Acute Myeloid Leukemia Diagnosis in the 21st Century

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Context.—Rapid advances in understanding the molecular biology of acute myeloid leukemia are transforming the approach to diagnosis, prognostication, and treatment of these cases.

Objective.—To briefly review the current state of AML classification with a particular emphasis on the role of molecular studies and their impact on the management of acute myeloid leukemia and other malignancies.

The advent of the new 2008 World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissue1 makes it clear that the entire field of pathology is changing very rapidly, and not just hematopathology. The new classification defines 108 new diagnostic entities in hematopathology, including 50 new or provisional leukemia entries. The classification of acute leukemias in particular has moved a long way from the French-American-British classification, which was entirely based on morphology and cytochemical stains. Today’s WHO classification requires not only morphology but also immunophenotyping, cytogenetics, and molecular studies for proper classification and prognostication.2

The goal of this article is to briefly review some of the basics of contemporary acute myeloid leukemia (AML) diagnosis under the 2008 WHO classification and illustrate how molecular studies are transforming the approach to diagnosis, prognostication, and choice of therapy. We have gone from almost complete ignorance of molecular abnormalities to having a reasonably useful set of molecular markers for the karyotypically defined subtypes. Even more strikingly, during the past several years, clinically useful molecular abnormalities have been identified in most cases of AML with normal karyotype, a heterogeneous group of disorders about which little was known at the molecular level.

DIAGNOSIS

Several excellent and more detailed reviews of acute leukemia diagnosis have been published recently. The WHO classification1 is especially good. The reader is also particularly encouraged to visit the United States & Canadian Academy of Pathology (USCAP) Web site3 (http://www.uscap.org/site--/98th/pdf/companion21h02.pdf; accessed June 4, 2010) and review the “Algorithmic Approach to the Classification of Acute Leukemia” by Daniel Arber, MD, which was a major inspiration for this overview. The starting point for diagnosis of leukemia is morphologic examination to document the presence of at least 20% blasts in bone marrow or in blood. In rare cases, the blast count is below 20%, but cytogenetic abnormalities are present that by convention warrant a diagnosis of AML (see cytogenetics section below). It is important to assess the degree of dysplasia in the different lineages, as the presence of multilineage dysplasia warrants the diagnosis of AML with multilineage dysplasia, which has adverse prognostic implications (Figure 1).4

Cytochemistry, which played a central role in older leukemia classification schemes, is no longer required, although in rare cases it can be helpful in the identification of monocytic differentiation. The current standard of care is to perform immunophenotyping by multicolor flow cytometry to further subclassify by lineage (eg, myeloid leukemia, B-cell leukemia, and T-cell leukemia) and to supplement the findings with other studies discussed below. Blasts are expressed as the percentage of nucleated cells and the count is typically based on a 200-cell count in peripheral blood and 500-cell count in the blood marrow. If there are more than 50% erythroid precursors, the erythroid progenitors are also excluded from the blast count. This is quite important in the diagnosis of acute erythroleukemia.

Monoblasts and promonocytes are included in the blast count as blast equivalents, while monocytes are excluded. The morphologic features of these 3 types of cells, especially if dysplastic, can be problematic. Monoblasts are quite large, with moderate amounts of cytoplasm that is often vacuolated and nuclei with delicate chromatin. Promonocytes are even larger cells, often with folded nuclei. Their chromatin is still quite fine, and it is common

Data Sources.—Current literature and experience of the authors.

Conclusions.—While morphology, immunophenotyping, cytogenetics, and clinical history continue to play an important role, an increasing number of molecular tests are now required to properly classify these cases. (Arch Pathol Lab Med. 2010;134:1427–1433)
to see vacuolated cytoplasm. Although the WHO emphasizes abnormal monocytes, these are not readily distinguished from normal ones. In general, their nuclei are larger and not as folded as those of their normal counterparts. In addition, it is important to distinguish promonocytes from the dysplastic promyelocytes of acute promyelocytic leukemia, which have variable cytoplasmic granulation, Auer rods, and characteristically bilobed nuclei.

Clinical findings play an important role in the 2008 WHO classification. The myeloid disorders associated with Down syndrome are now diagnosed separately from non–Down syndrome cases. If a patient with AML has an antecedent history of myelodysplasia, the leukemia is diagnosed as AML with multilineage dysplasia. Finally, if patients have been previously treated with cytotoxic chemotherapy, the leukemia is put into the therapy-related AML category. Cases of therapy-related AML with a specific recurrent abnormality are diagnosed as AML with the specific chromosomal abnormality (eg, therapy-related AML with t(9;11)) (Figure 1).

