Review

Molecular Diagnostics in Adult Acute Myeloid Leukemia

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Acute myeloid leukemia (AML) is a clinically and pathogenetically heterogeneous group of hematopoietic malignancies. Diagnosis, treatment choices and prognosis of AML have evolved from depending on evaluation of morphological and cytochemical features to relying heavily on cytogenetic profiling of leukemic cells by chromosome karyotyping and fluorescence in situ hybridization (FISH). However, given that at least 40% of all adult patients with AML lack identifiable cytogenetical abnormalities, there is a strong interest clinically in refining risk assessment as well as defining possible new targets for treatment. We review here some of the well studied molecular markers employed in the stratification of AML with normal cytogenetics, including the *Fms-Like Tyrosine Kinase 3 (FLT3)*, *nucleophosmin-1 (NPM1)* and *CCAAT/enhancer binding protein-α (CEBPA)* genes. We discuss other factors of potential interest, but less well characterized in the context of AML, including miRNA expression signatures. Technical aspects of molecular testing are also discussed.

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INTRODUCTION

AML is a group of malignant hematopoietic disorders characterized by maturation arrest and uncontrolled proliferation of early myeloid precursors in the bone marrow.[1] The estimated number of new cases of AML in the United States for the year 2011 is 12,950; the corresponding number of deaths due to AML is 9,050 (1). The current standard of care for AML consists of chemotherapy, or, in high risk patients, stem cell transplantation. Approximately 60% to 70% of adults under age 60 attain remission; however, less than 50% achieve a life expectancy of >3 years.[2] At this point, morphological subclassification of AML carries little prognostic significance. Instead, the cytogenetic profile at the time of diagnosis is the most important prognostic factor.[3-7] Seven of the entities listed in the most recent World Health Organization (2008) classification of AML are characterized by recurrent, balanced translocations and/or inversions. The presence of any of these seven structural aberrations, certain autosomal monosomies, or a complex karyotype with three or more clonal abnormalities allows for risk assessment. However, about 40-50% of adult AML cases lack identifiable cytogenetical abnormalities.[7] Studies of molecular abnormalities may hold more hope for better stratification of AML. For example, conditions associated with AML including both syndromic and non-syndromic familial disorders highlight the relevance of underlying molecular abnormalities to AML.[8-9] The current WHO classification lists two provisional AML entities that are characterized by abnormalities at the molecular level: AML with mutated *NPM1* and AML with mutated *CEBPA*.[10]

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In addition, it has been well documented that *FLT3* mutations (*FLT3-ITD*) are associated with poor prognosis.[11] This review aims to discuss some of the well studied and emerging molecular markers in their stratification and residual disease detection of adult AML, along with the technical advances of molecular testing.

MOLECULAR MARKERS

FLT3 (CD135)

Tyrosine kinases, many of which have dual functions of being a kinase and a receptor, have crucial roles in many cell signaling pathways. Tyrosine kinase receptors that are constitutively activated by mutations or rearrangements are present in several hematological malignancies. Well-characterized examples include the t(9;22) fusion transcript found in chronic myelogenous leukemia, the mutated JAK2 kinase seen in several myeloproliferative neoplasms, and the *platelet-derived growth factor receptor alpha* (*PDGFR-alpha*), *PDGFR-beta* and *fibroblast growth factor receptor 1*(*FGFR1*) rearrangements detected in myeloid and lymphoid neoplasms with eosinophilia. Tyrosine kinases are of great interest as therapeutic targets.

The proto-oncogene tyrosine kinase cytokine receptor FLT3 belongs to the PDGF family of growth factor receptors.[12] This class of receptors contains an extracellular ligand-binding domain, a membrane-spanning domain and a cytoplasmic tyrosine kinase domain.[13] Upon binding of its specific, ubiquitously expressed transmembrane protein ligand (FLT3L), the FLT3 receptor undergoes homodimerization, which activates the intrinsic tyrosine kinase. The kinase autophosphorylates the catalytic domain of the receptor, which results in conformational changes and activation of downstream signaling pathways including the

Ras/Raf/MAPK and PI3 cascades. The FLT3 receptor is encoded by the FLT3 gene on the long arm of chromosome 13. It appears to be expressed mainly on pluripotent hematopoietic progenitor cells and on blast cells in myeloid leukemia.[14-15] FLT3 signaling normally supports survival and proliferation of hematopoietic progenitor cells, as well as differentiation of macrophages and dendritic cells.

