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ORIGINAL ARTICLE

Molecular characterization and testing in acute myeloid leukemia

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Abstract Acute myeloid leukemia (AML) is a clinically and biologically heterogeneous group of neoplasms found in both the adult and pediatric populations. Many of the mutations that underlie AML pathogenesis have been elucidated and include both large-scale genomic events such as chromosomal additions, deletions, and translocations, as well as small-scale point mutations in tumorigenic genes. As patient prognosis and therapeutic decisions are largely determined by these genetic events, multifaceted genetic analysis of tissue sample from AML patients is required for proper diagnosis. Thus, an understanding of both the genetic events associated with AML and the testing modalities that assess these aberrations is essential for appropriate sample evaluation. Additionally, recognition of the molecular differences between adult and pediatric AML may provide insights into critical pathways involved in AML pathogenesis.

Keywords Acute myeloid leukemia · Molecular alterations · Testing modalities · Genetic events

Acute myeloid leukemia (AML) affects ~12,000 new patients in the USA each year [1]. Although this leukemia mainly occurs in older adults, a significant proportion of patients are infants and children, and roughly 800 pediatric cases of AML—accounting for approximately 20 % of pediatric leukemia—are diagnosed yearly in the USA [2–4].

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Both adult and pediatric AML are characterized by an overproduction of myeloid progenitor cells that are unable to develop into mature nonlymphoid hematopoietic elements [5, 6]. This typically (but not always) manifests as an overabundance of blasts that account for >20 % of total nucleated cells. The rather broad definition of AML clearly encompasses a wide variety of discrete pathologic entities that display highly variable phenotypic features. For instance, there is a spectrum of morphologic and immunophenotypic findings in AML, with tumor cells ranging from very immature CD34+ blasts to cells showing granulocytic, monocytic, erythroid, or megakaryocytic lineage commitment [6, 7]. Likewise, patients with AML show a wide of clinical outcomes, with some responding quite well to conventional chemotherapy and experiencing sustained remissions, while others require bone marrow transplantation for long-term survival, and still others display refractory disease with rapid demise [8–10].

The pathologic and clinical phenotypes of AML are driven, in large part, by the specific genotypic alterations harbored by the tumor cells. Since the unifying features of AML are increased proliferation of hematopoietic elements and the inability of these cells to differentiate, Gilliland and coworkers categorized mutations in AML as class I (those that are related to increased proliferation) and class II (those that are related to lack of maturation), and they surmised that these two classes of mutations must both occur to convert a normal hematopoietic cell into one giving rise to AML [11, 12]. Recently, a third class of mutations (class III), involving genes that regulate epigenetic modifications and chromatin structure, has also emerged as critical for AML pathogenesis [13]. Although these three types of mutations are not always identified in a single tumor, many mutations recurrently found in AML fall into one of these three categories. Additionally, this three-class model

of mutations is clearly not comprehensive, as large-scale sequencing studies have revealed mutations in other genes, such as splicing factors, that influence additional cellular properties [14].

Though these types of AML mutations underlie the general similarities among AML cases, the wide diversity of AML phenotypes suggests a similarly wide diversity of AML-associated mutations. Indeed, numerous recurrent driver mutations are found among AML patients, and not surprisingly, many of these mutations correlate with specific phenotypic features, including cytomorphology, immunophenotype, and clinical outcomes [5, 14–16]. The importance of the underlying mutational events driving both AML biology and the clinical course of patients has been codified in the WHO classification of hematopoietic tumors, and current clinical workups for proper AML categorization are largely dependent on multiple genetic tests of blood or bone marrow samples [6, 17]. In addition to the mutations formally used for AML subclassification, many novel AML driver mutations have recently been identified through large-scale genome sequencing projects [14, 18, 19]. As many of these mutations have prognostic implications, genetic testing at these additional will likely become standard in the diagnostic workup of both adult and pediatric AML.

This article describes many of the mutations found in AML, with particular attention paid to those currently used to define AML subclasses, as well as to those with prognostic significance in pediatric patients. Since identifying these mutations in AML requires multiple assays to be performed on blood, bone marrow, or other tissue specimens, a basic understanding of testing methodologies is essential for proper specimen triage. Thus, discussions of both standard and emerging clinical molecular assays are included. Finally, important molecular distinctions between pediatric and adult AML will be highlighted along with possible implications of these differences.

Karytopic ("large-scale") abnormalities in AML

Since the original method of examining the genome of normal and leukemic cells was by microscopic analysis of metaphase spreads, the earliest identified and most well-studied genetic abnormalities associated with AML are those involving whole chromosomes or large parts of chromosomes [10]. These abnormalities include numerical abnormalities (i.e., chromosomal gains and losses), as well as translocations and inversions. Many of these events not only correlate with the morphology or immunophenotype of the leukemic cells but also define patient prognosis and guide antileukemic therapy [5, 10, 20]. Due to the primary importance of karyotypic abnormalities in AML, the WHO classifies seven AML subtypes based on a defining chromosomal defect [6]. Furthermore, many of the numerical chromosomal abnormalities found in AML are important diagnostic findings for an additional WHO-defined subcategory of AML (i.e., AML with myelodysplasia-related changes).