THE IMPACT OF CYTOGENETIC ABNORMALITIES

About 45% of acute leukemias have an abnormal karyotype with a recurrent chromosomal alteration, and about 15% have 3 or more cytogenetic abnormalities (complex karyotype). Cytogenetic studies remain an extremely important prognostic indicator and define 3 general risk groups: favorable, intermediate, and adverse. The favorable-risk group includes acute promyelocytic leukemia with t(15;17) and is the only AML subtype that is associated with a specific therapy (ie, trans–retinoic acid). Acute myeloid leukemias with t(8;21) or inv(16)/t(16;16) tend to be in the favorable-risk category but are treated with the same agents (cytarabine, doxycyclin) as all the other subtypes. Although this type of leukemia is considered “favorable,” half of the patients still die from their disease, and the results are even worse in older patients. Cases with t(9;11) are associated with intermediate risk, along with cases showing gains of whole chromosomes or loss of the Y chromosome. The adverse-risk group includes t(6;9), inv(3)/t(3;3), and complex karyotype. Cases with multiple alterations tend to be observed with older patients and have an extremely poor prognosis. Until recently, it has been difficult to stratify the subset of AMLs (40%) with normal cytogenetics (CN-AML). The overall risk in this group is “intermediate”; however, the outcome in individual cases varies considerably and is related in part to the presence or absence of specific molecular abnormalities.

As discussed above, in rare cases the blast count is less than 20% but a recurrent cytogenetic abnormality is present. The WHO currently recommends that cases with a t(8;21)(q22;q22), inv(16)(p13.1q22), t(16;16)(p13.1;q22), or t(15;17)(q22;q12) abnormality be considered AML with that specific recurrent genetic abnormality. If the blast count is less than 20% but other recurrent cytogenetic abnormalities are present, then these cases are most commonly classified as myelodysplastic disorders with a cytogenetic abnormality. Time will tell whether other recurrent abnormalities will be considered pathognomonic. Acute myeloid leukemia cases with complex karyotype (3 or more cytogenetic abnormalities) have an extremely poor prognosis, with patients rarely surviving beyond 1 year. But even within this group, there might be some buried, but useful, information. Bob Löwenberg, MD, and colleagues in Belgium have conducted a very large study of adult patients with AML and put forward a new concept that may be more helpful in assessing the impact of cytogenetic abnormalities. This is the so-called monosomal karyotype, in which at least 2 autosomal monosomies or an autosomal monosomy, in the presence of 1 or more structural abnormalities, is present: for example, monosomy 5 and monosomy 7, or monosomy 5 with del(7q). The 4-year overall survival associated with the monosomal karyotype within the complex karyotype group is 3% compared to 26% for the other cases.
Increasingly, acute myeloid leukemia classification has become based on genetics and it parallels recent advances in our understanding of the underlying biology. As a result, it is now clear that this is a highly heterogeneous collection of diseases, each arising from the sequential acquisition of specific genetic alterations. Some of these, such as the recurrent cytogenetic abnormalities, have also proved to be important markers of prognosis. However, the lack of such aberrations in CN-AML has proved challenging for the management of this clinically variable group. During the last 10 years, nucleotide-level mutations that are undetectable by standard cytogenetics have been discovered within a growing list of genes. Like the recurrent cytogenetic aberrations, these can also carry important prognostic implications. This is no more evident than in CN-AML, for which gene mutations have revolutionized the prognostic stratification of this heterogeneous group. To date a wide assortment of mutations have been identified in CN-AML, the most frequent of which are FLT3 internal tandem duplications (ITDs), FLT3 tyrosine kinase domain mutations, MLL partial tandem duplications, and a variety of nucleotide-substitution mutations or short insertion or deletion mutations within the coding region of the NPM1, CEBPA, NRAS, and WT1 genes. Of these, NPM1, CEBPA, and FLT3-ITD mutations now have sufficiently well-established prognostic significance that testing is recommended for all patients with CN-AML who will receive treatment other than low-dose chemotherapy or best supportive care. These mutations and their clinical relevance are discussed further below. The remaining mutations (NRAS, MLL partial tandem duplications, WT1, and FLT3 tyrosine kinase domain mutations) either do not have prognostic relevance in the context of current therapies, or the clinicopathologic significance remains unclear at present and requires further investigation (Figure 2).1,2,8

### The Importance of Molecular Abnormalities

The affected gene targets are involved in key pathways that regulate cellular survival, proliferation, and hematopoietic differentiation. These discoveries have laid the foundation for the 2-hit model hypothesis in which leukemia-initiating cells acquire 2 classes of mutations that cooperate during leukemogenesis.9 The class I mutations (ie, FLT3, KIT, NRAS/KRAS, and PTPN11) confer a proliferation and survival advantage and frequently target key components of kinase signaling pathways. These occur late and are associated with AML progression. In contrast, class II mutations (ie, CEBPA, NPM1, and the recurrent chromosomal translocations/ inversions described above) lead to impaired myeloid differentiation by affecting genes involved in transcriptional regulation. These occur early during leukemogenesis and are stable throughout the disease course and have been proposed to be founder (initiating) mutations.

