Review

Genetic Tests To Evaluate Prognosis and Predict Therapeutic Response in Acute Myeloid Leukemia

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Management of patients with acute myeloid leukemia relies on genetic tests that inform diagnosis and prognosis, predict response to therapy, and measure minimal residual disease. The value of genetics is reinforced in the revised 2008 World Health Organization acute myeloid leukemia classification scheme. The various analytic procedures—karyotype, fluorescence *in situ* hybridization, reverse transcription polymerase chain reaction, DNA sequencing, and microarray technology—each have advantages in certain clinical settings, and understanding their relative merits assists in specimen allocation and in effective utilization of health care resources. Karyotype and array technology represent genome-wide screens, whereas the other methods target specific prognostic features such as t(15;17) *PML-RARA*, t(8;21) *RUNX1-RUNX1T1*, inv(16) *CBFB-MYH11*, 11q23 *MLL* rearrangement, *FLT3* internal tandem duplication, or *NPM1* mutation. New biomarkers and pharmacogenetic tests are emerging. The pathologist's expertise is critical in 1) consulting with clinicians about test selection as well as specimen collection and handling; 2) allocating tissue for immediate testing and preserving the remaining specimen for any downstream testing that is indicated once morphology and other pertinent test results are known; 3) performing tests that maximize outcome based on the strengths and limitations of each assay in each available specimen type; and 4) interpreting and conveying results to the rest of the health care team in a format that facilitates clinical management. Acute myeloid leukemia leads the way for modern molecular medicine.   *(J Mol Diagn 2010, 12:3–16; DOI: 10.2353/jmoldx.2010.090054)*

More is known about the molecular basis of leukemia than any other form of cancer, primarily due to the availability of abundant malignant cells for study and because translocations and other gross chromosomal changes are often visible by karyotype. Limited prognostic and predictive ability of traditional morphological, immunophenotypic, and cytogenetic tests has driven research to define more subtle nucleotide-level alterations that not only shed light on pathogenesis but also serve as tumor markers and, in some cases, impart valuable prognostic information. Better understanding of disease biology and pathogenesis is essential to cancer prevention and to design novel interventions that are personalized to the host and tumor genotype.

The World Health Organization classification scheme for acute myeloid leukemia (AML) provides a framework for clinical management. It was revised in 2008 to add three distinct forms of AML with recurrent cytogenetic abnormalities [t(6;9), inv(3) and t(1;22)] and two provisional categories with nucleotide level changes (involving *NPM1* and *CEBPA* genes). These revisions emphasize the importance of genetic test results to define clinically relevant disease entities in conjunction with morphology, immunophenotype, and other clinicopathologic features[1] (Table 1). Moreover, management guidelines of the National Comprehensive Cancer Network highlight the added value of genetic tests in combination with more traditional microscopic examination and immunophenotyping (*http://www.nccn.org/professionals/physician_gls/f_guidelines.asp*, accessed July 14, 2009).

The relevant genetic technologies include karyotype, fluorescence *in situ* hybridization (FISH), polymerase chain reaction (PCR), sequencing, and microarrays. These DNA or RNA assays are widely considered to be the most powerful tools for predicting behavior of AML in

Table 1.   2008 World Health Organization Classification of Acute Myeloid Leukemia and Related Tumors

AML with recurrent genetic abnormalities
  AML with t(8;21)(q22;q22) *RUNX1-RUNX1T1* (*CBFA-ETO*)
  AML with inv(16)(p13q22) or t(16;16)(p13;q22) *CBFB-MYH11*
  APL with t(15;17)(q22;q11–12) *PML-RARA*
  AML with t(9;11)(p22;q23) *MLLT3-MLL* and other balanced translocations of 11q23 (*MLL*)
  AML with t(6;9)(p23;q34) *DEK-NUP214*
  AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) *RPN1-EVI1*
  AML (megakaryoblastic) with t(1;22)(p13;q13) *RBM15-MKL1*
  AML with mutated *NPM1**
  AML with mutated *CEBPA**
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified
  AML with minimal differentiation
  AML without maturation
  AML with maturation
  Acute myelomonocytic leukemia
  Acute monoblastic/monocytic leukemia
  Acute erythroid leukemias
  Acute megakaryoblastic leukemia
  Acute basophilic leukemia
  Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome (+21)
  Transient abnormal myelopoiesis
  Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasms

Adapted from Swerdlow et al.[1]
*Provisional categories.

response to therapy. Automation and kits are becoming available to facilitate implementing standardized assays in clinical laboratories. Results not only identify disease-specific genetic alterations that are important for diagno-sis and upfront management but also provide a mecha-nism to monitor tumor burden in response to therapy and to detect minimal residual disease that could herald im-pending relapse.

Definitions of several terms are pertinent. A prognostic test is one used to assess the likelihood of response to standard therapy, while a predictive test is used to as-sess response to a particular nonstandard intervention. A pharmacogenetic test is a predictive test for a specific pharmaceutical agent or regimen. Worldwide consensus on best practices for managing AML is evolving, and optimal test strategies and intervention for a given patient depend on factors beyond genetic test results.

Genetic Technologies

A brief description of each genetic technology is pro-vided in Table 2. Gross translocations and numerical changes in chromosomes are readily detected by karyo-typing. Less commonly recognized (but certainly not less commonly present) are mutations, subtle deletions, and/or gene amplifications. Regardless of which genetic defects initiate tumor cell growth, these defects are passed down to all cellular progeny within a tumor clone. Certain defects are characteristic of distinct clinicopath-ologic subtypes of cancer, and these defects serve as markers of the malignancy that can be used to assist in diagnosis, classification, and monitoring residual disease after therapy. Furthermore, knowledge of the affected biochemical pathway could help identify therapy target-ing the underlying cause of malignant cell growth.