Three of the translocations that define AML subgroups are associated with relatively favorable patient outcomes [10, 15]. These are t(15;17)(q24;q21), t(8;21)(q22;q22.3), and inv(16)(p13.1q22) or t(16;16)(p13.1;q22). The resulting fusions disrupt transcription factors essential for myeloid differentiation.

The t(15;17) subtype occurs in ~10 % pediatric AML and essentially has a 1:1 correlation with acute promyelocytic leukemia (APL), also known as AML-M3 in the now obsolete FAB classification. The translocation fuses the alpha receptor for retinoic acid (*RARA*) on chromosome 17 to the inducer of promyelocytic leukemia (*PML*) locus on chromosome 15. RARA is a critical transcriptional regulator of myeloid differentiation, and the fusion creates a dominant negative form of the protein, leading to a differentiation block at the promyelocyte stage [21, 22]. Rapid recognition of this entity, cytologically characterized by an abundance of atypical promyelocytes with bilobed nuclei, and molecular confirmation of the translocation is essential for appropriate clinical management since patients with this AML subtype are at high risk for disseminated intravascular coagulation and devastating vascular events [5]. Treatment for patients with t(15;17) is unique in that pharmacologic dosing of all-trans retinoic (ATRA) is the mainstay of therapy [23]. Binding of ATRA to the PML-RARA fusion relieves the dominant negative inhibition of the fusion protein and leads its degradation by ubiquitin-mediated proteolysis. The use of ATRA in APL has completely changed the prognosis for patients with AML harboring the PML-RARA fusion. Prior to its use, AML with t(15;17) had a dismal prognosis, whereas current long-term remission rates exceed 90 % [24].

Almost all patients with APL morphology are positive for the *PML-RARA* fusion, yet there are variant RARA translocation partners that can lead to APL-like cytology. These include fusions with nucleophosmin (*NPM1*) [25], nuclear mitotic apparatus protein (*NUMA1*) [26], zinc finger and BTB-domain containing protein 16 (*ZBTB16*) [27, 28], and signal transducer and activator of transcription 5b (*STAT5B*) [29]. Although rare, these variants are important to recognize since a subset does not respond to ATRA therapy and alternative therapeutic strategies must be employed.

The other two translocations associated with a relatively favorable prognosis in both adults and children are t(8;21) and inv(16)/t(16;16) [3, 24, 30–32]. The former chromosomal defect, found in 10–15 % of pediatric AML, fuses the runt-related transcription factor 1(*RUNX1*, formerly known as the core binding factor subunit alpha 2) to its translocation partner, *RUNX1T1*, on chromosome 8. The latter inversion/translocation, found in ~5 % of adult and pediatric AML, fuses the

beta subunit of the core binding factor (*CBFB*) to the heavy chain of myosin 11 (*MYH11*) [33]. Both of these genetically defined subgroups have cytologic and immunophenotypic correlates, though they are not as strong as that between APL and t(15;17). Blasts with t(8;21) have characteristic salmon-pink cytoplasmic inclusions, thin Auer rods with tapered ends, and basophilic cytoplasmic rims and may express lymphoid-associated proteins, especially CD19 [5, 34]. These blasts are typically found in a background of myeloid maturation (i.e., FAB-M2). Additionally, there is a recognized correlation between t(8;21) and the presence of granulocytic sarcomas, and this correlation seems particularly strong in the pediatric population [35, 36]. The subgroup of AML with inv(16)/t(16;16) shows significant morphologic overlap with the FAB entity AML-M4 Eo, characterized by myelomonocytic differentiation and numerous immature cells with eosinophilic and basophilic granules [37, 38]. Although both *RUNX1-RUNX1T1* and *CBFB-MYH11* AML subclasses (collectively known as core-binding factor AML) are associated with a favorable prognosis, additional mutations can modify tumor aggressiveness. One such modifying event is an activating mutation in the v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT), which downgrades the prognosis of core-binding factor leukemias from good to intermediate [39]. This effect of KIT on prognosis, however, seems to be more profound in adult than in pediatric AML [40].