Mutations in the FLT3 gene are mainly concentrated to two regions: the juxtamembrane domain, which has negative autoregulatory activity, and codons 853-836 in the second tyrosine kinase domain (activation loop). The most common mutation, found in about 25% of all adult AML patients and up to 45% of AML patients with normal karyotype, is an internal tandem duplication (ITD) in exon 14 of the juxtamembrane region.[16] It results in a varying number (three to several hundred) of insertions, which are always in-frame, i.e. with potential to produce a functional protein. The duplicated sequence may rarely involve intron 14 and exon 15. The tandem insertion weakens the negative autoregulatory function of the juxtamembrane domain, leading to constitutive activation of the FLT3 receptor.[17-19] Several point mutations affecting the aspartate residue at codon 835 (found in about 7% of AML cases; 16), and rarely, other sites in the kinase domain, have been described in AML patients.[20-22] Both ITD at the juxtamembrane domain and the mutations at the kinase domain are also seen in a small percentage of myelodysplastic syndrome (MDS) and acute lymphoblastic leukemia (ALL) cases.[23-24] Both types of *FLT3* mutations result in constitutive ligand-independent activation of the receptor. The ITD mutation leads to strong constitutive activation of transcription factors Stat5 and FOXO, while the normal targets for the wild-type receptor (Ras/Raf/MAP kinase pathways and Akt) are only weakly activated.[25-27] In contrast, signaling from a receptor with a tyrosine kinase mutation is qualitatively similar to that of the wild-type receptor.[28]

It is still unclear whether point mutations in the tyrosine kinase domain are correlated with a specific AML type and whether they constitute independent prognostic factors.[29-35] However, the *FLT3-ITD* mutation is well established as an adverse prognostic factor in AML patients [36-38] with the exception of the acute promyelocytic leukemia (APL) variant.[38-39] Up to 5 different *FLT3-ITD* mutations of varying size and relative level have been reported to co-exist in individual patients.[37,40-42] Loss of the remaining wild-type *FLT3* allele indicates worse prognosis.[38-39] Increased levels of *FLT-ITD* have previously been seen in relapsed AML in several studies.[43-45] In a recent investigation a decreased remission rate was associated with an increasing number of *FLT3-ITD* mutants, but the relapse risk and overall survival did not differ significantly. The size of the ITD mutation (range 15-231 bp) had no impact on the clinical outcome in young *FLT3-ITD* positive patients.[40] Some studies have shown that the *FLT3* mutation status may change during the course of treatment; thus this marker should be used with some caution for minimal residual disease (MRD) detection.[43-44,46]

FLT3-ITD can be detected by qualitative PCR amplification of genomic DNA using primers that span the ITD region, followed by capillary electrophoresis to determine the number of repeats. The point mutation at codon 835 can be detected by PCR followed by polyacrylamide gel electrophoresis, or by real-time PCR. When referring to older literature, it should be noted that the nomenclature for the *FLT3* gene was revised in 2001 as described in.[47] There is a strong interest in developing target inhibitors of FLT3 for therapeutic use in patients with AML. The tyrosinase inhibitors AC220 and midostaurin are currently in clinical trials on AML patients with *FLT3* mutations.[48-50]

CEBPA

CEBPA is a member of the basic region leucine zipper family of transcription factors. It is crucial in the granulocyte maturation process.[51-52] The gene consists of a single exon and is located at chromosome 19q13.1. N-terminal frameshift mutations cause truncation of the full length transcript but allow for generation of a shorter 30-kd isoform from an alternative downstream in-frame start codon. This translational isoform has a dominant-negative effect on the normal activity of CEBPA. C-terminal mutations, on the other hand, are in-frame and act by disrupting the zipper region, which leads to impairment of dimerization and DNA binding.[51-54] CEBPA acts as a tumor suppressor. In AML, specific loss of the wild-type transcript leads to bypassing of the CEBPA-regulated coupling of lineage commitment and proliferation control during myeloid differentiation.[51]