Karyotype

The karyotype represents a genome wide screen for translocations and other numeric or structural defects

Table 2.   Genetic Test Methods

Laboratory Test	Description of Methods
Karyotype:	Whole chromosomes from cells in the metaphase stage of cell division are stained and visualized by microscopy.
Fluorescence *in situ* hybridization (FISH):	Whole chromosomes (metaphase from dividing cells or interphase from non-dividing cells) are hybridized to complementary probes and visualized on a fluorescence microscope.
Polymerase chain reaction (PCR):	DNA is isolated and a specific segment of it is copied a billion-fold for ease of detection and for further analysis. A variant method called reverse transcription PCR (rtPCR) converts RNA into complementary DNA (cDNA) prior to PCR amplification. A variant called quantitative PCR (Q-PCR) can measure the level of target DNA, usually by monitoring product accumulation during each cycle using one or more fluorescent internal probes, and then comparing the time course of product accumulation to a series of standards of known concentration. A fluorescent internal probe combined with "melt curve analysis" detects sequence variants within the amplicon.
DNA sequencing:	The nucleotide sequence is determined by replicating one of the DNA strands and monitoring the order in which labeled nucleotides are added.
Comparative genomic hybridization array (CGH array):	Patient DNA is hybridized to hundreds or thousands of probes arrayed on a solid surface, and gene dosage is determined for each locus on the array, thus identifying deletions, duplications, and gene amplifications. Single nucleotide polymorphism (SNP) arrays can additionally detect copy-neutral loss of heterozygosity (uniparental disomy).
Gene expression array:	Patient RNA is typically amplified and labeled, then mixed with control RNA labeled with a different fluorochrome and hybridized to hundreds or even hundreds of thousands of probes (eg, 60-mers) arrayed on a solid surface. Scans of each spot followed by data analysis permit evaluation of the gene expression profile in the tissue, which can be matched to the pattern of normal or diseased tissues for purposes of diagnosis, or to the pattern of clinical outcome variants to predict response to therapy.

that are present in about half of AMLs. Giemsa staining patterns (G-bands) are interpreted for each chromosome in at least 20 fresh dividing cells. Findings are further interpreted in the context of the patient's clinical and histopathological features to help diagnose and classify AML. Even nonspecific karyotypic changes can impact prognosis: a complex karyotype, usually defined as three or more concomitant defects, portends a poor prognosis in children and adults, including patients over 60 years old.[2–5] Recent data suggest that a "monosomal karyotype," defined by two or more autosomal monosomies or one monosomy plus one or more structural defects, for example, −5 with −20, or −7 with t(3;3)(q21;q26), is indicative of bad outcome in adults less than 60 years old, with an overall survival of only 4% at 4 years.[6]

Fluorescence in Situ *Hybridization*

FISH can be applied to either interphase (nondividing) or metaphase (dividing) cells where it is used to: 1) confirm a tumor-related karyotypic defect that can then be monitored over time in blood or marrow specimens, 2) detect cryptic translocation in a tumor suspected to harbor a particular defect based on clinicopathologic findings, 3) detect a deletion or duplication not evident by karyotype, and 4) discover critical cytogenetic information in specimens failing to grow in tissue culture media.

FISH probes are commercially available for relevant targets including t(15;17) *PML-RARA*, t(8;21) *RUNX1-RUNX1T1*, inv(16) *CBFB-MYH11*, and 11q23 *MLL*.[7] Various probe strategies (eg, single fusion, dual fusion, or break-apart) are used depending on the technical and clinical circumstances.[8] For example, a dual fusion strategy has better analytic sensitivity for finding low level *PML-RARA*, while a break-apart probe strategy detects any of the relevant *RARA* translocations irrespective of the partner gene. Centromere probes are used to enumerate chromosomal gains or losses such as trisomy 8 or monosomy 7. Published guidelines for validating FISH assays include recommendations on how to set a cutoff for interpreting results as normal versus abnormal.[8]

Sensitivity for detecting minimal residual disease depends on the probe strategy, specimen quality, and the number of cells that are scored. A typical interphase FISH is performed on 200 cells and reliably detects a leukemic clone involving at least 5% of cells in the specimen. This level of sensitivity is comparable with that of a 20-cell karyotype, although karyotype sensitivity varies depending on the relative growth rate of leukemic versus nontumor cells *ex vivo*. In dividing cells, metaphase FISH or hypermetaphase FISH can be applied to resolve complex karyotypes and identify partner genes fused by translocation.

A variation of FISH known as spectral karyotyping or multiplex FISH applies multiple labeled probes along whole chromosomes, essentially "painting" each of the 24 chromosomes a different color. Results are interpreted in conjunction with G-banded karyotype to sort out complex rearrangements. The gene copy number information is similar to, albeit with less resolution than, comparative genomic hybridization arrays.

Polymerase Chain Reaction

PCR has tremendous analytic sensitivity and has become a mainstay of molecular pathology. PCR can find "a needle in a haystack" because, after 30 cycles of amplification, each DNA or cDNA target sequence has been copied 2^{30} times, yielding a billion amplicons. These amplicons can be quantified using precise real-time instrumentation, and/or amplicons can be further evaluated using various analytic methods like sequencing, melt curve analysis, or electrophoresis. In typical clinical assays, a dilution of one leukemic cell per 100,000 normal cells is identifiable, facilitating detection of minimal residual disease. Paucicellular specimens and partially degraded nucleic acid can often be accommodated, which is helpful in unusual samples like cerebrospinal fluid or biopsied myeloid sarcoma. Molecular protocols have been published, and commercial primers and probes are available for selected fusion transcripts, mutations, and controls.[9–17]

DNA Sequencing

Determining the order of nucleotide bases is useful for genes like *CEBPA* having multiple alternative mutations at different nucleotide positions. Traditional assays relying on dideoxynucleotide incorporation can identify a variant comprising at least 20% of alleles in the specimen (equivalent to 40% of cells). Pyrosequencing, which detects pyrophosphate release, is potentially more sensitive and also more quantitative. High throughput sequencing methods are now being validated to expand coverage and in some instances to improve assay sensitivity.

Microarrays

Array technology is being validated for use in clinical laboratories so that many simultaneous analyses may be performed, such as gene expression profiling, gene copy number measurement, methylation, or allele-specific mutation detection. The massive amount of data generated by arrays requires bioinformatic tools to present the data in a manner that facilitates interpretation. Quality assurance and assay validation are especially challenging when so many tests are performed simultaneously.