On the other end of the prognostic spectrum from APL and core-binding factor AML are AML with t(6;9)(p23;q34), fusing the *DEK* proto-oncogene to a nucleoporin gene (*NUP214*), and AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) which juxtaposes ribophorin 1 (*RPN1*) to the MDS1 and EVI1 complex locus (*MECOM*) [24, 30, 41]. Both of these entities occur infrequently in the pediatric population, yet like the more common translocations described above, these, too, correlate with distinct cytologic features. AML with *DEK-NUP214* is associated with myelodysplasia in virtually all cases and frequently shows basophilia [42]. AML with inv(3)/t(3;3) also shows myelodysplasia with atypical megakaryocytes and giant platelets [43]. Interestingly, the clinical phenotype of this latter leukemia is likely related to overexpression of *MECOM*, as the translocation brings a *GATA2* enhancer element in proximity to the *MECOM* locus [44, 45]. As expected, both inv(3)/t(3;3) AML and AML with increased *MECOM* expression in the absence of the chromosomal rearrangements have dismal prognoses [46–49]. Since t(6;9) and inv(3)/t(3;3) AML are rare, they are not specifically targeted in routine diagnostic workups for pediatric AML; however, it is important to test for these alterations in cases with characteristic cytology given the association with aggressive disease.

At least three translocations are important for pediatric patients with AML displaying megakaryoblastic differentiation (AMKL). One translocation, t(1;22)(p13;q13), fuses RNA-binding motif protein 15 (*RBM15*) to megakaryoblastic leukemia (translocation) 1 (*MKL1*) and defines a WHO-described subclass of AML [6]. This translocation is typically seen in infant AMKL, and patients with this somatic translocation exhibit a more favorable clinical course than those who lack it. Two other AMKL-associated chromosomal rearrangements are not formally incorporated into the WHO classification. These are inv(16)(p13.3q24.3) which encodes a fusion between a core-binding factor gene (*CBFA2T3*) to a GLIS family zinc finger gene (*GLIS2*) and t(11;12)(p15;q35) encoding a fusion between a nucleoporin protein and lysine-specific histone methylase (*NUP98-KDM5a*) [50, 51]. These two translocations account for ~25 and ~10 % of pediatric AMKL cases, respectively. It should be noted that the *CBFA2T3-GLIS2* translocation is not restricted to AMKL, and one study has shown that half of the AML cases with this fusion does not show megakaryocytic differentiation [52].

Perhaps the most interesting locus involved in AML-associated translocations is the mixed-lineage leukemia (MLL) gene, now also known as lysine methyltransferase 2A (*KMT2A*) located at 11q23. MLL translocations are found in both myeloid and lymphoid acute leukemia [3]. For AML, 11q23 translocations are frequently (but certainly not exclusively) found in two clinical settings—primary infant acute leukemia and AML arising in patients previously treated with topoisomerase II inhibitors [53]. MLL encodes a protein that is part of a complex that regulates histone methylation (hence its new designation), and translocations involving *MLL* lead to dysregulated histone modification and chromatin accessibility [54]. Unlike most loci involved in AML-associated translocations, in which both fusion partners are unique or show rare variants, *MLL* has been found with more than 80 different partners [33]. The prognosis of patients with an *MLL* translocation varies depending on the partnering locus. One large study evaluating outcomes in children with *MLL*-rearranged leukemia found poor prognostic associations with translocations involving chromosomes 6q27, 10p11.2, and 4q21 (overall survival 5-year survival of 22–27 %), while all 27 children with a t(1;11) translocation were alive at 5-year follow-up [55]. The most common translocation partner of *MLL* in AML cases is *MLLT3*, located on chromosome 9. This t(9;11)(p22;q23) abnormality occurs in about half of *MLL*-rearranged AML cases and defines a WHO-described subcategory of AML. Some studies have suggested that children with this translocation carry a favorable prognosis, though others have shown intermediate outcomes [30, 32, 55, 56].

A few additional translocations are not included in the current WHO classification but are recurrently found in pediatric AML and merit discussion. Fusions involving *NUP98* (such as the *NUP98-KDM5A* fusion previously discussed) are recurrently found in pediatric AML. Like *MLL*, *NUP98* is nondiscriminatory and associates with multiple different partners

[57]. One such fusion involves *NUP98* and the nuclear receptor domain SET binding protein 1 gene (*NSD1*), which results from t(5;11)(q35;p15.5) [58–60]. This translocation is cytogenetically cryptic and has been found in 16 % of karyotypically normal childhood AML. Interestingly, patients with this translocation frequently harbor concomitant internal tandem duplications in the fms-related tyrosine kinase 3 (*FLT3*) gene and have a relatively poor prognosis. Likewise, AML with a t(8;16)(p11;p13) translocation is also associated with a poor prognosis [61, 62]. This translocation is seen infrequently in cases of infant AML and has the characteristic cytologic finding of blasts with hemophagocytosis. Despite the relatively aggressive nature of this AML, a subset of patients shows spontaneous regression and long-term remission of their leukemia, a phenomenon somewhat reminiscent of the transient abnormal myeloproliferation seen in a subset of infants with Down syndrome.