Mutations in the *CEBPA* gene are seen in about 10% of patients with AML, and in up to 15% - 18% of individuals with normal karyotype AML.[51,52] In AML with a normal karyotype, *CEBPA* mutations confer a favorable prognosis.[55-57] The 2008 WHO provisional diagnostic category "AML with mutated *CEBPA*" includes sporadic as well as familial AML with mutated *CEBPA* (defined as germ-line *CEBPA* mutation identified in a family where several members have been diagnosed with AML).[58-59] In a majority of AML patients with sporadic *CEBPA* mutations, the mutations are bi-allelic. The most common combination is one N-terminal together with one C-terminal mutation. The presence of two *CEBPA* mutations seems to confer a more favorable prognosis than single mutations.[51-55,60] Mutations in other AML associated genes such as *FLT3* or *NPM1* are relatively infrequently observed in the same patient; the most frequent "second" genomic abnormality in a patient with one *CEBPA* mutation is an additional *CEBPA* mutation.[51]

CEBPA mutations are more common in patients with the French-America-British (FAB) subtypes 1 and 2.[51] AML patients with *CEBPA* mutations display a specific gene expression profile,[55,59,60,61] showing upregulation of genes involved in erythroid differentiation, while genes coding for factors in proliferation signaling pathways and genes involved in myeloid differentiation are down regulated.[51]

The expression and activity of CEBPA in AML has been shown to be regulated by a number of events and factors, including hypermethylation of the *CEBPA* promoter,[62] and

activation of the RNA-binding protein calreticulin which blocks translation of CEBP proteins.[63] Inhibition of phosphorylation of CEBPA may be involved in the differentiation block seen in blasts in AML with FLT3 activating mutations.[64] The PML-RARA fusion protein has been shown to decrease CEBPA activity; treatment of patients with APL with all-trans retinoic acid induces *CEBPA* mRNA expression.[65]

Restoring CEBPA function would be of great interest in the treatment of AML. Both MAP/ERK kinase inhibitors and FLT3 tyrosine kinase inhibitors have been shown to decrease phosphorylation on a specific serine residue of CEBPA, thereby increasing the function of the CEBPA protein.[51]

Testing for mutations in the *CEBPA* gene can be performed by sequencing analysis of the single exon. The most common approach includes PCR amplification by several primer pairs, followed by fragment analysis by capillary electrophoresis of the amplicons. This protocol screens for changes in amplicon size produced by insertions and deletions of the gene. Fragments of aberrant length are then subjected to sequencing.[66] Previously described polymorphisms in the *CEBPA* gene need to be taken into account when results are interpreted.[67-68]

CEBPA mutations should be evaluated in familial AML.[69] Testing for the germline mutation should be performed on cells that are not involved by leukemia, such as buccal swab specimens. Traumatic sampling with risk for admixture of blood should be avoided.

NPM1

The *NPM1* gene is located at 5q35.1. Several pseudogenes and alternative splicing transcripts have been identified. The *NPM1* gene encodes a phosphoprotein which is normally localized to the nucleolus but has the ability to shuttle between the nucleus and the cytoplasm. The protein is involved in protein chaperoning and a multitude of other cellular processes involving ribosomes, centrosomes, and histones. It also has a role in regulation of the tumor suppressor ARF/p53 pathway.[70] The mutated form found in AML is sequestered to the cytoplasm.

Aberrations at the chromosomal level involving NPM1 are seen as translocations in anaplastic large cell lymphoma, APL and MDS. However, the molecular defects associated with AML consist of small insertions, most commonly TCTG, in exon 12. So far, the TCTG mutation has not been seen in any other hematologic malignancy.[71-72] This frame-shift mutation produces a disruption of the nucleolar localization signal in the C-terminal portion of the protein. Furthermore, a new signal is created causing the aberrant accumulation of NPM1 in the cytoplasm.[73-74] *NPM1* mutations are present in about 50% of AML cases with normal cytogenetics. The exact mechanisms underlying *NPM1* associated leukemogenesis are unknown, but may include disruption of normal interactions with tumor suppressors p14ARF and p53.[75] As opposed to the *FLT3-ITD* mutation, the *NPM1* mutations are generally seen in the entire leukemic cell population. Furthermore, in instances where both *FLT3* and *NPM1* mutations are found, the *NPM1* precedes the *FLT3* mutation. The *NPM1* mutation is therefore assumed to represent an early event in leukemogenesis, while *FLT3* mutations are associated with progression of disease.[40,76] Apart from *FLT3*, cooperating secondary mutations include those in the *NRAS*, *IDH1*, and *TET2* genes. In rare cases the leukemic stem cells with *NPM1* mutations may acquire a recurrent chromosomal abnormality as a second hit.[70]