Prognostic Applications of Genetic Technologies

Karyotype Is Prognostic

Karyotype is recommended in every suspected AML for proper diagnosis and classification. In the 2008 World Health Organization classification scheme, over two-thirds of AML cases are categorized based on genetic tests compared with only one-third in the 2001 World Health Organization scheme.[1] Even when blast percentage in blood or marrow does not exceed the 20% usually required for diagnosis of AML, presence of t(15;17), t(8;21),
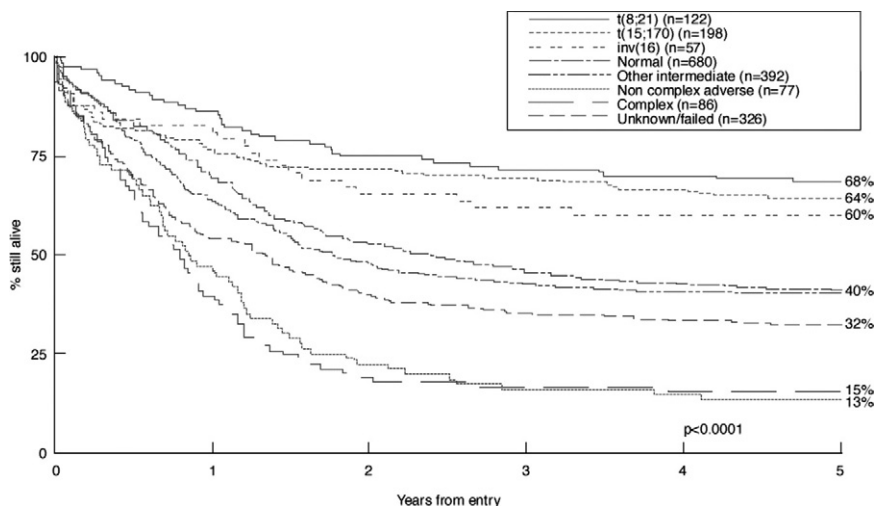
Figure 1. Prognosis in AML strongly correlates with cytogenetic findings.[18–21] Favorable prognosis is associated with t(15;17), t(8;21), or inv(16) whether alone or in combination with other chromosomal abnormalities, with the possible exception of inv(16) or t(8;21) with complex karyotype. An intermediate prognosis is associated with normal karyotype or select abnormalities: +6, +8, −7q, −9q, −12p, +21, +22, −Y. Adverse outcome is associated with abnormal 3q, 11q, 17p, 20q, 21q, or with −5, −5q, −7, −7q, −9q, t(6;9), t(9;22), or with complex karyotype. Abnormalities of the *MLL* gene on 11q23 impart a dismal prognosis.[22,23] In children, poor survival is associated with monosomies or complex karyotype.[5] (Adapted from Grimwade[18] with permission).

or inv(16) in the face of abnormal hematopoiesis is considered sufficient for a diagnosis of AML.

National Comprehensive Cancer Network guidelines refer to cytogenetics as the "single most important prognostic factor for predicting remission rate, relapse, and overall survival" (Figure 1). The choice of which therapy to deliver rests largely on grouping of AMLs into one of three categories: favorable, intermediate, or unfavorable risk.[18,24–26] Among 1213 AML patients treated on CALGB protocols, the 5-year survival rate was 55% for favorable, 24% for intermediate, and 5% for unfavorable cytogenetic categories.[27] Outcomes have improved since 2002 when these data were published, and now it has become routine to supplement karyotype with assays for molecular-level defects as described below.

Assays for Cryptic Translocation and MLL Gene Defects

In the ~45% of adult AML having a normal karyotype, FISH or rtPCR may help detect cryptic translocation whenever the clinical presentation (eg, DIC), morphology and immunophenotype is suggestive of one of the three favorable subtypes, t(8;21), t(15;17), or inv(16).[7,28,29] Another defect that is missed by karyotype involves partial tandem duplication of the *MLL* gene on chromosome 11q23.[30,31] Whether *MLL* is altered by partial tandem duplication or by translocation with any of >70 partners, *MLL* rearrangement portends a poor outcome in AML, except for t(9;11), which imparts an intermediate risk.[32–34] Patients with poor prognosis *MLL* rearrangement or partial tandem duplication are candidates for allogeneic stem cell transplant.

Acute Promyelocytic Leukemia with t(15;17) PML-RARA

Acute promyelocytic leukemia (APL) is an archetypal example of how genetic technologies are used in cancer diagnosis and management. It is important to recognize leukemia harboring t(15;17) *PML-RARA* because of the

unique treatment strategies and monitoring assays that are available to affected patients. Retinoic acid receptor α (*RARA*) gene structure is altered by translocation to thwart RARA transcription factor function and arrest differentiation at the promyelocyte stage. The molecular defect in most cases can be overcome by treating with high doses of retinoic acid (all-*trans*-retinoic acid, ATRA), providing a prime example of cancer therapy specifically targeting a gene product involved in tumorigenesis. Introduction of retinoic acid therapy represented a paradigm shift in managing leukemia because this drug operates by overcoming the effect of the translocation rather than by eliminating the malignant clone. To diminish the likelihood that secondary mutation renders the tumor resistant to single agent ATRA, combination therapy with an anthracycline-based regimen or with arsenic trioxide is recommended and is curative in about 80% of cases, representing one of the greatest advances in the history of cancer therapy.

A small subset of patients with morphological and clinical features overlapping those of classic t(15;17) APL have variant translocations: t(11;17)(q23;q12) *ZBTB16-RARA* (previously called *PLZF-RARA*), t(5;17)(q35;q12) *NPM1-RARA*, t(11;17)(q13;q12) *NUMA1-RARA*, t(4;17)(q12;q21) *FIP1L1-RARA*, interstitial duplication of chromosome 17 resulting in *STAT5B-RARA* fusion, or occult rearrangement of chromosome 17 resulting in *PRKAR1A-RARA* fusion.[35–37] Unraveling the genetics has therapeutic implications since defects involving *ZBTB16* or *STAT5B* may be resistant to ATRA. Nonetheless, when a morphological diagnosis of APL is made, it is reasonable to begin targeted therapy pending genetic testing, and adjust the therapeutic regimen if a relevant genetic defect is not identified.[38]

More than 95% of APLs harbor a *PML-RARA* translocation detectable by karyotype, FISH, or rtPCR.[28,39–41] The most sensitive of these is rtPCR in which RNA extracted from blood or marrow is converted to cDNA and then primers flanking the *PML-RARA* breakpoint specifically amplify the chimeric sequence (Figure 2). As with any assay targeting RNA, special precautions are needed to avoid RNA degradation. Negative results are
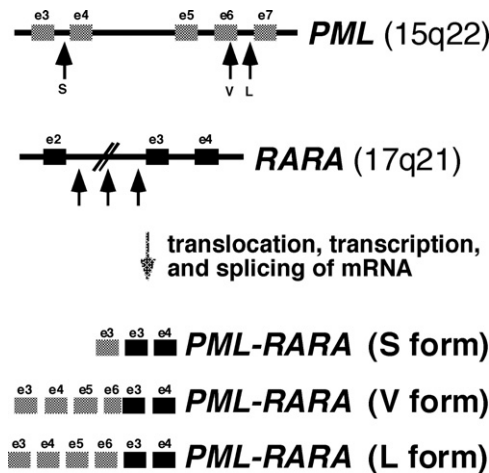
Figure 2. The *PML* and *RARA* genes are shown, along with three forms of *PML-RARA* fusion transcripts. Breaks in *PML* are clustered into three regions: intron 3, the distal half of exon 6, or intron 6. *RARA* breakpoints are spread across intron 2. In the *PML-RARA* fusion transcript, either exon 3 or exon 6 of *PML* is juxtaposed with exon 3 of *RARA* to produce short (S), long (L), or variable (V) length coding sequence. The short form, also called the bcr3 isoform, may carry a worse prognosis.

