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Seek and Ye Shall Find: Subclonal Anaplastic Lymphoma Kinase Mutations

Rani E. George

Bellini and colleagues demonstrate the importance of next-generation sequencing to uncover subclonal anaplastic lymphoma kinase (*ALK*) mutations in neuroblastoma. Although the significance of these subclonal aberrations is not yet understood, deep sequencing could identify patients whose tumors may respond to ALK inhibitors. *Clin Cancer Res; 21(21); 4747–9. ©2015 AACR.*

See related article by Bellini et al., p. 4913

In this issue of *Clinical Cancer Research*, Bellini and colleagues (1) demonstrate that targeted deep sequencing of neuroblastoma tumors identifies subclonal mutations in the anaplastic lymphoma kinase (*ALK*) receptor tyrosine kinase that may have gone undetected using conventional Sanger sequencing. Since the original identification of activating somatic mutations in *ALK* in neuroblastoma, multiple sequencing studies have validated this kinase as the main targetable molecular aberration in this disease (2–5). In neuroblastoma, the majority of *ALK* mutations are found in the kinase domain and promote constitutive, ligand-independent activation of the receptor. These alterations are sensitive to small molecule inhibitors of ALK and lead to down-regulation of its activity and that of its downstream targets with consequent growth inhibition of neuroblastoma cells (4). *ALK* is also activated via translocation events in other malignancies, including non–small cell lung cancer, anaplastic large-cell lymphomas, and inflammatory myofibroblastic tumors, where it is thought to contribute to tumorigenesis and progression (6).

In the study by Bellini and colleagues (1), 276 neuroblastoma samples were analyzed by Sanger sequencing, revealing a mutation rate of 4.3% (12 of 276). To confirm the suspicion that conventional Sanger sequencing may not detect subclonal *ALK* mutations because of the limits of detection and background noise inherent in this technology, the authors resequenced these samples using the more sensitive two-step PCR procedure and HiSeq technology (Illumina). Targeted resequencing of exons 23 and 25, which contain the two most frequently observed mutational hotspots, *F1174* and *R1275*, respectively, identified subclonal *ALK* mutations (defined as <20% of the cell population, with up to 50% contamination from normal tissue) in an additional 15 tumors, yielding an overall mutation frequency of approximately 10% (27 of 276). *ALK* mutations at *F1174* were observed in 15 samples, 13 with a mutation leading to the amino acid change *F1174L*, while 2 samples showed *F1174C* and *F1174V* with the mutated allele fractions ranging widely, from 0.5% to 40%. The *R1275* locus was mutated in 12 cases, 11 with the *R1275Q* and one with the *R1275L* mutation; the proportion of cells with the mutated allele ranged from 0.8% to 73%. This wide range of mutated allele fractions was present even when corrected for tumor cell content and chromosome 2p copy-number status.

There was no association between clonal versus subclonal mutations and important clinical prognostic parameters, such as patient age, tumor stage, or outcome (1). Although on univariate analysis there was a worse overall survival in patients whose tumors expressed mutated or amplified *ALK*, especially those with the *F1174* mutation, this was not borne out when other factors were taken into consideration. In multivariate analysis, advanced-stage disease and *MYCN* amplification were the only independent prognostic variables, underscoring the secondary role of mutated *ALK* in determining treatment outcome in this patient cohort.

The authors report a close correlation between mutated *ALK* and amplified *MYCN* with enrichment of *F1174L* in tumors showing *MYCN* amplification (1), attesting to the demonstrated cooperative effect of both alterations in neuroblastoma (7, 8). Although *ALK* mutations were either clonal or subclonal, *MYCN* status was homogeneous throughout the tumor cell population. These observations support the general conclusion that *MYCN* deregulation is the initial event in neuroblastoma tumorigenesis, with *ALK* mutations occurring later in tumor development. An interesting observation was the percentage of clonal and subclonal *ALK* aberrations in the *MYCN*-amplified versus *MYCN*-nonamplified tumors. The majority of *MYCN*-amplified tumors (13 of 15) contained only subclonal populations of *ALK*-mutated cells, while in *MYCN*-nonamplified tumors the majority of *ALK* mutations (10 of 12) were clonal and only 2 of 12 contained subclonal *ALK* (1). This indicates that in most *MYCN*-nonamplified tumors with an *ALK* mutation, the kinase aberration appears to be dominant.

The significance of these subclones is far from clear. They could simply reflect the characteristic genetic heterogeneity of neuroblastoma or they could signal the presence of cell populations with the potential to expand and cause relapse (Fig. 1; ref. 9). The first scenario seems less likely, as almost all of the *ALK* mutations reported in neuroblastoma are activating (6). However, to date,

Department of Pediatric Hematology and Oncology, Dana-Farber Cancer Institute and Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts.