Numerical abnormalities involving gains or losses of chromosomal material are also particularly important in determining prognosis of patients with AML. Complex karyotypes (currently defined as three or more chromosomal abnormalities not otherwise associated with an AML subtype) as well as a monosomy karyotype (defined as monosomy of two chromosomes or a single monosomy in the context of other structural abnormalities) both portend a poor prognosis [33, 63, 64]. In adults, loss of chromosome 7 or 7q is also associated with aggressive disease [65]. These last abnormalities occur infrequently in children and should not be seen as a single entity since children with 7q loss have a significantly better prognosis than those with loss of the entire chromosome 7 [66]. Moreover, additional chromosomal abnormalities when present with −7, especially inv(3), −5, and +21, have a significant negative impact on patient outcome.

Clinical assessment of "large-scale" changes in AML

Many of the genomic changes in AML discussed above were initially recognized by standard metaphase chromosomal analysis [10]. Despite remarkable advances in profiling human genomes, this seemingly antiquated technique remains a cornerstone of diagnostic testing for patients with AML [67]. The technique is well-established and relatively straightforward yet requires highly skilled cytogeneticists to perform the assay and interpret the data. Most translocations in AML can be identified cytogenetically, though cryptic or noncanonical translocations requiring alternative methodologies for detection are not infrequent. Perhaps the greatest utility of cytogenetic analysis is that it gives a relatively unbiased view of the entire genome. Large-scale abnormalities may be detected, regardless if they were expected prior to performing the assay. This broad evaluation of tumor cell chromosomes is especially useful in diagnosing AML with complex karyotypes for which multiple abnormalities can be visualized with a single cellular preparation.

A second method for analyzing large-scale genomic alterations is fluorescence in situ hybridization or FISH [68]. With this technique, fluorescent DNA probes are hybridized to the loci of interest within the nuclei of sample material. The resulting fluorescent signals reflect the absolute number and the relative chromosomal location of the loci in question. Standard probes used clinically for translocation analysis can show juxtaposition to two loci (fusion probes), splitting of a single locus (break-apart probes), or both (dual-fusion probes). For diagnostic samples, in which there is a preponderance of tumor cells, FISH is the current gold standard for detecting most translocations since its sensitivity approaches 100 % and can detect cryptic fusions potentially missed by standard cytogenetics or reverse transcription polymerase chain reaction (RT-PCR)-based assays [68, 69]. An additional useful characteristic of chromosomal analysis by FISH is that it does not require cells to be alive and cycling, whereas standard cytogenetics does. Thus, diagnostic samples that are unable to grow in the laboratory or that are formalin fixed can still be analyzed for recurrent genetic abnormalities by FISH [70]. Broad genomic analysis by FISH, however, is much more limited than by standard cytogenetics. A general overview of the chromosomal composition of AML cells is not possible with standard diagnostic FISH techniques since only a restricted set of FISH probes is used. FISH in isolation, therefore, may allow for detection of specific—and suspected—genetic events but can easily miss other unsuspected—and perhaps prognostically important—chromosomal abnormalities. Clearly, the sensitivity of FISH and the broad chromosomal analysis of standard cytogenetics complement one another, and both techniques are (and should be) parts of a standard workup of diagnostic samples from patients with AML.

Despite the diagnostic sensitivity of these two techniques (especially FISH) in identifying chromosomal lesions, their analytic sensitivity is quite low. In a sample being analyzed by FISH, usually only 200–500 cells are examined microscopically for aberrant signals [68]. Standard karyotypes involve examination of only 20 cells. However, detection of minimal residual disease, which is clinically meaningful with blast percentages of 1 in 10,000 cells, obviously requires analysis of many more cells than can be reasonably done using either of these two techniques [71]. Thus, both FISH and cytogenetics are invaluable tools at initial diagnosis and during frank cytologic recurrence (when blast percentages are relatively high), but they have virtually no role for monitoring patients who are in cytologic remission.

A commonly used alternative method for detecting translocations involves RT-PCR with primer pairs specific for the two loci involved in the translocation [72, 73]. With this method, the presence of the fused loci results in the production of a

fusion-specific PCR amplicon. Detection of the PCR product can be accomplished by multiple methodologies including gel or capillary electrophoresis and real-time amplification, the latter of which also allows for quantification of a relative amount of fusion DNA and may be useful for following patients in cytologic remission. A number of methodologies use multiplex PCR to screen for many common translocations in a single reaction. Products can be distinguished from one another by the size differences of the resulting amplicons or by hybridization to product specific oligonucleotides covalently coupled to fluorescent microspheres [74, 75].

PCR techniques have a number of advantages over both FISH and standard cytogenetics to identify chromosomal translocations. PCR has the fastest turnaround time of these three techniques, so is the method of choice when a rapid diagnosis is essential (e.g., AML with PML-RARA). PCR also has the highest analytic sensitivity, so these methods can be used to detect leukemic cells during and after treatment, when evaluation of minimal residual disease is a central clinical question [76].