Figure 3. Relative frequencies of common recurrent genetic abnormalities in acute myeloid leukemia.

reported only when a control test of a "housekeeping" transcript shows that amplifiable cDNA was achieved, and results are reported in the context of assay sensitivity so that it is clear whether the assay can detect minimal residual disease.

After chemotherapeutic induction, consolidation therapy is used to achieve a durable molecular remission. Patients are likely to relapse if they do not achieve molecular remission in the marrow as assessed by rtPCR after consolidation.[42] National Comprehensive Cancer Network guidelines suggest that a positive rtPCR test result should be confirmed, and repeat positivity is treated as if the patient had hematological relapse (AML Clinical Practice Guideline Version 2009.1, accessed at *http://www.nccn.org/professionals/physician_gls/f_guidelines.asp* on July 14, 2009) In contrast, patients having consistently negative results after consolidation therapy enjoy prolonged survival and may even be cured.[26,43]

To detect early relapse, the National Comprehensive Cancer Network guidelines recommend that rtPCR be performed on either blood or marrow at 3-month intervals for the next 2 years, and then every 6 months for 2 to 3 years. Confirmed positive results, as defined by persistent positive rtPCR within a month of the first positive result, with at least one of these positive results being in marrow, warrant treatment for relapse. Patients who achieve molecular remission (negative by rtPCR) after being treated for relapse have better outcomes than those who remain positive. Readers are referred to alternative practice guidelines for further opinions on testing strategies in patient management.[38,44–47]

Prognosis of AML with Normal Karyotype

Studies have implicated a number of molecular abnormalities as being prognostic in cytogenetically normal AML (Figure 3). The various defects are not necessarily mutually exclusive, implying that each tumor probably
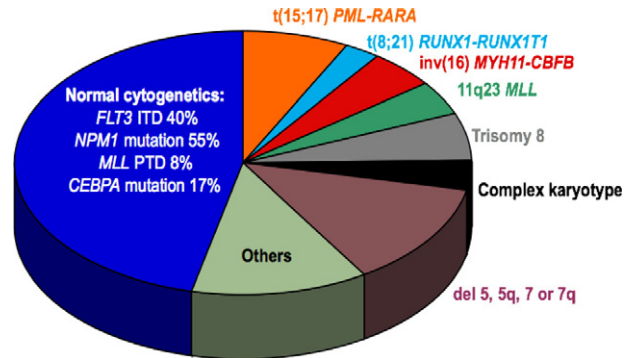
harbors multiple genetic abnormalities, and any one lesion may be insufficient for malignant transformation.[48,49] The combinations of abnormalities are not random, suggesting that co-acquisition of selected defects synergizes in leukemogenesis. One defect blocking differentiation and a second defect inducing proliferation appears to be a potent recipe for acute leukemia.[50–52] Sorting out the pathogenic defects from benign "passenger defects" is a daunting task given the propensity of cancers to acquire secondary genetic alterations. Some abnormalities are prognostic only in certain subsets of cancer (Table 3).

Molecular tests are increasingly applied to reveal tumor characteristics that refine prognosis in cytogenetically normal AML. Test results are used to select those patients who may benefit from chemotherapy and those who should be considered for an allogeneic stem cell transplant if a suitable donor is available and if patient age, comorbidities, and other factors are amenable for allografting.[48]

FLT3 Internal Tandem Duplication Confers a Worse Prognosis

Activating mutation of the FMS-related tyrosine kinase 3 (*FLT3*) gene is associated with a higher risk of relapse and a worse prognosis.[53,54] The relevant mutation is an in-frame internal tandem duplication (ITD) within the coding sequence of the juxtamembrane domain that causes constitutive activation of the encoded FLT3 tyrosine kinase. Signaling through the MAPK, PI3K, and STAT5 pathways contributes to proliferation, resistance to apoptosis, and blocked differentiation. *FLT3* ITD testing is recommended in all cytogenetically normal AML patients who are candidates for allogeneic transplantation or investigational therapies.[55] *FLT3* ITD is also present in a subset of APLs, but the implications for patient management are unclear.[56–58]

To identify the *FLT3* ITD, DNA from leukemic cells is first amplified using PCR and then sized by capillary electrophoresis to detect an abnormally large amplicon associated with ITD[59] (Figure 4). The extent of amplicon enlargement varies from 3 to about 400 bp, and the insertion is always "in frame" to preserve the translation and function of the remaining *FLT3* domains. A high ratio of mutant to wild-type *FLT3* has been linked to a worse outcome.[60]

Table 3. Prognostic Genetic Characteristics in Acute Myeloid Leukemia

Favorable risk factors
  t(15;17)(q22;q12) *PML-RARA*
  t(8;21)(q22;q22) *RUNX1-RUNX1T1*
  inv(16)(p13;q22) or t(16;16)(p13;q22) *CBFB-MYH11*
  NPM1 mutation when *FLT3* internal tandem duplication
    is absent and cytogenetics are normal
  CEBPA mutation (correlates with erythroid
    differentiation and higher hemoglobin)
Intermediate risk group
  Normal karyotype*
  FLT3 internal tandem duplication with *NPM1* mutation
    and normal cytogenetics
  KIT mutation with t(8;21) or inv(16)
  +8 only
  t(9;11) *AF9-MLL* only
  Abnormalities not otherwise listed
Unfavorable risk factors
  Complex karyotype (≥3 abnormalities)
  Monosomal karyotype (≥2 autosomal monosomies, or
    a single one plus ≥1 structural defect)
  −5, −7 or other autosomal monosomy
  del(5q) or del(7q)
  11q23 *MLL* translocation, excluding t(9;11) *AF9-MLL*
  MLL partial tandem duplication with normal
    cytogenetics
  inv(3)(q21;q26) or t(3;3)(q21;q26*) RPN1-EVI1* or
    MDS1-EVI1
  EVI1 overexpression
  17p abnormality or *TP53* mutation
  FLT3 internal tandem duplication when *NPM1* mutation
    is absent and cytogenetics are normal
  t(9;22)(q34;q11) *BCR-ABL1*
  t(6;9)(p23;q34) *DEK-CAN*
  ERG overexpression without *FLT3* ITD when
    cytogenetics are normal
  BAALC overexpression with normal cytogenetics
  MN1 overexpression with normal cytogenetics
  WT1 mutation with normal cytogenetics
  TET2 mutation

Prognostic categorization may vary by analytic method, patient population, study design, and other variables.
*Loss of X or Y chromosome is not considered an abnormality for purposes of prognosis.