Corresponding Author: Rani E. George, Department of Pediatric Oncology, Dana-Farber Cancer Institute, Dana 640E, 450 Brookline Avenue, Boston, MA 02215. Phone: 617-632-5281; Fax: 617-632-4850; E-mail: rani_george@dfci.harvard.edu

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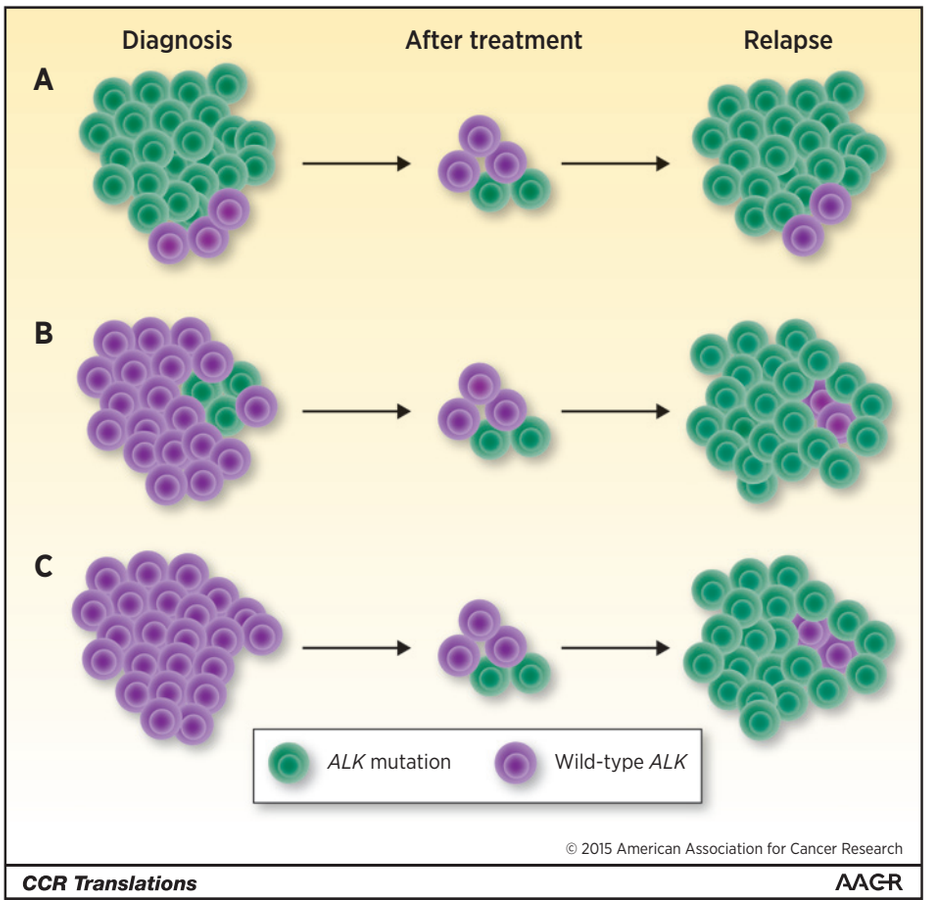

Figure 1.
Possible origins of *ALK* mutations detected at relapse. A, *ALK*-mutated cells can be clonal and form the dominant transformed population. These mutations can be identified through conventional sequencing methods. At relapse, these clonal populations retain their dominance, although the possibility of their replacement by clones bearing new mutations cannot be excluded. B, *ALK*-mutated cells are subclonal at diagnosis and could be either eradicated or evolve and expand at relapse to form the dominant transformed population. These mutations at diagnosis can be missed through conventional sequencing and will need to be searched for through advanced sequencing techniques. C, tumors without *ALK* mutations at diagnosis but in which ALK mutations arise *de novo* after treatment.

there is no experimental evidence that tumors containing malignant subclones have different growth properties or respond differently to *ALK* inhibitors. The numbers of patients used to determine clinical correlations in the Bellini study was relatively small, and repeat tumor biopsies were not available at relapse, eliminating opportunities to track the fate of subclonal *ALK* mutations that were uncovered in the diagnostic samples. Two earlier studies reported a higher frequency of *ALK* mutations at relapse, some of which were present at diagnosis but were below the limits of detection using standard sequencing methods (10, 11). For example, Schleiermacher and colleagues (10) compared 54 paired tumors at diagnosis and relapse and identified 14 *ALK* mutations, 5 of which were not detected at diagnosis. Deep sequencing revealed subclonal *ALK* mutations in 2 of 4 diagnostic samples. Moreover, in a recently published study by Eleveld and colleagues (11), in which whole-genome sequencing of 23 paired diagnostic and relapse samples was performed, 10 cases with *ALK* mutations were identified, of which 3 were not detected in the primary tumor. Ultra-deep sequencing and PCR-based methods identified two of the three being present at low frequency in the primary tumor (11). These examples, as well as the reported establishment of a fully *ALK*-mutated cell line from a primary tumor containing a subclonal *ALK*-mutated population (10), are suggestive of clonal evolution, but need to be validated in larger cohorts and through experimental methods. Therefore, whether the higher frequency of *ALK* mutations seen at relapse reflects those already present at diagnosis or whether they emerged during the development of relapse is a key question that needs to be addressed. It is possible that subclonal *ALK* mutations at diagnosis may not be effectively targeted by standard chemotherapy and therefore continue to evolve, eventually contributing to relapse.

