



REVIEW

Acute myeloid leukemia in the era of precision medicine: recent advances in diagnostic classification and risk stratification

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ABSTRACT

Acute myeloid leukemia (AML) is a genetically heterogeneous myeloid malignancy that occurs more commonly in adults, and has an increasing incidence, most likely due to increasing age. Precise diagnostic classification of AML requires clinical and pathologic information, the latter including morphologic, immunophenotypic, cytogenetic and molecular genetic analysis. Risk stratification in AML requires cytogenetics evaluation as the most important predictor, with genetic mutations providing additional necessary information. AML with normal cytogenetics comprises about 40%-50% of all AML, and has been intensively investigated. The currently used 2008 World Health Organization classification of hematopoietic neoplasms has been proposed to be updated in 2016, also to include an update on the classification of AML, due to the continuously increasing application of genomic techniques that have led to major advances in our knowledge of the pathogenesis of AML. The purpose of this review is to describe some of these recent major advances in the diagnostic classification and risk stratification of AML.

KEYWORDS

Acute myeloid leukemia; myeloid neoplasms; genomics; precision medicine; next-generation sequencing; cytogenetics; mutations

Introduction

The diagnosis and classification of myeloid neoplasms has been described in the revised World Health Organization (WHO) classification of hematopoietic neoplasms, published in 2008¹. The WHO classification was originally published in 2001, and at that time, had represented a paradigm shift by incorporating genetic information into the diagnostic algorithms^{2,3}. According to the WHO classification, disease entities are defined by a combination of clinical, morphologic, immunophenotypic and genetic features. Myeloid neoplasms include the following major subgroups: (1) acute myeloid leukemias (AML) and related neoplasms; (2) myelodysplastic syndromes (MDS); (3) myeloproliferative neoplasms (MPN); (4) myelodysplastic/myeloproliferative neoplasms; and (5) myeloid/lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDFRB*, or *FGFR1*¹. All myeloid neoplasms are considered to be clonal

diseases arising from hematopoietic stem cells, with AML diagnosed as an acutely presenting disease with >20% bone marrow or circulating myeloid blasts, and the latter 4 subgroups as chronic diseases. MDS are considered pre-leukemic disorders, characterized by ineffective hematopoiesis, cytopenias, often with detected clonal cytogenetic abnormalities, <20% bone marrow blasts, and a propensity to secondarily progress to AML (secondary AML).

Since 2008, additional major advances in our knowledge of the molecular genetic features of hematologic neoplasms, in particular for myeloid neoplasms, are leading to another paradigm shift in the diagnosis and clinical management of these neoplasms in the era of precision medicine, as was also evident by papers presented at the recent annual meeting of the American Society of Hematology (ASH) in December, 2015. Due to these continuing major advances, a revision of the 2008 WHO classification of hematopoietic neoplasms has been proposed, including for AML and other hematopoietic neoplasms. This classification is due to be released in 2016, for AML and all other hematopoietic neoplasms, as per conference presentations by the WHO classification authors at the Society for Hematopathology session at the March 2015 Annual Meeting of the United States and Canadian Academy of Pathology in Boston, MA⁴, and at an educational

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session at the recent ASH meeting for the proposed 2016 classification criteria for MDS⁵. The purpose of this review is to describe some of the recent major advances that have occurred due to the impact of genomics in the diagnostic classification and risk stratification of AML.

Acute myeloid leukemia

Acute myeloid leukemia is a genetically heterogeneous disease that occurs primarily in adults, with an increasing incidence in the U.S., due primarily to longer survival, including after treatment for prior non-hematologic malignancies⁶. In 2015, 20,830 new cases of AML were expected to occur in the U.S., including 12,730 in males and 8,100 in females⁷. The original classification of acute leukemias by the French-American-British group was based on morphology and cytochemical stains⁸, followed by the WHO classification in 2001², with revision in 2008 including the categories for AML, as shown in **Table 1**¹. The WHO classification recognized distinct clinicopathological entities characterized by the underlying specific cytogenetic or molecular genetic abnormalities. Since the 2001 WHO criteria, the diagnosis of an acute leukemia requires >20% bone marrow or peripheral blood blasts, with >20% myeloid blasts for a diagnosis of AML, or <20% myeloid blasts in the presence of recurrent cytogenetic abnormalities specific for AML. Diagnostic criteria for each of the categories were described in multiple monographs in the 2008 WHO 4th edition⁹, along with the rationale for the categories¹⁰.