While the ITD is strongly prognostic in both adults and children, point mutation in the kinase domain of *FLT3* does not seem to have a major influence on outcome.[61,62] Mis-sense mutations can rarely occur in the juxtamembrane domain and may confer activation similar to that of the ITD.[63] Testing for activating point mutations in *FLT3* is currently recommended only in clinical trials where efficacy of a putative FLT3 inhibitor is being examined.

NPM1 Mutation Confers a Better Prognosis

Nucleophosmin (*NPM1*) mutation is associated with a good prognosis when *FLT3* ITD is absent and an intermediate prognosis when *FLT3* ITD is present.[60] The *NPM1* mutation results from insertion (or combined insertion and deletion) in one allele of *NPM1*. The gene encodes a nuclear shuttle protein that, when mutated, aberrantly localizes to the cytoplasm, affecting its regulation of the ARF-p53 pathway.[64,65] *NPM1* mutation is found in 30% of all adult AML,[66] and it is enriched for in those with
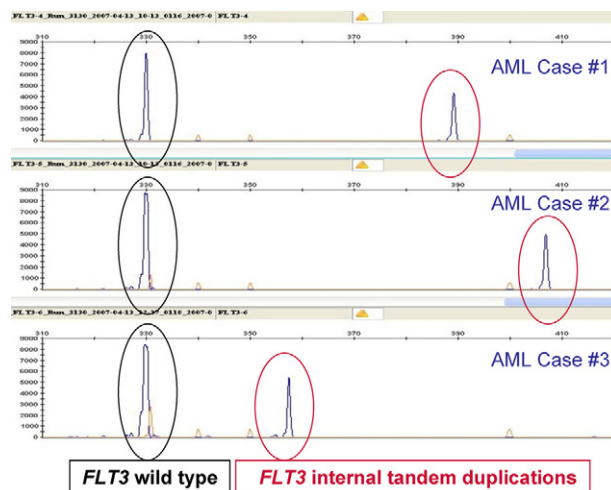

Figure 4. FLT3 internal tandem duplication is identified in genomic DNA that has been amplified across exons 14 and 15 using PCR and then sized by capillary electrophoresis. Amplicons representing the wild-type allele are 325 bp in length (circled in black). In each of three AML cases, a larger amplicon is also seen (circled in red), consistent with *FLT3* internal tandem duplication in the leukemic cells.

normal karyotype (55% of cases), which is the recommended target population for prognostic testing. It is much less common in childhood AML (8%) where it is most informative of outcome in cytogenetically normal tumors without *FLT3* ITD.[67,68]

Two predictive aspects of *NPM1* mutation have been proposed and must now be independently validated: 1) Patients with *NPM1* mutation without *FLT3* ITD do not necessarily benefit from allogeneic stem cell transplant following conventional anthracycline and cytarabine-based induction therapy[54]; and 2) older patients with *NPM1* mutation without *FLT3* ITD might benefit from adding ATRA to their chemotherapy regimen.[69]
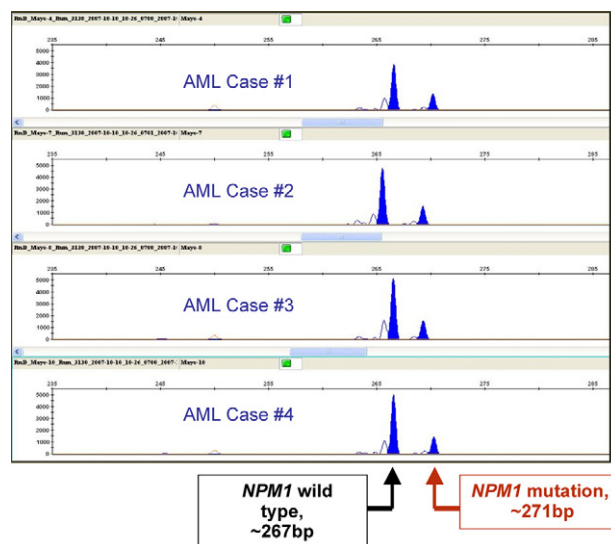

Figure 5. NPM1 mutation is identified in genomic DNA from four cases of AML by amplification of a segment of exon 12 followed by sizing of the amplicons using capillary electrophoresis. Amplicons representing the normal, wild-type allele are 267 ± 1 bp in length, while abnormal amplicons are usually 4 bp larger, consistent with insertional mutation in the leukemic cell DNA.

Laboratory testing for *NPM1* mutation typically relies on PCR followed by capillary electrophoresis to detect a small insertion in one allele in exon 12[70] (Figure 5). Alternative molecular methods are feasible.[12,71,72] At least 40 molecular variants exist, most of which result in a 4-bp enlargement, although rarely an amplicon up to 11 bp larger than normal is seen. Frameshift mutations interfere with the nucleolar localization motif in the C-terminal end of the protein. Mislocalized NPM1 protein can often be visualized using immunohistochemistry except in blasts having scant cytoplasm. Molecular testing for *NPM1* mutation is a reasonable alternative to immunohistochemistry when one considers that interpretation of *NPM1* results is usually done in conjunction with *FLT3* results, and *FLT3* is tested using molecular methods.

In adult AML, type A *NPM1* mutation (a 4-base TCTG duplication) comprises three quarters of mutated cases, while two alternate 4-bp insertions at the same position (type B is CATG, and type D is CCTG) comprise an additional 15% of mutated cases. Type B insertion is more common in children. Each of these common variants has been targeted using allele-specific amplification to detect minimal residual disease and to predict relapse.[73–75] Unfortunately, lack of the mutation in 10% of relapsed AML patients limits the reliability of these allele-specific assays for monitoring tumor burden over time.[73]

CCAAT/Enhancer Binding Protein α (CEBPA) Mutation Confers a Better Prognosis

"AML with a mutated *CEBPA* gene" is a provisional category of the World Health Organization classification comprising 17% of cytogenetically normal AML. *CEBPA* mutation is a favorable prognostic indicator when it is biallelic and when it occurs in isolation of other prognostic genetic defects.[76–78] Moreover, *CEBPA* mutation portends a better prognosis than does wild-type *CEBPA* in AMLs with *FLT3* ITD.[78] Despite the absence of *FLT3* ITD, the subset of patients with so called "triple negative" results (negative for *CEBPA* mutation, *NPM1* mutation, and *FLT3* ITD) do poorly and may be considered for allogeneic transplant.[54]

CEBPA can be silenced by either mutation or by promoter hypermethylation,[76,79] implying that DNA sequencing in combination with methylation analysis is required to capture all of the relevant prognostic information. *CEBPA* encodes a transcription factor important in neutrophil differentiation. Mutation down-regulates *HOX* gene expression leading to decreased expression of myeloid differentiation factors, induction of *miR181*, and increased expression of erythroid differentiation genes leading to elevated hemoglobin.[80,81] There is slow uptake of *CEBPA* testing, in part because rather extensive sequencing and methylation analysis is required to detect the relevant defects.