The *NPM1* mutations seen in AML confer a favorable prognosis.[71,76] In fact, mutated *NPM1* defines a subgroup of AML with a distinct gene expression and microRNA profile.[77-79] A majority of AML cases with *NPM1*-mutations are negative for CD34.[70,80-81] Patients who have an *NPM1* mutation but lack a *FLT3-ITD* mutation have a favorable prognosis and may not benefit from stem cell transplant in first remission.[40,56,73,76,82,83] The high degree of responsiveness to induction chemotherapy seen in patients with *NPM1* mutations has been suggested to correlate with the quantity of *NPM1* mutation.[84] Monitoring of the mutation by quantitation could potentially predict relapse at an early stage similarly to *BCR-Abl* monitoring. The presence of *NPM1* mutations appears to be stable over time even when the AML spreads to extramedullary locations, making them attractive targets for MRD detection.[76,85-89] In comparison, *FLT3-ITD* may be lost at relapse in about 9% (4-27%) of cases, which should be taken into account when using FLT3-ITD for MRD detection.[44,90]

The *NPM1* mutation can be studied with molecular diagnostic methodology as well as by immunohistochemistry targeting cytoplasmic expression of nucleophosmin.[70] Although immunohistochemistry may be conducted in laboratories without access to molecular methodology, the result may be difficult to interpret in blasts with a minimal cytoplasmic compartment, and it is not suitable for quantitation/MRD detection.[83] Cytoplasmic NPM1 can also be detected by flow cytometry.[91]

miRNA

MicroRNAs (miRNAs) are short (18-25 nucleotides), non-coding RNAs that act as regulators of gene transcription by hybridizing to complementary mRNA (messenger RNA). Each miRNA has the capacity to regulate many different genes.[92] MiRNAs are involved in cellular differentiation, proliferation and development of cancer.[93]

Over the past couple of years, several studies have specifically addressed the role of miRNAs in AML. Associations between up- or downregulation of various miRNAs and the *NPM1, FLT3-ITD, and CEBPA* mutations, as well as overexpression of *BAALC* and *MN1*, has been shown.[94]

One study identified a miRNA expression signature that distinguishes a group of *NPM1* mutation positive patients from cases without *NPM1* mutations.[95] Interestingly, several of the down-regulated miRNAs in this study are located in

the *HOX* gene cluster. Upregulation of *HOX* genes has previously been seen in cases with *NPM1* mutations.[73] In a different study, miRNA expression was shown to predict *NPM1* mutation status with 92.5% accuracy.[77] Another investigation showed upregulation of miR-155 in *FLT3* mutation positive AML patients.[94,96] A recent study which used quantitative real-time PCR to measure a selected group of miRNAs showed correlation between miR-181a and morphological subtypes M1 and M2 (FAB), and an association between expression of miRNAs and *HOXA* and *HOXB* genes.[94,97-98]

A previous study defined a miRNA signature including down-regulation of members of the miRNA-181 family which was associated with clinical outcome.[99] Expression of miR-181 family members has been suggested to correlate with aggressiveness of disease; a different investigation found up-regulation of miR181 family members to be included in a miRNA signature associated with *CEBPA* mutations.[100] Isolated miRNA-181a expression was associated with favorable clinical outcome in two different patient cohorts, which suggests the possible usefulness of this marker in prognosis as well as in future treatment strategies.[99,101]

Technically, many issues remain to be resolved, including those related to reproducibility, sensitivity and, importantly, to processing of large amounts of data. Substantial validation work will be needed before miRNA expression arrays may potentially become part of the routine clinical evaluation.