The majority of AML occur *de novo*, and few occur secondary to another myeloid disorder (secondary AML), or may occur after cytotoxic or other leukemogenic therapy (therapy-related), which are differentiated by clinical history. In AML, clonal chromosomal aberrations detected by cytogenetics at presentation were, in the past, recognized as the most important prognostic variable for AML, with three main (low, intermediate and high) prognostic risk groups¹¹, with the intermediate risk cytogenetically normal AML (CN-AML) comprising the largest group. Since then, numerous studies have identified additional molecular abnormalities leading to modification of the previous solely cytogenetic risk groups to now be defined by combined cytogenetic and molecular abnormalities, with only a few cited here¹²⁻²⁰. The combined cytogenetic and molecular abnormalities are now incorporated into the National Comprehensive Cancer Network (NCCN) guidelines for AML²¹.

Risk stratification in AML

As per current NCCN guidelines Version 2.2014, the better

Table 1 Acute myeloid leukemia and related myeloid neoplasms

Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); <i>RUNX1-RUNX1T1</i>
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); <i>CBFB-MYH11</i>
APL with t(15;17)(q22;q12); <i>PML-RARA</i>
AML with t(9;11)(p22;q23); <i>MLL3-MLL</i>
AML with t(6;9)(p23;q34); <i>DEK-NUP214</i>
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); <i>RPN1-EVI1</i>
AML (megakaryoblastic) with t(91;22)(p13;q13); <i>RBM15-MKL1</i>
Provisional entity: AML with mutated <i>NPM1</i>
Provisional entity: AML with mutated <i>CEBPA</i>
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Pure erythroid leukemia
Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasms

risk group includes AML with recurrent translocations, including the t(8;21)(q22;q22), inv(16)(p13.1q22) or t(16;16)(p13.1;q22) and t(15;17)(q22;q12). It is important to note that not all AML with recurrent translocations/inversions are in the low risk category, as described in the WHO classification²². The t(6;9) and t(3;3) translocations, and inv(3) are in the high risk group, which also includes AML with complex karyotypes (>3 clonal chromosomal abnormalities), monosomal karyotype (at least two autosomal monosomies, or one autosomal monosomy and

one structural chromosomal abnormality), -5, 5q-, -7, 7q-, 11q23 – non t(9;11), and t(9;22). Clinically, the high risk group includes therapy-related AML and those secondary to MDS or MPNs. The intermediate risk group includes AML with normal cytogenetics (CN-AML), +8 alone, t(9;11), and other non-defined clonal aberrations. In addition, molecular abnormalities further redefine the above-described cytogenetic risk groups as follows, as per NCCN guidelines: (1) for t(8;21), inv(16) and t(16;16), the risk becomes intermediate with the presence of a *KIT* mutation, the commonest of which (in codon D816) shows no response to the tyrosine kinase inhibitor, imatinib; (2) the presence of *NPM1* mutation in the absence of *FLT3*-ITD or an isolated biallelic *CEBPA* mutation in AML with normal cytogenetics, moves the risk from intermediate to better risk category; and (3) *FLT3*-ITD mutation with normal cytogenetics moves the risk from intermediate to poor risk category. For an update on therapeutic management of AML, the reader is referred to reviews elsewhere^{23,24}.