ERG, BAALC, WT1, EVI1, MN1, microRNA, and Integrated Panels of Prognostic Factors

In addition to the gene rearrangements and mutations described so far, transcriptional dysregulation of selected genes (eg, *ERG, BAALC, WT1, EVI1, MN1, miR181*) seems to confer prognostic information.[51,82] For example, *EVI1* overexpression in AML (or rearrangement of the *EVI1* gene on 3q26) is associated with lack of response to current treatments and a dismal prognosis.[82]

Applying a large panel of microRNAs reveals patterns of expression that are associated with outcome in AML.[80] The microRNA signature has been purported to add prognostic value beyond what is achievable with *FLT3* and *NPM1* testing. A panel of just seven microRNAs could distinguish the major karyotypic categories of AML.[83] A panel of 12 microRNAs can divide cytogenetically normal AML into poor and intermediate risk categories independently of *FLT3* ITD.[84]

The list of prognostic factors seems to be growing rapidly, making it difficult to discern which prognostic factors are independent of the others, and which panel of tests to perform in a given patient. Prognostic factors are most useful when they impact on therapeutic response, in other words, when they are predictive of outcome.[54,55] It would be helpful to have a tiered algorithm for ordering various genetic tests based on cost effectiveness data in various clinical scenarios that account for the available therapeutic options (eg, stem cell transplant). Alas, long lists of putative prognostic tests and therapeutic regimens, combined with a paucity of clinical trial data for various settings, render it difficult to achieve international consensus on a testing algorithm. A useful integrated predictor has recently been proposed by Dutch/Belgian/Swiss investigators that relies on combined *FLT3, NPM1, ERG, CEBPA,* and *BAALC* genotypes to place patients into one of four groups with respect to the risks and benefits of stem cell transplantation.[85]

Predictive Applications of Genetic Technologies

KIT Mutation and Drug Responsiveness

KIT is a receptor tyrosine kinase that functions in normal hematopoiesis. Gain of function mutations in *KIT* have been found in 2% of AML overall and in a third of the "core binding factor" leukemias [AML with t(8;21) or inv(16)], as well as in systemic mastocytosis and several non-hematopoietic malignancies.[86] *KIT* mutation (especially D816V encoded by exon 17) is associated with a worse prognosis in AML with t(8;21) *RUNX1-RUNX1T1,* in contrast to the good prognosis normally associated with t(8;21).[20] Some *KIT*-mutated malignancies respond to tyrosine kinase inhibitors, although response depends on the type of *KIT* mutation and on the mechanism and site of action of the drug.[87] A number of tyrosine kinase inhibitors such as imatinib, dasatinib, and PKC412 are being tested for efficacy against *KIT*-mutated AML.[88]

KIT mutation is generally detected by sequencing exons 8 and 17 in leukemic cells. Alternatively, allele-specific PCR can detect exon 17 D816V, the most relevant mutation in AML.[89]

RAS Mutation and Drug Responsiveness

Among the *RAS* family of genes, *NRAS* and *KRAS* are more frequently mutated in AML than is *HRAS*.[90] Overall, *RAS* mutation is present in about 15% of AMLs and is enriched for in cases having inv(16) or inv(3).[91,92] *RAS* mutation may enhance response to high dose cytarabine,[54,93] while response to a farnesyl transferase inhibitor (tipifarnib, a drug that shuts down activated RAS) was predicted by the *RASGRP1* to *APTX* gene expression ratio.[94]

Emerging Array Technologies

Microarray-Based Gene Expression Profiles

As the list of prognostic and predictive tests becomes longer, it is reasonable to consider whether massive parallel transcriptional analysis might be more cost effective than panels of disparate ancillary methods (eg, karyotype, FISH, mutational analysis, immunophenotype). Gene expression profiling uses arrays to detect and semiquantify expression of all ~25,000 human genes at once. Smaller or custom arrays can be created to order. The microarray method requires extracting RNA from a fresh or frozen specimen containing a high proportion of malignant cells to identify patterns of gene expression that are characteristic of prognostic or predictive subsets of disease. Distinct profiles are seen in most AML categories of clinical importance, such as t(8;21), t(15;17), inv(16), and the monocytic subclasses.[95–98] *CEBPA* mutation or methylation-related silencing has a distinct profile, highlighting the ability of arrays to detect both genetic and epigenetic forms of *CEBPA* dysfunction. Not surprisingly, the group of AMLs with a complex karyotype lacks a distinct profile, in keeping with the diversity of the genetic defects comprising complex karyotypes. Interestingly, *NPM1* and *FLT3* defects are not readily identified by array signatures, suggesting that mutational effects are diverse or else the same effects are seen in non-mutated cases.[97]

Once validated, it is likely that expression profiles will be used for patient care, either in place of traditional assays or to select the next round of ancillary tests that are most appropriate for managing that patient initially and after treatment when residual disease testing is relevant. Novel array-based prognostic algorithms have been proposed. In one study of adult AML, a 133-gene algorithm predicted survival independent of the usual clinical predictors.[99] In another study, 86 probes targeting 66 genes yielded a prognostic score that was independent of *FLT3* and *NPM1* status in cytogenetically normal AML.[100] MicroRNA signatures are also informative and may complement mRNA signatures in classifying AML and predicting outcome.[95,98,101,102] It is likely that array signatures will be interpreted in combination with more traditional clinical data (eg, age, cell counts) and independent prognostic factors. Expression profiling does not currently have a role in monitoring residual disease; however, this does not exclude a role for expression profiles in monitoring response to treatment.