Class I and class II mutations occur together in very specific patterns. For example, FLT3-ITD with concurrent NPM1 mutation is common and represents a collaboration of both enhanced proliferation (class I) and a block in differentiation (class II). In contrast to this, class II mutations generally do not coexist in AML. This mutual exclusivity, along with the high stability of these mutations at relapse, is consistent with the proposition that class II aberrations are founder mutations. Each mutation within this class is also frequently associated with characteristic clinicopathologic features, suggesting that each defines a unique and biologically distinct entity. Further support comes from recent studies highlighting specific gene-expression, microRNA, and DNA methylation signatures that are associated with each class II subtype.10–12 These collective observations provided the rationale for the inclusion of AML with mutated NPM1 and AML with mutated CEBPA as provisional entities in the 2008 WHO classification scheme for AML with recurrent genetic abnormalities (Table).1 Accordingly, NPM1 and CEBPA mutation testing is recommended for cases lacking recurrent chromosomal translocations or inversions. As described above, FLT3-ITD mutation testing should also be performed in this group owing to its strong prognostic relevance (Figure 3).1,2,8

Gene mutations identified thus far have limited prognostic utility in AML subtypes other than CN-AML. One possible exception is in the core-binding factor leukemias (ie, t(8;21) and inv(16)), which demonstrate a relatively high frequency of KIT exon 8 and 17 gene mutations (20%–40%).13 Recent studies14,15 have consistently associated KIT exon 17 mutations, especially D816V, with an inferior outcome in the t(8;21) subset. In one study,15 the median survival was reduced from 1836 days
to 304 days when KITD816 mutation was present in t(8;21) AML. This association, and also the relevance of KIT mutations in inv(16) AML, needs to be clarified with a larger set of patients before routine testing is warranted. In the 15% of AML cases with complex karyotype, p53 abnormalities may now stratify even these high-risk patients. As in solid tumors, p53 mutations in acute leukemia are a very poor prognostic finding.17

Of practical consideration are logistics related to molecular test ordering. In most instances the specific array of molecular tests that are ordered can be restricted to specific cytogenetic risk groups (Figure 3). The most effective method to accomplish this is to collect a peripheral blood or marrow specimen for molecular testing at the same time other diagnostic specimens are collected. This can be submitted to the molecular laboratory with an order to “extract DNA and hold for authorization.” Once the cytogenetic determination is complete, then the relevant molecular tests can be authorized. This procedure ensures that a high-quality DNA specimen is obtained, since AML molecular tests usually require fresh and not archived blood or marrow specimens. If the molecular laboratory does not extract and hold DNA specimens, then the panel of molecular tests should be ordered and tested immediately upon collection of the specimen.

**FLT3-ITD Mutation: Unfavorable Risk Marker**

FMS-related tyrosine kinase 3 (FLT3) is a membrane-bound receptor tyrosine kinase that, when activated by its ligand, supports the proliferation and survival of hematopoietic progenitors. Internal tandem duplication mutations occur across a wide range of cytogenetic subsets, including about 30% of CN-AML cases, and are strongly associated with poor outcome, including shorter relapse-free and overall survival.17 The ITDs result from the duplication and tandem insertion of a small, variably sized (3–400 nucleotides) fragment of the gene. Mechanistically, this is a gain-of-function mutation that leads to ligand-independent constitutive activation of the receptor. Identification of ITD mutations generally involves a polymerase chain reaction (PCR)-based test that can detect the larger PCR amplification products indicative of the duplication (Figure 4, A).18