AML with normal cytogenetics

AML with normal cytogenetics (CN-AML) comprises about 40%-50% of all AML. CN-AML has been studied intensively, in an effort to identify molecular abnormalities that can predict for a better or worse prognosis than intermediate risk, due to the absence of chromosomal aberrations in this group of AML that define the risk in better or poor risk categories. In CN-AML, the most commonly identified mutations currently known to provide additional clinical significance are in the following genes: fms-related tyrosine kinase 3 (*FLT3*) gene, the nucleophosmin gene (*NPM1*), the CCAAT/enhancer binding protein (C/EBP), alpha (*CEBPA*) gene, the runt-related transcription factor 1 (*RUNX1*) gene, the myeloid-lymphoid or mixed leukemia (MLL) *KMT2A* gene, the neuroblastoma RAS viral oncogene homolog (*NRAS*) gene, the Wilms tumor 1 (*WT1*) gene, and the genes encoding for NADPH-dependent isocitrate dehydrogenase 1 (*IDH1*), isocitrate dehydrogenase 2, mitochondrial (*IDH2*), DNA methyltransferase 3 alpha (*DNMT3A*), and additional sex combs like transcriptional regulator 1 (*ASXL1*). The most common recurrent molecular abnormalities in adult CN-AML are mutations in *NPM1* (45%-60%), *FLT3*-ITD (28%-34%), *DNMT3A* (30%-37%), *IDH1* and *IDH2* (25%-30%), *ASXL1* (5%-12%), *TET2* (9%-23%), and *RUNX1* (8%-16%)²⁵.

Mutations in pathogenesis of AML

In AML leukemogenesis, mutations of several different

classes co-operate, with Class I mutations, such as *FLT3* and *NRAS* activating mutations and *BCR-ABL* fusion, conferring a proliferative advantage, and co-operating with Class II mutations, such as *CEBPA*, *NPM1* and *RUNX1* that primarily impair hematopoietic differentiation and subsequent apoptosis²⁶. Currently, the molecular markers *NPM1*, *CEBPA* and *FLT3* are widely used clinically, dividing the large group of CN-AML into two subsets: molecularly favorable (*NPM1* mutated without *FLT3*-ITD, or biallelic *CEBPA* mutation) and unfavorable (*FLT3*-ITD with or without mutant *NPM1*, or wild-type *NPM1* without *FLT3*-ITD), according to the European LeukemiaNet recommendations¹⁷. Epigenetic regulators are a third category of genes (Class III), mutations in which have emerged as clinically significant co-operators for the pathogenesis of AML, and these genes include *DNMT3A*, *IDH1*, *IDH2*, and *ASXL1*²⁷. *TET2* (tet methylcytosine dioxygenase 2), also an epigenetic regulator, is mutated in a subset of CN-AML, but currently, sufficient evidence does not appear to exist for *TET2* to be considered a clinically relevant prognostic marker in AML²⁵. The fourth category (Class IV), comprised of tumor suppressor genes, includes the *TP53* and *WT1* genes²⁷, with *TP53* aberrant AML having a dismal prognosis²⁸. Genes in the fifth category (Class V) are needed for RNA maturation²⁷, and include the RNA splicing genes, *SF3B1*, *SRSF2*, *U2AF1*, and *ZRSR2*. Mutations in these Class V genes currently appear to be more clinically significant in chronic diseases such as MDS and MPNs than in *de novo* AML.

Due to several next-generation sequencing (NGS) platforms now available to detect hundreds of gene mutations in the same analysis, and with the increasing application of these genomic techniques in the clinical setting, our knowledge about the clinical significance of the detected mutations is continuously increasing. The Cancer Genome Atlas (TCGA) study of 200 *de novo* AML showed at least one driver mutation in 199 (>99%) cases, with thirteen coding mutations (single nucleotide variants and indels) on average per patient, and recurrent mutations in 23 genes²⁹. Human AML is a multi-step genetic process, with a complex clonal structure that evolves much before clinical presentation with AML³⁰. Different subtypes of AML appear to have different latency periods³⁰. For example, therapy-related AML, which are classified as therapy-related myeloid neoplasms³¹, may have a shorter (25-26 months)^{32,33}, or longer (60 months)³⁴ latency period after the initiating event. Also, mutation rates have been shown to be different within different subclones within the same AML, indicating that numbers of mutations alone may not correspond to the duration of the latency period³⁵. Clonal cell populations

appear to evolve during the latency period, as suggested by a genomic sequencing study of AML cases at diagnosis and relapse, that showed several clones present at diagnosis of AML³⁶. Clinically, increased clonal heterogeneity at diagnosis contributes to resistance to chemotherapy in any cancer, including in AML³⁷, with relapse often occurring due to clonal expansion of a previously present resistant subclone. In the whole genome sequencing study mentioned above, AML relapse originated in 3 of 8 patients from a single major clone present at AML diagnosis³⁶.