Microarray-Based Gene Copy Number Variants and Whole-Genome Sequencing

While normal cells have two copies of every gene (one inherited maternally and the other paternally), many AMLs have fewer or more copies of a given gene, of a whole chromosome, or of an intermediate sized region. While karyotype can detect large additions or deletions, array technology can, depending on the design of the probes and their density, detect smaller copy number changes and even point mutations or segmental uniparental disomy. Uniparental disomy can result in duplication of a mutated locus while the normal locus on the other allele is lost, causing copy neutral loss of heterozygosity with the potential for complete alteration of gene function.

Copy number variation studies show that AMLs contain many alterations that were occult by traditional karyotype. Even balanced translocations are often identifiable based on subtle hybridization variation at the breakpoints in DNA (or in fusion transcripts).[103–105] Identification of microdeletions or duplications, as well as cryptic translocations, may add value when interpreted in combination with results of traditional metaphase cytogenetics.[106]

Full-genome sequencing is now feasible and may reveal novel factors responsible for tumor initiation and progression. Full-genome sequencing of an AML showed 10 acquired mutations, two in known AML-related genes and another eight that were unexpected.[107] Full genomic or exonic sequencing is considered a discovery tool at this time, but more targeted sequencing or SNP arrays approaches may be useful for finding druggable pathways and markers for monitoring tumor burden.

A Practical Approach to Specimen Allocation and Testing

Given the ever-expanding panels of tests now available, it is timely and important to describe a rational approach to ordering genetic tests at initial diagnosis and in follow-up. For any marrow suspected of harboring AML, karyotype must be done up front.[26] If the karyotype is normal, but morphology and immunophenotype suggest one of the prognostically favorable karyotypes, FISH or rtPCR should be done to detect cryptic rearrangement of the relevant locus. This genetic workup is considered sufficient for initial therapeutic decision-making.[26] Additional testing to refine prognosis is useful for decision-making at the time of first remission or relapse.[108–111]

Normal karyotype AML patients may benefit from an additional panel of prognostic tests (eg, *FLT3* ITD, *NPM1* mutation) to assist with downstream clinical decisions such as whether to prioritize transplant in first remission.[54,109–111] Activating *KIT* mutation (exons 8 or 17) negatively influences prognosis in t(8;21) cases, but otherwise such patients tend to do well with standard high-dose ARA-C-containing consolidation regimens and are not considered candidates for allogeneic transplant in first remission.[20] In unfavorable prognosis AML, the benefits of allogeneic transplant may outweigh the risks.[109,110]
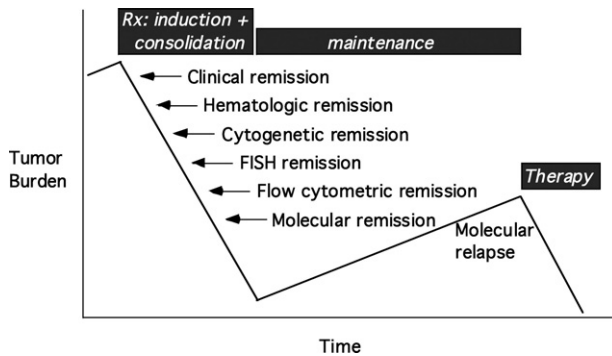
Figure 6. Genetic and phenotypic abnormalities that are unique to the tumor provide a marker by which to measure tumor burden. Up to a billion leukemic cells remain in a patient who is in hematological (morphological) remission. Sensitive assays can detect and measure residual disease to permit early intervention when tumor burden is rising.

The Dutch-Belgian-Swiss clinical trial experience in adults under age 60 was recently summarized in the context of risk categorization using modern panels of cytogenetic and molecular tests.[85,108]

Tumor burden can be monitored over time if the malignant clone has a distinct feature that can be accurately and sensitively measured in blood or marrow.[26,112] Fusion transcripts, antigen receptor gene rearrangement, and point mutation are examples of these biomarkers. Figure 6 shows the relative sensitivity of various detection methods. Amplification assays can often detect very rare tumor cells in a specimen containing upwards of 100,000 normal cells, permitting relapse to be predicted well before the patient becomes symptomatic.[9–11] Early detection and treatment of relapsing disease restricts the number of cell divisions and thus limits the risk for secondary genetic hits that might render the tumor less responsive to therapy.

A positive molecular test may be the first sign of impending relapse. A negative test result can be interpreted with certainty only when the test is known to detect a valid tumor marker for that patient. Therefore, it is wise to assess tumor markers upfront when the tumor is abundant. A cost-effective alternative is to save residual leukemic specimens for later testing. Assuring proper handling and storage of specimens is critical.

Specimen Collection and Storage

The best available specimen should be used for genetic analysis. Fresh marrow or blood should be collected and handled according to the testing laboratory's recommendations. While heparin is the preferred anticoagulant for cytogenetics, heparin can interfere with DNA amplification, so EDTA is preferred for PCR and rtPCR-based assays. Processing for cytogenetics should be initiated as soon as possible, preferably within 24 hours of collection. Any residual cells remaining after karyotype can be stored for further analysis once the karyotype is known. These leftover cells are typically fixed in Carnoy's solution (methanol and acetic acid) and refrigerated to preserve target analytes for FISH or rtPCR.

Smears and touch preparations are amenable to interphase FISH, and these may be air dried and saved unstained at 4°C for several weeks before analysis, thus allowing for completion of morphological, immunophenotypic, and karyotype studies before ordering FISH. Although interphase FISH can be done on paraffin sections, smear and touch preparations containing single layers of whole nuclei are more readily interpreted compared with paraffin sections where parts of nuclei are often missing.

It should be emphasized that virtually all PCR-based assays are more robust when applied to fresh or frozen cells as compared with fixed cells, since formalin-mediated cross-linking renders nucleic acid less amenable to hybridization. Formalin preservation, although not ideal, is preferred over B5 and other mercury-based fixatives. Decalcification results in acid-mediated degradation of nucleic acid, so clot sections are preferred over decalcified biopsy sections.

RNA is a particularly labile molecule, so handling specifications should be strictly followed and specimens should be delivered promptly to the testing laboratory. Tests for minimal residual disease, whether they target DNA or RNA, require special care to prevent degradation before analysis. As a check on specimen quality, test results are interpreted in conjunction with results of a control assay demonstrating amplifiable housekeeping DNA or cDNA, as appropriate.