**NPM1 Mutation: Favorable Risk Marker**

Nucleophosmin (NPM1) is a multifunctional phosphoprotein that shuttles between nuclear compartments and the cytoplasm. In its normal state, NPM1 is predominately located in the nucleolus where, among other functions, it is implicated in ribosome assembly and regulation of ARF and p53 tumor suppressor function.19 Mutations in NPM1 were first discovered in AML owing to the cytoplasmic mislocalization of the mutated NPM1 protein.20 These cases were previously referred to as NPM1c positive and were associated with CD34− immunophenotype. NPM1 mutations are now considered the most common genetic lesion in AML, occurring in about 30% of adult de novo cases and in 50% to 60% of AMLs with normal cytogenetics. For patients with the latter condition, NPM1 mutation is associated with a good response to induction therapy and a favorable prognosis. Importantly, however, the favorable impact of NPM1 mutation is highly dependent on FLT3-ITD status. Only cases that have an NPM1 mutation without FLT3-ITD (NPM1mut/ FLT3-ITDneg) are associated with a favorable outcome.17,21–24 For these patients, the prognosis is similar to that associated with the favorable t(8;21) and inv(16)/t(16;16) core-binding factor leukemias. In contrast, the NPM1WT/FLT3-ITD+ genotype confers an unfavorable outcome. Because FLT3-ITD mutation status can affect the prognostic impact of NPM1, both should be tested for concurrently and their prognostic impact interpreted collectively.

More than 40 different mutations have been identified within exon 12 of the NPM1 gene, with 3 types (A, B, and D) constituting approximately 92% of these mutations.25 Despite this variety, virtually all of the mutations lead to a net insertion of 4 nucleotides. As with FLT3-ITD testing,
**NPM1** mutation has gained utility as an independent prognostic marker and is significantly associated with lower relapse rates and improved overall survival. These patients have a favorable prognosis that is similar to that of **NPM1**/**FLT3**-ITD AML. **CEBPA** mutations span the whole protein coding region, but largely fall within 2 types: N-terminal frameshift mutations that lead to truncation of the full-length p42 **CEBPA** protein and C-terminal in-frame mutations that affect both the full-length p42 **CEBPA** protein and a shorter p30 isoform that is normally coexpressed from an alternate internal start codon. The C-terminal mutations generally occur in the bZIP domain region and lead to proteins with impaired dimerization and DNA binding activities. Testing for **CEBPA** mutations requires a technology capable of detecting the wide range of nucleotide alterations (duplications, insertions, deletions, substitutions) that occur throughout the entire coding region. Thus, the preferred method is DNA sequencing, which is relatively laborious and requires specialized expertise. Consequently, this testing is currently only available in a limited but growing number of clinical laboratories (the test is offered at the University of Michigan through MLabs).

The significance of a positive **CEBPA** mutation test result requires careful interpretation. Positive cases typically harbor both N- and C-terminal mutations simultaneously, with each occurring on a different allele. The presence of such biallelic mutations is consistent with complete loss of **CEBPA** function. In contrast, up to one-third of CN-AML cases exhibit only a single mutation (monoallelic). While the functional significance of single **CEBPA** mutations is not clear, it is important from a prognostic standpoint to distinguish biallelic from monoallelic cases. This is emphasized by several recent studies demonstrating that only biallelic **CEBPA** mutations are associated with a favorable clinical outcome in CN-AML.

Recently, a series of AML cases have been identified that show silencing rather than mutation of **CEBPA**, as a result of high levels of CpG methylation. Interestingly, these are frequently associated with mutations in the transcription factor NOTCH, which are common in T-acute lymphoblastic leukemia and often show expression of T lineage in addition to myeloid markers. In contrast to the favorable outcome associated with **CEBPA** mutation, silencing of **CEBPA** is associated with a distinctly poor prognosis.

**EMERGING APPLICATIONS OF AML GENETICS: OPPORTUNITIES AND CHALLENGES**

**Prediction of Transplant Benefit**

Beyond their utility as markers of prognosis, gene mutations have been shown to also have predictive value. In a large meta-analysis of CN-AML by Schlenk et al., the genotype **NPM1**/**FLT3**-ITD predicted a lack of benefit from allogeneic stem cell transplant at first remission. In contrast, transplant improved outcome in patients with either the **FLT3**-ITD or **NPM1**/**CEBPA** genotypes. If independently verified, these findings have important implications for future therapeutic decision-making algorithms in CN-AML. While hematopoietic stem cell transplants are usually limited to AML with high-risk and not low-risk cytogenetics, their role for the

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**Figure 4.** Molecular tests for acute myeloid leukemia prognosis. A, **FLT3** internal tandem duplication (**FLT3**-ITD) mutation analysis. Note the second peak of larger size, which is consistent with the presence of an internal tandem duplication mutation. B, **NPM1** mutation analysis. A positive result is indicated by a mutant peak that is usually 4 nucleotides larger than the wild-type peak. C, **CEBPA** mutation analysis. Overlapping peaks in the DNA sequence chromatogram indicate the presence of a mutation. In this case it is a 1-nucleotide deletion (G) occurring in the N-terminal region. A second mutation was also observed in the C-terminal region (not shown).
intermediate-risk CN-AML group has not been well defined. The ability to predict benefit for patients in this group permits this high-risk procedure to be restricted to those who can benefit from it.