Although PCR-based techniques have certain advantages, they are not entirely without flaws. The exquisite analytic sensitivity of PCR, one of the main assets of the technique, is ironically one of its drawbacks as well. Low levels of AML-associated fusions can be detected in DNA samples from patients without leukemia, and patients can remain in long-term remission while continuing to display PCR-positivity. Thus, a positive PCR result is neither specific for leukemia nor does it not indicate treatment failure [72, 77–80]. Along these same lines, the presence of residual blasts below 1 in 10,000 may not be clinically relevant, so the ability of PCR to detect cells as low as one in a million may be excessive. Additionally, the use of PCR with translocation-specific probes to follow patients during treatment is restricted to patients who have a detectable abnormality at diagnosis; these represent only a subset of AML patients. Given these drawbacks, multiparameter flow cytometry, which can detect immunophenotypic abnormalities in most cases of AML and can do so with an appropriate analytic sensitivity of 0.01 %, is typically used to follow pediatric patients with AML during their treatment [71].

Submicroscopic (small-scale) mutations in AML

Initial identification of genomic abnormalities in AML was based on rather blunt techniques for assessing the human genome. As techniques for the rapid assessment of nucleotide sequence have become widespread, numerous additional mutations have been found to be repeatedly associated with AML [6]. Like the large-scale genomic alterations, many of these small-scale mutations, which include point mutations and small insertions and deletions, have prognostic implications, and a few have been incorporated into formal classification schemata.

Of these small-scale mutations, two can be used for defining subclasses of AML (albeit provisional) using the most recent WHO classification guidelines. These are AML with mutations in nucleophosmin (*NPM1*) and AML with mutations in the gene encoding the CCAAT/enhancer-binding protein alpha (*CEBPA*) [6].

Recurrent mutations in *NPM1* in AML were first recognized by Falini and colleagues [81]. *NPM1*, which is largely found in the nucleolus, is thought to have a role in ribosomal biogenesis and may also have a link to tumor suppression by aiding in nucleolar sequestration of the MDM2 oncoprotein [82–84]. Mutations in NPM1, which are typically found in the C-terminus, abrogate the nucleolar localization signal, and the protein is allowed to migrate to the cytoplasm. Indeed, a rapid test for determining if an NPM1 mutation exists is a simple immunohistochemical stain against NPM1 protein and determining microscopically if the protein localizes outside of the nucleus [85].

NPM1 mutations are found in ~30 % of adult AML, yet only about 10 % of pediatric AML cases possess *NPM1* mutations [86–88]. Mutations in *NPM1* are most commonly found in AML with a normal karyotype, and a significant overlap can be found between *NPM1* mutations and activating mutations in the *FLT3* gene [89]. This relationship between *NPM1* and *FLT3* mutations is important with regard to patient prognosis. AML with an *NPM1* mutation portends a rather favorable prognosis, but only when a concomitant activating internal tandem duplication in *FLT3* is not detected (see below) [15, 87, 90, 91]. Further studies have indicated that *NPM1* mutations are associated with favorable prognosis only when they occur simultaneously with isocitrate dehydrogenase (*IDH*) mutations [15], yet these results remain to be verified in independent AML cohorts.

The second entity with provisional diagnostic status in the most recent WHO classification is AML with *CEBPA* mutations [6]. This gene encodes a transcription factor that is critical for myeloid differentiation [92], and consistent with the lack of maturation seen in AML blasts, mutation abrogates *CEBPA* function [93]. In adults, tumors with *CEBPA* mutations also have a relatively good prognosis, but this seems only true for cases with biallelic mutations [93–95]. These prognostic features have been difficult to validate in the pediatric population, as the mutation is present in only ~5 % of childhood AML, but recent studies are consistent with survival benefit of a biallelic *CEBPA* mutation [96].

Many of the growth-promoting mutations in AML are activating point mutations in receptor tyrosine kinases or downstream molecules. These include activating mutations in *KIT*, *NRAS*, and *KRAS*. Of particular interest in AML, particularly due to the relatively high mutational frequency, impact on prognosis, and recent development of small-molecule

inhibitors, are activating mutations in the *FLT3* gene. Mutations in *FLT3* are found in ~25 % of AML and are common in AML with normal karyotype [97, 98]. AML-associated *FLT3* mutations result in constitutive kinase activity, but occur in two different flavors. The majority of mutations are internal tandem duplications (termed *FLT3*-ITD) in the juxtamembrane domain, while a minority are point mutations in the kinase domain (*FLT3*-KD) [90, 97, 99]. These mutations are somewhat puzzling with regard to patient outcome and disease biology. *FLT3*-ITD is clearly associated with poor patient outcomes [97, 98, 100–102]; surprisingly, the negative prognostic implications do not hold for *FLT3*-KD mutations as a number of studies have shown that *FLT3*-KD-mutant tumors are not as aggressive as their *FLT3*-ITD counterparts [103, 104]. These results suggest that constitutive kinase activity may not be the only disruption caused by the internal duplication.