A summary of acceptable specimen types for various molecular tests is shown in Table 4. While PCR is typically used to detect point mutations and small duplications or insertions (eg, *NPM1* mutation or *FLT3* ITD) in extracted DNA, rtPCR is typically used to detect fusion transcripts representing translocations or inversions in extracted RNA. The optimal strategy for designing an assay relies on a thorough understanding of the relevant technologies, specimen types and handling parameters, genetic

Table 4. Tissue Requirements for Genetic Tests

Laboratory procedure	Recommended sample types
Karyotype	Heparinized marrow aspirate (preferred) or blood or fresh biopsy
FISH	Fresh cells for metaphase analysis; for interphase analysis, alcohol-fixed cells, smears, touch preparations, or formalin-fixed, non-decalcified tissue sections
Southern blot analysis, gene expression array, rtPCR	Fresh blood or marrow (EDTA), frozen nucleated cell pellet, frozen tissue
PCR, DNA sequencing, CGH array, microRNA	Fresh blood, marrow aspirate, or body fluid; frozen or paraffin-embedded tissue

Refer to collection and handling requirements for each assay in each testing laboratory.

Table 5.   Genetic Predisposition to Acute Myeloid Leukemia

Heritable syndrome	Gene symbol	Gene name	Locus
Fanconi anemia A	*FANCA*	Fanconi anemia, complementation group A	16q24.3
Fanconi anemia C	*FANCC*	Fanconi anemia, complementation group C	9q22.3
Fanconi anemia D2	*FANCD2*	Fanconi anemia, complementation group D2	3p26
Fanconi anemia E	*FANCE*	Fanconi anemia, complementation group E	6p21-p22
Fanconi anemia F	*FANCF*	Fanconi anemia, complementation group F	11p15
Fanconi anemia G	*FANCG*	Fanconi anemia, complementation group G	9p13
Fanconi anemia J	*BRIP1*	BRCA1 interacting protein C-terminal helicase 1	17q22
Fanconi anemia N	*PALB2*	Partner and localizer of BRCA2	16p12.1
Familial AML	*CEBPA*	CCAAT/enhancer binding protein (C/EBP), alpha	19q13.1
Familial platelet disorder with propensity to AML	*RUNX1*	Runt-related transcription factor 1	21q22.3
Schwachman-Diamond	*SBDS*	Schwachman-Bodian-Diamond syndrome protein	7q11
Bloom	*BLM*	Bloom syndrome	15q26.1
Li-Fraumeni	*TP53*	Tumor protein p53	17p13
Down			trisomy 21
Ataxia telangiectasia	*ATM*	Ataxia telangiectasia mutated	11q22.3

target(s), and intended use of the assay. Guidelines for assay validation were recently published.[113]

Reporting and Quality Assurance of Genetic Tests

College of American Pathologists' recommendations for reporting molecular test results include specifying the technology used and the gene targets.[114] To ensure that everyone uses the same term for a given gene, the Gene Nomenclature Committee of the Human Gene Organization has developed a database of the name and symbol for each gene, searchable at *http://www.genenames.org* (accessed July 14, 2009). Cytogenetic nomenclature rules are used to designate karyotype and FISH findings, while nucleotide-level alterations are described in comparison with a reference sequence.[114] So that reports may be deciphered by a general physician, a written explanation of the results and their clinical significance is essential. A molecular genetics pathologist is well suited to interpret and convey results by virtue of expertise in the relevant technologies as applied to diagnosis, prognosis, and prediction.

Molecular results should be interpreted in conjunction with morphological and clinical information to maximize the value for clinical decision-making.[114] The 2008 World Health Organization book states, "because of the multi-disciplinary approach required to diagnose and classify myeloid neoplasms it is recommended that the various diagnostic studies be correlated with the clinical findings and reported in a single, integrated report."[1] This synthesis is typically done by the consulting hematopathologist at initial diagnosis. In follow-up specimens, the ordering physician (whether the clinician or consulting pathologist) assures that testing is medically necessary and that results are synthesized with relevant clinicopathologic findings. Molecular pathologists and other physicians overseeing laboratory testing are responsible for assisting with test selection, interpreting results, and assuring quality and relevance of laboratory work.

Heritable Syndromes Predisposing to AML

Inherited predisposition to AML should be considered in patients having a strong family history of cancer (Table 5). The heritable defect often involves a DNA repair protein, in which case affected patients should minimize exposure to radiation and chemotherapeutic drugs that induce DNA damage. Therapy-related leukemia is associated with heritable polymorphisms in drug metabolizing enzymes such as glutathione-*S*-transferase M1 or T1, *N*-acetyl transferase 2, quinone oxoreductase, or cytochrome p450 (CYP1A1).[115]

Interestingly, studies of families inflicted with multiple myeloid malignancies uncovered rare instances of germ-line defects in *CEBPA* or *RUNX1*, the same genes that are somatically altered in some leukemias.[116] The 2008 World Health Organization classification system includes a new category designated as "myeloid leukemia associated with Down syndrome," defining a group of tumors with distinct clinicopathologic correlates (eg, frequent *GATA1* mutation) and altered prognosis compared with sporadic leukemia.

Summary and Future Directions

Genetic technologies are powerful ancillary tools for diagnosing, classifying, and managing acute leukemia. Over 150 different recurrent lesions have been described in AML, and dozens of these influence patient management. The Association for Molecular Pathology Test Directory (*http://www.amptestdirectory.org/,* accessed July 14, 2009) displays information about testing laboratories, and the following websites (accessed July 14, 2009) also contain reliable information linking cancer genotype and phenotype: *http://atlasgeneticsoncology.org* (Atlas of Genetics & Cytogenetics in Oncology & Hematology); *http://www.ncbi.nlm.nih.gov/Literature/index.html* (medical literature); *http://www.ncbi.nlm.nih.gov/sites/entrez?db=cancerchromosomes* (cancer genetics database of the National Center for Biotechnology Information). On the horizon are pharmacogenetic tests estimating likelihood of response to spe-

cific therapy based on knowledge of a patient's germline and/or leukemic genotype. Arrays evaluating dozens to thousands of analytes, when applied to RNA, can survey numerous biochemical pathways by assaying panels of transcripts or miRNAs. When applied to DNA, arrays can simultaneously detect mutations, deletions or amplifications, epigenetic changes, and sometimes even balanced translocations. Prediction scores will emerge as cost-effective strategies for managing reams of disparate clinical and laboratory information. Array results may suggest which additional ancillary test to perform for purposes of identifying a tumor marker to track over time. Finally, arrays hold promise for unraveling the complexity of tumor heterogeneity in a way that drives development of novel therapies and companion assays to predict and track therapeutic efficacy. Classification schemes will regroup patients based on improved understanding of pathobiology and shared response to given interventions. Sensitive genetic tests will evaluate the success of therapy and trigger further intervention when relapse is imminent.

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