**Molecularly Targeted Therapies**

The ability to identify specific patients who may benefit from molecularly targeted therapies holds great promise in this emerging era of personalized medicine. By testing for the presence of genetic aberrations that are targeted by specific drugs, treatments can be tailored to the molecular characteristics of the individual patient’s disease. Indeed, one of the first examples of targeted therapy was the selective use of all-trans-retinoic acid in acute promyelocytic leukemia. This treatment, as later discovered, specifically blocked PML-RARA fusion protein function and revolutionized the prognosis and treatment of patients with this previously fatal disease. Other leukemias may respond particularly to TKIs. Kinase signaling pathways in particular are attractive targets owing to the high frequency of kinase-activating gene mutations, including FLT3 and KIT. These have been explored in various clinical trials, with much attention focused on FLT3 owing to its high mutation frequency and the current availability of tyrosine kinase inhibitors (TKIs), including suitinib, midostaurin, and lestaurtinib, which have established in vitro activity against FLT3 mutants. Ongoing trials are exploring combination therapy with TKIs and conventional chemotherapy for patients with FLT3 mutations in the hope of achieving a synergistic cytotoxic effect.

KIT mutations are common in the core-binding factor leukemias. In this instance, the specific KIT mutation needs to be considered owing to the differential sensitivity of the particular mutations to TKIs. Imatinib, for example, has activity against KIT proteins with exon 8 mutations, but not the D816 mutations found in exon 17. Other TKIs, such as dasatinib or midostaurin, can target D816 mutations. Still another opportunity for targeted therapy is provided by AMLs that show widespread DNA methylation abnormalities, such as cases with CEBPA silencing. These leukemias may respond particularly well to demethylating agents. Trials evaluating the activity of decitabine and azacitidine in AML are underway.

**Disease Monitoring**

The stability of the leukemic-specific fusion genes make them excellent markers for trending treatment response and detecting minimal residual disease. Quantitative molecular tests, with sensitivities as low as $10^{-5}$ to $10^{-9}$, offer the ability to assess both early treatment response and low levels of posttreatment disease. A minimal reduction or a persistence of the marker may indicate an impending relapse. This form of highly sensitive monitoring provides the opportunity for early intervention in the form of additional chemotherapies or allogeneic stem cell transplant months before there is morphologic evidence of disease. Studies in acute promyelocytic leukemia (PML-RARA) and the core-binding factor leukemias (RUNX1-RUNXIT1 and CBFB-MYH11) have provided encouraging results for the utility of quantitative monitoring of these markers for risk of relapse.

The lack of recurrent gene fusions in CN-AML has until recently made the application of genetics to disease monitoring difficult for this large group of patients. FLT3 gene mutations were a potentially attractive marker owing to the high mutation frequency across this and other AML subtypes. However, these mutations can occur in leukemic subclones and the mutation status can change during treatment, making their detection of limited clinical utility. NPM1 mutations, on the other hand, show promise for minimal residual disease, as they are highly stable and generally persist at relapse. Preliminary studies have correlated decreasing mutant NPM1 copy numbers with treatment response and persistence of posttransplant NPM1 mutations with relapse. For these reasons, it is likely that NPM1 mutations will become important markers for disease monitoring in patients with CN-AML. An alternative approach involves measuring mRNA expression levels for genes that are widely overexpressed in AML blasts. Of these, WTI has been extensively evaluated, with encouraging results for the use of this marker in predicting risk of relapse.

**CONCLUSION**

Acute myeloid leukemia is the focus of intense research, with major efforts into whole-genome sequencing for mutation discovery, array comparative genomic hybridization to identify submicroscopic genomic deletions and amplifications, and DNA methylation arrays to detect epigenetic modifications. Additional molecular markers will undoubtedly be identified that have prognostic and therapeutic significance. This will create new challenges for molecular pathology laboratories, informatics infrastructure, and hematopathologists to stay abreast of this rapidly moving field. The demand for sophisticated testing will further increase with the more widespread introduction of targeted AML therapies. This is truly an exciting time to be in the field of pathology and to witness the translation of molecular discoveries into better patient care.

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**References**

2. World Health Organization Classification of Tumours; vol 2.