EMERGING MARKERS AND NEW TECHNOLOGY

As stated earlier, the main purpose of molecular diagnostic testing in AML is risk stratification, in particular of the large group of AML patients classified as intermediate risk, since these patients do not harbor any of the defined recurrent cytogenetic changes. As of now, *FLT3*, *NPM1* and *CEBPA* mutations are relatively well-established molecular markers for AML; however, a significant number of karyotypically normal AML cases do not demonstrate changes in any of these specific genes. Analysis of other genes such as *KIT* and *WT1*,[102-103] or of the *WT1* polymorphism rs16754,[103] may become clinically useful when more knowledge accumulates and/or new therapeutic strategies are developed.[10] In addition, in the past couple of years, a number of other potential prognostic markers have been identified in AML with normal karyotype, including acquired somatic mutations in *MLL*,[104-105] *NRAS*,[106] *IDH1/2*,[107-112] *IDH1 SNP rs11554137*,[113] and *DNA methyltransferase 3A (DNMT3A)*[114] as well as changes in expression levels of *MN1*,[115-116] and *BAALC*.[117] Aiming at understanding the interaction between different markers, both clinical and molecular, a recent study created an integrative prognostic risk score (IPRS) including age, white blood cell count, mutation status of *NPM1, FLT3-ITD, CEBPA, SNP rs16754*, and expression levels of *BAALC, ERG, MN1*, and *WT1* in individuals with AML with normal karyotype.[118] However, several of these markers are not clinically applicable at this point and more extensive validation is needed.

In the future, microarray profiling may become a clinically relevant tool for molecular subclassification and prognostication of AML, as well as for identification of novel therapeutic targets.[119-120] Large-scale interrogation of gene mutations (genotyping) and gene expression analysis by microarray has already increased our understanding of the pathogenesis of AML.[121] The most commonly used DNA hybridization microarray type features specific nucleotide sequences (oligonucleotide probes) attached to a matrix on a solid surface. Nucleic acid isolated from the patient sample to be interrogated is hybridized to the probes. The probe-target complexes can be detected and quantified by various techniques including chemiluminescence. Several microarray platforms exist. Furthermore, microarrays can be customized, i.e. manufactured to contain different sets of oligonucleotide probes depending on the intended application. It should be noted that even if the same type of microarray is used, algorithms for handling of raw data (clustering of samples, filtering of signals, data normalization and setting of thresholds during analysis) may vary between protocols and affect reproducibility between different laboratories. In addition, variation in the size and composition of the population under study, as well as technical issues related to sample collection, nucleic acid isolation, and the hybridization process itself may affect results.[122] Transcriptional profiling entails quantitative analysis and is inherently more complex than genotyping.[123] Reproducibility is thus a crucial issue that needs to be addressed when considering clinical use of microarrays. For example, a recent meta-analysis comparing 25 published reports of gene expression profiling (GEP) in AML found a total of 4,918 reported genes. In this study, about one third of the total number of genes and only a minority of reported prognostically-associated genes (9.6%) were replicated in at least one other study.[124] However, the partial lack of overlap of expression profiles seen using different microarray platforms may not be an insurmountable problem, since different sets of markers may be equally informative for prognosis.[121,125] This was recently demonstrated in the context of breast cancer profiling as two different GEP predictors with limited overlap were identified by two separate research groups. Both profiles were externally validated, and one is now converted into an FDA cleared commercially available test (Mammaprint).[121] However, it is clear that much standardization work remains before microarrays may be used in clinical hematology practice. The MicroArray Quality Control (MAQC) project spearheaded by the FDA aims to develop standards and quality measures for microarray and next-generation sequencing technologies.[126]

The first application of microarray-based gene expression profiling for prognosis in AML used an unsupervised class discovery approach (i.e. did not use external information such as mutations or karyotypic subtypes to build profiles). This microarray accurately classified an initial group of test cases into AML vs. acute lymphoblastic leukemia. A derived class predictor algorithm was then able to determine the class of an independent data set. These experiments also demonstrated the potential of gene expression profiling in the discovery of new subclasses of AML.[127] Since then, several

investigators have used microarrays to define molecular signatures for AML. Specific gene expression patterns for known translocations, and previously unrecognized subtypes have been demonstrated.[128-131] Defined expression patterns have been linked to known molecular markers such as *NPM1* and *CEBPA.*[132] For instance, one study demonstrated that only *CEBPA* homozygotes, not heterozygotes, displayed a specific gene expression profile in AML.[133] Another investigation suggested the existence of a new distinct subgroup of AML characterized by epigenetic *CEBPA* silencing and an immature myeloid/T-lymphoid phenotype.[134] Attempts at predicting *FLT3-IDT* status in patients with normal karyotypes have produced varying results in regard to sensitivity and specificity. In a recent study a GEP based predictor for *FLT3-ITD* mutation status which had been built using samples from patients with AML and normal karyotype showed a sensitivity of 73% and a specificity of 85% in the validation group. However, in terms of predicting clinical outcome, this expression profile claimed to outperform presence of the FLT3 mutation as analyzed by standard molecular methodology.[135]

