sequencing</th>
<th>ALK Detection by PGM (% of total reads)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLB_Ma</td>
<td>9</td>
<td>4</td>
<td>Abdominal tumor</td>
<td>Bone marrow</td>
<td>16</td>
<td>DOD</td>
<td>MNA</td>
<td>F1174L</td>
<td>TTC&gt;TAA</td>
<td>Neg</td>
<td>Pos</td>
<td>0.027</td>
</tr>
<tr>
<td>CLB_Ba</td>
<td>27</td>
<td>4</td>
<td>Bone marrow (80%)</td>
<td>Bone marrow</td>
<td>117</td>
<td>NED</td>
<td>MNA</td>
<td>F1174L</td>
<td>TTC&gt;CTC</td>
<td>Neg</td>
<td>Pos</td>
<td>6.609*</td>
</tr>
</tbody>
</table>

Abbreviations: DOD, dead of disease; INSS, International Neuroblastoma Staging System; MNA, MYCN amplification; NED, no evidence of disease; Neg, negative; PGM, Personal Genome Machine; Pos, positive.

*Signifies a base frequency as detected by PGM with a statistically significant difference from the controls.
at the time of relapse. This finding is of utmost clinical importance given the possibility of targeted treatment with ALK inhibitors and the fact that ALK mutations are considered an important predictive molecular marker.21

Sanger sequencing is reported to detect 20% to 30% of mutated alleles in a wild-type background.23,24 For sensitivity estimation, contamination with normal cells should be considered. For this study, contamination of tumor samples by normal cells of up to 50% was

Fig 1. ALK status in different samples of NB1224. (A) Macroscopic and microscopic aspect of the tumor at different time points and results of Sanger sequencing searching for ALK mutation. The position corresponding to the mutation R1275Q is underlined (chr2:29432664). At diagnosis, a localized International Neuroblastoma Risk Group Staging System L2 neuroblastoma, stroma poor, poorly differentiated, with high mitosis-karyorrhexis index (MKI), was observed. Sanger sequencing showed the presence of an R1275Q (CGA→CAA) mutation (diagnostic sample, NB1224-D). The patient received two courses of chemotherapy and was then observed. The patient experienced local progression 4 months after first-line chemotherapy. At relapse, a biopsy of the tumor was first performed (relapse sample, NB1224-R2). After additional courses of chemotherapy, surgical resection revealed a postchemotherapeutic tumor classified as a peripheral neuroblastic tumor, not otherwise specified, according to INPC recommendations. The postchemotherapy effects were minimal, without any necrosis being observed, and this tumor was indeed histologically composed of a stroma-rich component (relapse sample, NB1224-R3) and of several stroma-poor nodules with numerous neuroblasts, poorly differentiated; in one nodule, the MKI was low (relapse sample, NB1224-R4); in another nodule, the MKI was high (relapse sample, NB1224-R5). At progression, the R1275Q (CGA→CAA) mutation was found only in the nodule corresponding to neuroblastoma, stroma poor, poorly differentiated, MKI high (NB1224-R5), and not in the other nodule or in the stroma-rich component. (B) Results of Personal Genome Machine (PGM) sequencing of case NB1224 at position chr2:29432664. Diagnostic sample: NB1224-D; relapse samples: NB1224-R2, NB1224-R4, and NB1224-R5. The observed frequencies of bases at the studied position are indicated in the graph. For the controls, the mean of the base percentages for the four control cell lines is indicated. Statistically significant differences between the sample and controls of base frequencies for the base A, corresponding to the mutation, are circled, and the results of the Fisher’s exact tests are indicated. PGM sequencing confirmed the results observed with Sanger sequencing, with presence of an R1275Q (CGA→CAA) mutation in samples NB1224-D and NB1224-R5. The base corresponding to the mutated allele did not exceed the background variability in the other tumor fragments analyzed (NB1224-R2 and NB1224-R4).
tolerated, and thus, it is expected that in these samples heterozygous mutations occurring in all tumor cells, present in 25% of all analyzed DNA fragments, would be at the limit of detection by Sanger sequencing. To search for ALK mutations with a higher sensitivity than that of Sanger sequencing, which has a low sensitivity due to the high background variability of the observed DNA fragments, would be at the limit of detection by Sanger sequencing.

Future technologic improvements may reduce this background variability. In this high-coverage analysis, the overall error rate in lower coverage experiments, including mismatches, deletions, and insertions, has been reported to be approximately 0.019%, which might vary strongly according to the genome structure and the presence or absence of homopolymers and might be linked to sequencing errors as a result of polymerase slippage, errors in the chemistry, or other errors. Future technologic improvements may reduce this background variability. In this high-coverage analysis, the background variability was 0.034% per base, resulting in a sensitivity of 0.17%, 100-fold that of Sanger sequencing.

This deep-sequencing approach indicated that ALK mutations might occur as subclones at neuroblastoma diagnosis with secondary progression. Our data also suggest that, in some instances, ALK mutations occurring in all tumor cells, present in 25% of all analyzed DNA fragments, would be at the limit of detection by Sanger sequencing. To search for ALK mutations with a higher sensitivity than that of Sanger sequencing, which has a low sensitivity due to the high background variability of the observed DNA fragments, would be at the limit of detection by Sanger sequencing.

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**ALK Mutations in Relapse of Neuroblastoma**

This study also reveals the important observation, in one case, of two different ALK mutations at diagnosis and relapse, both leading to the same AA change. Possible explanations include the presence of the second mutation in a minor subclone at diagnosis below the detection limit of our technique; spatial heterogeneity throughout the tumor, with one mutation occurring in one tumor section and another in a different tumor section; or a new occurrence of a mutation not present at diagnosis. The same AA change having potentially occurred independently twice in tumor development suggests that these mutations emerged as a result of a possible addiction to the functional modifications linked to the ALK mutation.

The detailed knowledge of the ALK status in neuroblastoma is important in view of the availability of targeted therapy, with new-generation, more selective, higher affinity ALK-specific agents currently being developed. The identification of an ALK mutation in a tumor sample can be considered as a positive predictive marker for efficacy of ALK-targeted treatment. Additional studies are now necessary to determine how the presence of ALK mutations in tumor subclones might influence ALK-targeted treatment efficacy.

Our study leads to two crucial conclusions. First, the observation of five of 34 new ALK mutations at relapse suggests that the frequency of ALK mutations may be higher at relapse than at diagnosis, requiring further validation in larger cohorts. Second, subclones harboring ALK mutations may contribute to tumor evolution and relapse. This has major clinical implications. Our findings provide proof of principle that the systematic application of new, more sensitive deep-sequencing techniques in neuroblastoma is of clinical interest and should be considered on diagnostic samples. Furthermore, ALK mutations should be searched for not only at diagnosis but also at relapse when considering ALK-targeted therapies. Thus, although clinicians historically have been reluctant to prescribe invasive procedures for relapse in high-risk neuroblastoma, our findings implicate a change in medical practice in favor of tumor sampling even at relapse, and repeated tumor sampling should become a new standard of care.
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