The presence of subclonal *ALK* mutations at diagnosis becomes especially important in cases with *MYCN* amplification, where the majority of *ALK*-mutated cells were subclonal (1). If these patients are to benefit from the administration of an ALK inhibitor at diagnosis, to prevent *ALK*-mutated clones from becoming dominant at relapse, it is critical that subclonal *ALK* mutations are not missed during the initial analysis of the tumor. Sanger sequencing has been used widely in clinical laboratories for the analysis of mutations, but its sensitivity is such that only 20% to 30% of mutated alleles in a wild-type background are detected (12). As more sophisticated technologies become available, such as droplet digital PCR and next-generation sequencing techniques with the potential of detecting mutated allele fractions as low as 0.2%, as previously reported by the authors (10), it should be possible to replace Sanger and other conventional methods for identifying subclonal *ALK* mutations. Most importantly, this enhanced capability will make it possible to accurately track subclonal *ALK* mutations from diagnosis to relapse through repeated tumor sampling to establish their clinical relevance. For instance, if the

findings indicate the persistence and expansion of *ALK*-mutated subclones in a substantial fraction of patients, there would be justification for adding effective new *ALK* inhibitors early in the treatment course or at relapse. Whatever the outcome, the study by Bellini and colleagues (1) has provided the impetus to study more closely the pathogenic role of neuroblastoma subclones bearing *ALK* mutations and hence their potential value as therapeutic targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Bellini A, Bernard V, Leroy Q, Rio Frio T, Pierron G, Combaret V, et al. Deep sequencing reveals occurrence of subclonal *ALK* mutations in neuroblastoma at diagnosis. Clin Cancer Res 2015;21:4913–21.
2. Chen Y, Takita J, Choi YL, Kato M, Ohira M, Sanada M, et al. Oncogenic mutations of ALK kinase in neuroblastoma. Nature 2008;455:971–4.
3. Janoueix-Lerosey I, Lequin D, Brugieres L, Ribeiro A, de Pontual L, Combaret V, et al. Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. Nature 2008;455:967–70.
4. George RE, Sanda T, Hanna M, Frohling S, Luther W 2nd, Zhang J, et al. Activating mutations in ALK provide a therapeutic target in neuroblastoma. Nature 2008;455:975–8.
5. Mosse YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF, et al. Identification of ALK as a major familial neuroblastoma predisposition gene. Nature 2008;455:930–5.
6. Hallberg B, Palmer RH. Mechanistic insight into ALK receptor tyrosine kinase in human cancer biology. Nat Rev Cancer 2013;13:685–700.
7. De Brouwer S, De Preter K, Kumps C, Zabrocki P, Porcu M, Westerhout EM, et al. Meta-analysis of neuroblastomas reveals a skewed ALK mutation spectrum in tumors with MYCN amplification. Clin Cancer Res 2011;16:4353–62.
8. Berry T, Luther W, Bhatnagar N, Jamin Y, Poon E, Sanda T, et al. The ALK (F1174L) mutation potentiates the oncogenic activity of MYCN in neuroblastoma. Cancer Cell 2012;22:117–30.
9. Greaves M, Maley CC. Clonal evolution in cancer. Nature 2012;481:306–13.
10. Schleiermacher G, Javanmardi N, Bernard V, Leroy Q, Cappo J, Rio Frio T, et al. Emergence of new ALK mutations at relapse of neuroblastoma. J Clin Oncol 2014;32:2727–34.
11. Eleveld TF, Oldridge DA, Bernard V, Koster J, Daage LC, Diskin SJ, et al. Relapsed neuroblastomas show frequent RAS-MAPK pathway mutations. Nat Genet 2015;47:864–71.
12. Ihle MA, Fassunke J, Konig K, Grunewald I, Schlaak M, Kreuzberg N, et al. Comparison of high resolution melting analysis, pyrosequencing, next generation sequencing and immunohistochemistry to conventional Sanger sequencing for the detection of p.V600E and non-p.V600E BRAF mutations. BMC Cancer 2014;14:13.

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