Clonal abnormalities may occur in normal individuals³⁸, and recent studies have shown clonal hematopoiesis in normal individuals without evidence of a blood disorder³⁹⁻⁴². Interestingly, abnormalities identified as clonal in normal individuals are age-related, with clonal hematopoiesis identified in 0.8% of individuals under 60, rising to 19.5% in those >90 years of age, in a study of 4,219 individuals with investigation of 15 mutation hot spots in blood DNA using ultra-deep sequencing⁴². In this study, *DNMT3A*-R882 mutations, which occur often in CN-AML, were most commonly identified, even in age <25 years, but with normally increased prevalence with age. In contrast, mutations of the spliceosome genes (Class V) *SF3B1* and *SRSF2*, which occur commonly in MDS, were identified only in individuals aged >70 years in this same study⁴². Further, the 61 of 112 clones with *DNMT3A* mutations identified in this study had a low variant allele frequency of <3%⁴². Notably, *NPM1* mutations were almost completely absent in this cohort of >4,000 individuals, despite a highly sensitive assay designed specifically for their detection for this study⁴². Since *NPM1* mutations co-occur frequently with *DNMT3A* mutations in AML, the investigators suggested that *NPM1* mutations appear to be closely related to the evolution of AML, and that *DNMT3A* mutations may allow the *NPM1* mutant clones to be founded and expand towards AML⁴². In addition to explaining the high incidence of these clonal myeloid neoplasms with age, and clarifying the role of mutations in AML, these studies also have implications for the interpretation of mutational analysis findings in AML in clinical management.

Currently, many institutions and groups are analyzing AML samples upfront with multi-gene mutational profiling using NGS at diagnosis of AML, for prognosis, risk stratification and to aid therapeutic decisions. Molecular monitoring of post-therapy disease started initially with quantitative PCR-based assays. Now, multi-gene mutational profiling using genomic techniques is also being increasingly evaluated post-therapy for AML, at remission, for monitoring and at relapse, with clinically important

information provided by these studies to aid in clinical management. Persistence of a mutation such as *DNMT3A* may occur even in clinical remission⁴³ as would be expected since *DNMT3A* mutations may be present in normal individuals⁴². Earlier, an analysis of 18 genes in a study of 398 AML patients less than 60 years of age had suggested that mutational profiling could potentially be used for risk stratification, with internal tandem duplication in *FLT3* (*FLT3*-ITD), partial tandem duplication in *MLL* (*MLL*-PTD) and mutations in *ASXL1* associated with reduced overall survival, while *CEBPA* and *IDH2* mutations were associated with improved survival⁴⁴. Only the *IDH2* R140Q mutations had a favorable effect in these patients⁴⁴. Recently, in a study of 28 recurrently mutated genes in 271 patients with *de novo* AML, complex molecular genetic abnormalities involving three or more genetic mutations were found to be a strong independent prognostic factor, even among patients <65 years of age who were also negative for *FLT3*-ITD⁴⁵.

Secondary AML

In a study of 93 clinically defined secondary AML (AML occurring after MDS or chronic myelomonocytic leukemia), with sequencing of 82 genes that are known to be recurrently mutated in myeloid malignancies, 353 single nucleotide variants and small insertions or deletions affecting 40 genes were found, with at least one mutation detected in 90/93 (97%) cases⁴⁶. Mutations in 8 genes, including 4 spliceosome genes, *SRSF2*, *SF3B1*, *U2AF1*, and *ZRSR2*, 3 chromatin modifier genes, *ASXL1*, *EZH2*, and *BCOR*, and one cohesion complex gene, *STAG2*, showed >95% specificity for secondary AML as compared to *de novo* AML⁴⁶. Since these 8 genes are also commonly mutated in MDS⁴⁷⁻⁵⁴, it was suggested that these mutations may primarily drive the myelodysplastic disease and not overt AML. Correlation with clinicopathologic features of the antecedent MDS (extent of bone marrow dysplasia or lineages involved by cytopenias) was not given in this study⁴⁶. The investigators also identified 3 additional aberrations, *NPM1* mutations, *MLL*/11q23 rearrangements, and core binding factor (CBF) rearrangements, which occurred significantly less in secondary AML as compared with *de novo* AML, and therefore, called them *de novo* AML type mutations⁴⁶. In addition, 14 (15%) of their secondary AML cases had *TP53* mutations, and these cases had more complex karyotypes and reduced overall survival as compared with their secondary AML cases without *TP53* mutations⁴⁶. Further, in their 17 paired studied cases of antecedent MDS followed by AML, they noted that newly acquired mutations leading to the