The largest microarray gene expression profiling study in AML so far was performed by eleven different laboratories affiliated with the international multi-institutional Microarray Innovations in Leukemia (MILE) research study group using a customized AmpliChip Leukemia microarray. Analyzing 2,096 samples, this stage I retrospective study achieved 92.2% classification accuracy for all 18 distinct AML classes investigated. A second prospective study comprising 1,152 patients showed an overall accuracy of 88.1% in classification of 14 subgroups.[136]

The clinical utility and feasibility of using microarrays in the context of diagnostics and prognosis of AML still needs to be defined. It has been suggested that microarray gene expression analysis may replace or complement cytogenetic analysis in cases where metaphases are not available or of insufficient quality.[136] In the future, microarray assays that comprise a large number of defined molecular markers may replace multiplexed PCR/sequencing as a more cost-efficient alternative.[119] However, before implementation of gene expression profiling assays by microarray as a diagnostic assay, proper clinical validation comprising prospective analysis of large patient groups is needed. Also, added value beyond what is contributed by traditional markers including currently used molecular markers needs to be shown.[125, 137, 138]

Tremendous progress has recently been seen in the development of high-throughput technologies (next-generation sequencing) which enables analysis of large amounts of data. So far, application of next-generation amplicon deep-sequencing has been investigated in the context of MRD monitoring for patient-specific *RUNX-1* and *FLT3* mutations.[139-140] Although much work remains before such applications can be validated and used in the clinical laboratory, improved technology allowing simultaneous interrogation of a multitude of mutations representing multiple signaling pathways, transcription factors and miRNAs involved in pathogenesis, together with evaluation of epigenetic changes, as well as computerized analysis that integrate these data with traditional prognostic parameters will enable health care providers to provide personalized treatment options and improved care for patients with AML.

PRACTICAL ASPECTS OF MOLECULAR TESTING

In clinical practice, when selecting a molecular assay, one needs to consider whether the goal is to detect a specific mutation with high sensitivity, or to have the potential to detect a wide range of mutations. In addition one needs to determine whether quantitation of the mutation is necessary. For a diagnostic assay the blast count is assumed to be over 20%. In this case, the ability to detect all known mutations in the target gene may take precedence over the ability to quantitate/detect very low levels of these mutations. Both PCR amplification followed by fragment analysis of the resulting amplicons, and the more sensitive high resolution melting (HRM) analysis may serve as a screening assay for length mutations. Implicated amplicons are subjected to Sanger sequencing for further characterization.

On the other hand, for assays designed to monitor MRD, high sensitivity and the ability to quantitate are of great importance.[141-142] Real-time quantitative PCR assays claiming to detect specific mutations with extreme sensitivity (down to 0.001% of the cell population) have been described. The prerequisite is that the patient-specific mutation must be known. Pending external standardization of quantitative assays, great care should be taken when comparing mutation level results produced in different laboratory settings. Another caveat in the context of MRD monitoring is the stability of the mutations during disease evolution. As pointed out previously, *NPM1* mutation status appears to be stable over time; however, there are indications that the opposite may be true for *FLT3.*[143-144] All assays that use DNA as starting material as opposed to RNA need to address the issue of pseudogenes by careful design of primers. However, given current patent related restrictions on *FLT3* mutation detection, and the fact that many small and medium-sized academic laboratories do not have access to capillary electrophoresis instrumentation for fragment analysis and Sanger sequencing, molecular hematology tests are often performed as send-out tests to larger reference laboratories.

SUMMARY

Study of molecular markers has contributed significantly to the management of AML, especially in the case of AML with normal karyotype, and in the detection of MRD.[145-147] With the advancement of molecular technology and increasing understanding of existing and emerging markers, we may be able to further stratify and treat AML cases accordingly.

Conflict of Interest

The authors have no conflict of interest to disclose.

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