FLT3 mutations are thought to be acquired late during tumorigenesis; therefore, the mutational burden of *FLT3*-ITD can vary widely since only a subset of the original clones possesses the mutation. In fact, prognosis of *FLT3*-ITD tumors in both the adult and pediatric population has been shown to correlate with allele burden [90, 97]. Additionally, discrepancies in *FLT3*-status between diagnostic and relapse samples may exist, in that some patients with *FLT3*-ITD diagnostic samples will lack the mutation at relapse and vice versa [105]. Given that *FLT3*-ITD is typically associated with an aggressive phenotype, gain of the mutation at relapse may be expected but its loss at relapse is rather surprising.

Interestingly, studies in which cases of AML were segregated based on either transcriptional or DNA methylation profiling identified subgroups with significant enrichment of *CEBPA* mutation and *NPM1* mutations [106, 107]. Additional subgroups enriched for chromosomal abnormalities [such as t(15;17) and t(8;21)] were also identified. By contrast, *FLT3* mutations were not confined to certain subgroups but were found across the sample sets. These studies are consistent with the hypothesis that *FLT3* mutations are acquired late in tumorigenesis and that the recurrent translocations and mutations of *CEBPA* and *NPM1* are disease-initiating events, while *FLT3* mutations modify tumor aggressiveness (Tables 1 and 2).

Given the prognostic discrepancies between *FLT3*-ITD and *FLT3*-KD mutations, the cases of tumor recurrence with loss of *FLT3*-ITD, and the relatively late acquisition of *FLT3* mutations in tumorigenesis, it is tempting to hypothesize that a *FLT3* mutation may not be a driver mutation in AML, but may actually represent a marker aggressive disease. Arguing against this model, Smith et al., in a number of elegant experiments, showed that patients with *FLT3*-ITD blasts and treated with FLT3 inhibitors acquired additional mutations in *FLT3* that rendered the kinase insensitive to inhibition [117]. These results suggest that *FLT3*-ITD (and its inhibition) exerts a selective pressure on AML blasts and is, therefore, partially driving the disease rather than merely acting as a disease marker. The fact that *FLT3*-ITD is likely a driver mutation in AML, coupled with the high mutation frequency and its effect on patient prognosis, has made it a key target for development and testing of AML therapeutics in both the adult and pediatric populations [4, 118].

Since the publication of the WHO classification in 2008, many additional recurrent mutations in AML have been discovered. This wave of novel AML mutations has been driven in large part by the advent of next-generation sequencing technology, which allows relatively rapid exome or genome-wide screening of many AML samples. Of the recently discovered AML-associated mutations, much attention has focused on loci that are involved in the regulation of epigenetic events, such as DNA (cytosine-5-)-methyltransferase 3 alpha (*DNMT3A*), tet methylcytosine dioxygenase 2 (*TET2*) and *IDH1*, and *IDH2* [14–16, 18, 108, 115]. All of these mutations cause dysregulation of DNA methylation. DNMT3A is a maintenance DNA methyltransferase. Mutations in this gene, found in ~20 % of adult AML, dominantly abrogate methyltransferase function [17, 119]. TET2 is involved in DNA hydroxymethylation, an intermediate step in demethylation of DNA, and mutations in *TET2* also alter DNA methylation profiles [109]. IDH1 and IDH2 mutations are rather interesting since they create a neo-enzymatic activity that increases the cellular production of 2-hydroxyglutarate (2-HG) [120, 121]. 2-HG inhibits the function of TET2, similarly altering the methylation profiles of AML. As would be expected with mutations in the same functional pathway, the subset of AML cases with *TET2* mutations is almost entirely nonoverlapping with the subset of tumors with *IDH1* or *IDH2* mutations [109]. Prognostically, patients with *TET2* mutations seem to have more aggressive disease compared to those patients without these mutations [15, 113, 114]. The data are less clear for *DNMT3A*, *IDH1*, and *IDH2* mutations [108].

Next-generation sequencing not only has allowed for the identification and study of mutations at specific loci but also has allowed the entire mutational profile of multiple AML cases to be cataloged. In fact, TCGA studies lead by the sequencing group at Washington University have recently completed the mutational status of 200 adult AML diagnostic samples [14, 122]. These studies have revealed interesting insights into both the number and the types of AML mutations, particularly when compared to other tumor classes. AML samples, on average, have significantly fewer mutations than most other adult tumor types including lung, colon, breast, and melanoma [122]. In fact, the total number of mutations in adult AML is more similar to those of pediatric tumors such as neuroblastoma. These large-scale sequencing studies have not only revealed common mutations in AML (such as *DNMT3A*) but have also highlighted what mutations

