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COMMENTARY

Molecular Diagnostics of Acute Myeloid Leukemia

It's a (Next) Generational Thing

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In the United States, acute myeloid leukemia (AML) develops in approximately 13,000 individuals annually and accounts for approximately 9000 cancer-related deaths per year.[1] Although epidemiologically AML is considered a single disease, it is certainly not a uniform clinicopathologic entity. This fact is evidenced by the distinctly different outcomes observed in the clinical setting, with some patients responding well to chemotherapeutic regimens, others requiring hematopoietic stem cell transplants, and yet others rapidly dying of the disease. Although survival of patients with AML depends in part on tumor-extrinsic factors such as age and performance status, the major factors influencing prognosis are the molecular characteristics of the tumor cells themselves, in particular translocations and other genetic mutations, epigenetic abnormalities, and mRNA and miRNA expression.[2–6] Indeed, AML can be divided into subcategories based solely on the genomic, epigenomic, or transcriptomic characteristics of the neoplastic cells. Such subgroups are associated with significantly distinct outcomes, indicating that the molecular changes involved in AML unequivocally contribute to overall prognosis. Thus, identifying the spectrum of prognostically important aberrancies that occur within the leukemic cells is an area of intense basic research, and the use of clinical assays that probe these abnormalities is required for optimal treatment of patients with these malignancies.

The first recognized and best-studied recurrent genetic lesions in AML are large unsubtle chromosomal anomalies, which are usually detected by metaphase cytogenetic analysis.[4] These include translocations and inversions such as t(8;21), inv(16), and t(15;17), which confer a relatively good prognosis, and chromosomal gains and losses such as monosomy 7, which confers a poor prognosis. However, some AML cases do not display any abnormalities recognized by either traditional karyotyping or by its younger and more sensitive cousin, fluorescence *in situ* hybridization (FISH), and these cases with normal karyotypes account for approximately 40% to 50% of AML diagnoses.[7]

Despite the absence of karyotypic abnormalities, normal-karyotype AMLs are clearly not genetically normal, and they harbor mutations that distinguish the malignant cells from their physiological counterparts. In the past few years, numerous recurrent mutations that are too small to be detected by karyotyping or FISH have been discovered. This ever-expanding list includes mutations in *FLT3*, *NPM1*, *CEBPA*, *WT1*, *KRAS*,

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TET2, *DNMT3A*, *IDH1*, *IDH2*, *ASXL1*, and *PHF6*.[5,8] Many of these mutations have been demonstrated to have prognostic and sometimes related diagnostic implications for patients with AML. As a result, the latest edition of the World Health Organization fascicle on the classification of hematopoietic neoplasms includes provisional AML entities defined by mutations in *NPM1* and *CEBPA*, and recognizes the importance of testing for *FLT3* mutations (although the classification does not elevate AML with *FLT3* mutations to an entity).[9] The latest National Comprehensive Cancer Network (NCCN) clinical practice guidelines for AML recommend testing for mutations in these three genes at diagnosis.[10] In this regard, the article by Spencer et al[11] in this issue of *The Journal of Molecular Diagnostics* is significant in that it outlines a novel method, based on next-generation sequencing (NGS), by which *FLT3* mutations can be detected. Although their report focuses solely on *FLT3* mutation detection, the main implication of the article is that other mutations, such as those in *NPM1* and *CEBPA*, could be detected simultaneously.