Table 1 Recurrent translocations found in AML discussed in the text

Genetic abnormality	Frequency in adult AML	Frequency in pediatric AML	Comments	References
PML-RARA t(15;17)(q24;q21)	~10 %	~10 %	Strong correlation with FAB M3 (APL) Rare variant translocations Rapid diagnosis essential Treatment with ATRA Favorable prognosis	[10, 20, 24–30, 32]
RUNX1-RUXN1T1 t(8;21)(q22;q22.3)	5–10 %	10–15 %	Correlation with FAB M2 Correlation with granulocytic sarcomas Favorable prognosis	[10, 20, 30, 32, 35, 36]
CBFB-MYH11 inv(16)(p13.1q22) or t(16;16)(p13.1;q22)	5–10 %	Correlation with FAB M4eo	Favorable prognosis	~5 % [10, 20, 30, 32, 37, 38]
KMT2A previously *MLL* 11q23	~5 %	15–20 %	Most frequent in infants Many translocation partners Prognosis varies with translocation partner Usually poor prognosis	[10, 20, 30, 32, 55, 56]
DEK-NUP214 t(6;9)(p23;q34)	~1 %	~1 %	Frequent myelodysplasia and basophilia Poor prognosis	[41, 42]
RPN1-MECOM inv(3)(q21q26.2) or t(3;3)(q21;q26.2)	1–2 %	Rare	Frequent myelodysplasia and atypical megakaryocytes Poor prognosis	[46–49]
RBM15-MKL1 t(1;22)(p13;q13)	Rare	~15 % of AMKL cases	Mostly infants with AMKL Relatively favorable prognosis	[6, 30, 32, 50]
NUP98-KDM5A t(11;12)(p15;p13)	NA	~10 % of AMKL cases [50]	Limited studies No clear association with prognosis	[50]
CBFA2T3-GLIS2 inv(16)(p13.3q24.3)	NA	15–25 % AMKL	Limited studies Initial report showed poor prognosis	[50, 51]
NUP98-NSD1 t(5;11)(q35;p15.5)	1–2 %	4–7 %	Cytogenetically cryptic Poor prognosis	[58–60]
MYST3-CREBBP t(8;16)(p11;p13)	NA	<1 %	Mostly in infants Associated with erythrophagocytosis Few cases show spontaneous remission Poor prognosis	[61, 62]
Loss of chromosome 7	~5 %	2–5 %	Poor prognosis	[64–66]
Complex karyotype	10–15 %	5–10 %	Poor prognosis	[10, 20, 32]

are noticeably underrepresented. Strikingly, the mutational spectrum of AML—dominated by the loci discussed above—is remarkably distinct from the mutational spectrum of solid tumors, which is significantly weighted toward the well-described tumor suppressors *TP53*, *CDKN2A*, and *ATM*. These studies indicate that the selective pressures involved in forming AML are distinct from those involved in generating a solid tumor, and they should provide insight for designing therapeutics to target genes involved in tumor selection.

Table 2 Recurrent mutations found in AML discussed in the text

Mutated gene	Frequency in adult AML (%)	Frequency in pediatric AML (%)	Comments	References
NPM1	~30	~10	Mutation results in cytoplasmic localization of protein Common in AML with normal karyotype Associated with FLT3-ITD Favorable prognosis if FLT3 is wild-type	[85–91]
FLT3	~25	~15	Two types of mutations: ITD and KD FLT3-ITD associated with poor outcomes Allele burden influences prognosis Many cases with discrepant FLT3-ITD status a diagnosis and relapse	[97–102]
CEBPA	5–10	~5	Favorable prognosis if both alleles mutated	[92–96]
IDH1/2	~20	<5	Mutations create neo-enzymatic activity Inhibits hydroxymethylation of DNA Alteration of DNA methylation profile of AML Conflicting data on correlation with outcome Mostly mutually exclusive with TET2 mutations	[14, 108–112]
DNMT3A	~20	<5	Mutations create dominant negative form of DNMT3A Alteration of DNA methylation profiles	[14, 18, 110, 111, 113, 114]
TET2	5–10	<5	Influences hydroxymethylation and demethylation of DNA Similar DNA methylation profiles as IDH1/2 mutant AML Possibly associated with poor prognosis	[14, 110, 111, 115, 116]

Clinical assessment of "small-scale" changes in AML

Detection of point mutations or small insertions and deletions in specific loci relies heavily on PCR amplification. The choice of PCR primers and the method of analysis of PCR amplicons influence both the diagnostic and the analytic sensitivity of the chosen assay. PCR primers can be designed to be specific for the mutant allele (i.e., allele-specific oligonucleotides or ASOs) so that a product is formed only when the mutant exists [123]. This assay method is virtually identical to PCR analysis of specific translocations and is subject to the same advantages and disadvantages. The analytic sensitivity is quite high, especially if real-time detection methods are used for analysis, yet its diagnostic sensitivity is not 100 % and care should be used when interpreting positive results as they may not entirely reflect clinical status.