The fms-related tyrosine kinase 3 gene (*FLT3*) encodes a class III receptor tyrosine kinase that is required for normal hematopoiesis.[12] Recurrent mutations in the *FLT3* gene have been found in approximately 20% of AML cases overall, and in approximately 30% of normal-karyotype AML cases.[7] These mutations fall into two broad categories: internal tandem duplications (ITDs) within the juxtamembrane domain and point mutations within the kinase domain.[13] Although both types of mutations render the kinase constitutively active, only the ITD mutation has been definitively shown to correlate with prognosis. The presence of an *FLT3* ITD confers a poor prognosis, and patients with an *FLT3* ITD usually require hematopoietic stem cell transplant, because most are not expected to be to be cured with chemotherapy alone.[14] A few recent studies have shown that the FLT3 ITD protein is indeed a leukemogenic molecule that drives the formation of aggressive tumors. *FLT3* ITD can cause aggressive AML in mice of a specific genetic background with 100% penetrance.[15] Additionally, *FLT3* ITD-positive patients treated with an FLT3 inhibitor who become resistant to treatment acquire activating mutations within the *FLT3* kinase domain.[16] However, because i) not all of the leukemic blasts from a single *FLT3* ITD-positive patient harbor the insertion,[17] because ii) the *FLT3* ITD can be present at initial diagnosis and subsequently lost at relapse, or vice versa,[18] and because iii) *FLT3* ITD tumors do not fit into distinct tumor subclasses in unbiased clustering analyses,[3] the *FLT3* ITD is thought to be a secondary, rather than an initiating, mutation. It is therefore not, as we have already noted, afforded designation as a separate entity in the World Health Organization classification of AML.[9]

Regardless of the underlying biology of *FLT3* mutations, it is apparent that determination of *FLT3* ITD status has prognostic as well as therapeutic implications for patients with AML. Thus, *FLT3* testing is routinely performed on diagnostic AML specimens. Typically, the assay is performed by PCR using primers that flank the site of the potential ITD, followed by capillary electrophoresis.[19] Not only can this test identify the *FLT3* ITD, but it also allows for determination of the length of the inserted sequence as well as an approximate allelic ratio of *FLT3* ITD to wild-type *FLT3*, both of which have been shown to affect patient outcome.[17]

The molecular test for *FLT3* status is performed as a single, independent assay. Additional separate assays are required to determine whether the tumor has a recurrent translocation, whether it harbors chromosomal gains or losses, and whether other prognostically important loci are mutated, such as *NPM1* and *CEBPA* (or many other novel genetic candidates). As the number of loci that influence prognosis and treatment of AML expands, such monoplex assays are becoming increasingly inefficient and costly. Thus, there is certainly a growing need to develop and validate clinical assays that can probe multiple mutations simultaneously. Consolidation of numerous independent analyses into a single multiplexed assay could reduce i) the cost of performing molecular tests, ii) the amount of patient specimen needed for molecular prognostication, iii) the turnaround time for reporting test results, and iv) perhaps also sample misidentification. A number of proprietary tests (eg, those that perform multiplex RT-PCR for seven recurrent acute leukemia translocations) have capitalized on this need for multiple mutation analyses.[20] Next-generation sequencing, in which DNA sequence for multiple loci (or indeed the entire genome) can be obtained simultaneously with a single procedure is definitely a modality that can be used to probe multiple loci simultaneously. Not surprisingly, NGS serves as the basis for rapidly evolving assays to meet this diagnostic need.

In this issue, Spencer et al[11] show the feasibility of using a NGS approach to evaluate the presence of *FLT3* ITDs in tumor samples. To test this methodology, the authors initially obtained DNA from peripheral blood, bone marrow, and formalin-fixed tissue samples. They then randomly fragmented the DNA, ligated specific adapters to the ends of these fragments, and amplified the ligated products. Amplified products from specific genomic locations were then selected from the entire genomic pool by hybridization to biotinylated cRNA capture probes that were then isolated by binding to streptavidin-coated magnetic beads. After enrichment for the captured fragments, the targeted libraries were subjected to standard NGS sequencing protocols using a HiSeq 2000 sequencing system (Illumina, San Diego, CA). Primary analysis resulted in base calls and corresponding quality scores for each of the millions of reads generated. The resulting data were aligned to the reference human genome sequence (hg19, NCBI build GRCh) using Novalign (Novocraft, Selangor, Malaysia) and were queried for the presence of *FLT3* ITDs using multiple analytic algorithms. The authors compared the results they obtained from NGS analysis to those obtained from the standard *FLT3* ITD analysis (performed by PCR followed by capillary electrophoresis).