PCR with primers that flank regions of interest followed by amplicon detection by methods that can distinguish between wild-type and mutant alleles is another method of determining the mutational status of AML-associated loci [124, 125]. This method is typically used for *FLT3*-ITD evaluation, in which primers flanking the ITD are used in the PCR, and electrophoresis is used to show an increase in amplicon size when the ITD is present. These methods have a higher diagnostic sensitivity than ASO-based methods, as they are not restricted by a specific mutation. However, since they produce an amplicon regardless of mutational status, the wild-type allele can dominate the analysis in samples with low mutational burden. Thus, the analytic sensitivity of the assay is lower than that of ASO-PCR.

The above PCR methods are typically used with a single or relatively few loci. Given the growing array of mutations involved in AML, many institutions have recently begun to explore the utility of using massively parallel, next-generation sequencing (NGS) to evaluate the entire mutational profile of AML cells [126]. Since most of the mutations in AML are known, and only a subset of these are actionable or have prognostic relevance, sequencing panels are used by many institutions. Limiting the number of loci evaluated, rather than broadly evaluating the entire exome or genome, increases both the speed and analytic sensitivity of mutational assessment and limits the costs involved

Most of the information that can be gleaned from multiple other molecular techniques can be obtained from simply using a relatively restricted panel of loci in a NGS assay. Clearly, point mutations, small insertions, and deletions can be detected by this sequencing methodology. Using read counts of each locus, the allele frequency and locus copy number (which may indicate chromosomal gains and losses) can be determined. Similarly, since allele frequency is determined digitally through read counts and is not subject to the normal allele dilutional effects of analog methods, NGS with high read-

depth can be used for determination of minimal residual disease. Sequences that span translocation breakpoints can be used to determine the presence of translocations and inversions. Given these characteristics, NGS assays are likely to supplant traditional single-locus assays for mutational analysis of AML.

Mutational analysis and development of tests that probe DNA mutations has clearly dominated the field of molecular analysis of AML. Mutations, however, likely do not account for all of the dysregulation involved in tumorigenesis and probing other, non-mutational events may have significance in guiding treatment or determining the prognosis of patients with AML. As briefly mentioned above, dysregulation of both the transcriptional and epigenetic profiles is characteristic of AML and multilocus assessment of gene transcription, or DNA methylation has been shown to segregate tumors into specific subgroups with unique genetic and clinical features [106, 107, 127, 128]. Indeed, studies of DNA methylation in adult AML have shown that methylation patterns have prognostic significance independent of currently used clinical and genetic parameters. Although methods for assessing transcriptional profiles and DNA methylation status are not currently used in clinical evaluation of patients with AML, they are used for patients with other types of tumors [129, 130], and a number of groups are actively pursuing methods for non-mutational molecular tests in leukemic samples [131, 132].

The research on the molecular events involved in AML leukemogenesis has far outpaced the translation of important findings into clinical decision-making. Current recommendations of genetic testing in pediatric AML include karyotypic analysis, targeted assessment of critical translocations [i.e., t(15;17), t(8;21), inv(16)/t(16;16), and 11q23] as well as *NPM1*, *FLT3*, and *CEBPA* mutational status [133]. Further testing based on specific clinical or cytologic features (such as hemophagocytic blasts or megakaryocytic differentiation) should also be pursued on a case-by-case basis.

Pediatric AML vs. adult AML, are they identical?

Studying the effects of age on the biology and the clinical aspects of tumors is generally restricted to relatively small age ranges since most tumors occur either in the pediatric setting (e.g., neuroblastoma) or in the adult setting (e.g., non-small cell lung carcinoma) alone. The incidence of AML across all ages, however, provides a unique opportunity to study the effect of age on tumorigenesis and the mutational events that are prevalent in specific age-defined populations. Cytologically, adult and pediatric AML have similar features and, for the most part, do not vastly differ in their prevalence of cytogenetic alterations [134].

However, the distinction between adult and pediatric AML with regard to mutations in genes that influence the epigenome is quite striking. As noted earlier, the MLL gene, which is involved in histone modifications, shows a high prevalence of mutation in infant AML. Mutations in *DNMT3A* occur in approximately 20 % of adult AML but are rarely found in pediatric AML [110, 111, 134]. Similarly, *IDH1* and *IDH2* mutations are found in 10–15 % of adult AML but are much rarer in pediatric AML. Since mutations in epigenetic modifiers are likely early events in leukemogenesis, one can hypothesize that alteration of the epigenetic landscape is an initial requirement for leukemogenesis. The discrepant frequencies of these mutations between adult and pediatric AML may indicate that these are fundamentally distinct sets of tumors when viewed epigenetically. Alternatively, one could hypothesize that the epigenetic landscape of the physiologic hematopoietic stem cell is different between adults and children, and that different mutations are initially required to establish a common leukemic epigenetic profile. Establishing the underlying reasons for the striking differences in the mutational profiles of adult and pediatric AML would certainly add insight into leukemogenic pathways and could guide the modulation of treatment paradigms depending on patient age.

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