AML most commonly involved genes encoding myeloid transcription factors or genes in the RAS/tyrosine kinase signaling pathway, in contrast with the mutations in the 8 genes specific for secondary AML, or mutations in *TP53*, which were present in the MDS but not at the time of development of the secondary AML. They grouped mutations into (1) those occurring as early events, such as *DNMT3A*, *TET2* and *TP53*, or the secondary type mutations in the 8 genes given above, and (2) the driver mutations (RAS or tyrosine kinase pathway, transcription factors) that led to the emergence of AML, and found that at morphologic remission, the latter (driver) type mutations were preferentially lost, while the former group of mutations persisted⁴⁶.

In another recent study of 76 paired samples from 38 patients with MDS and subsequent progression to secondary AML that were examined by chromosome banding analysis and a 33 gene molecular panel⁵⁵, *FLT3*-ITD was significantly associated with MDS than with secondary AML (16% vs. 0%, $P=0.025$), and while not statistically different, *FLT3*-TKD, *NRAS* and *KRAS* were more often mutant in secondary AML than in MDS⁵⁵. Interestingly, 26 cases acquired mutations and of these, 18 remained cytogenetically normal, including all 12 patients with acquired *KRAS* or *NRAS* mutations. In comparing these MDS patients who transformed to AML, with their control cohort of MDS patients from a previous study who did not progress to AML⁵⁴, the investigators found that mutations in *ASXL1*, *ETV6*, *GATA2*, *IDH2*, *NRAS*, *RUNX1*, and *SRSF2* genes were more frequent in the MDS cases that progressed to AML, than in the control MDS cohort, suggesting that mutations in these genes predispose to transformation to secondary AML⁵⁵.

Minimal residual disease detection by molecular approaches in AML

Detection of subclinical levels of leukemic cells by molecular based approaches provides powerful independent prognostic information⁵⁶. Due to the extreme molecular genetic heterogeneity in AML, and due to difficulties in distinguishing normal benign myeloid blast cells from malignant (leukemic) blasts, detection of minimal residual disease (MRD) has been typically restricted to acute lymphoblastic leukemias. Nonetheless, MRD detection is now also being increasingly recognized as an important variable to be assessed in the clinical management of those categories of AML where a molecular abnormality present at diagnosis can be tracked after induction or consolidation therapy, as an early predictor of relapse. Further, risk

stratification in AML, which typically has been used at the time of diagnosis for initial treatment of AML, now also includes MRD detection for therapeutic decisions after initial therapy, including for stem cell transplantation.

Quantitative PCR assays are used for AML patients with balanced translocations such as *PML-RARA*, or for mutations in *NPM1*⁵⁶⁻⁵⁸. However, the degree of complexity of genetic aberrations in AML, as shown both in The Cancer Genome Atlas study of *de novo* AML²⁹, and in the recent study of targeted sequencing analysis of 194 patients with secondary or therapy-related AML⁴⁶, presents a major challenge in establishing molecular assays for multiple single genetic abnormalities in AML. Therefore, NGS is being increasingly utilized for detecting minimal levels of leukemic cells⁵⁹ and can detect resistant disease when PCR assays are not informative⁶⁰. Recently, Klco et al.⁶¹ used whole genome or exome sequencing on paired diagnostic and morphologic remission samples from 50 AML patients to show that the detection of persistent leukemia-associated mutations in at least 5% bone marrow cells at 30-day morphologic remission samples was associated with a significant risk of relapse, and reduced overall survival (19.3 months for persistent mutations versus 46.8 months for cleared mutations, $P=0.02$). Further, in their study, mutations in *DNMT3A*, *TET2*, *IDH1* and *IDH2* were rarely eradicated by conventional chemotherapy⁶¹, as would be expected for *DNMT3A* and *TET2* since mutations in these two genes have been shown to occur as early events in clonal hematopoiesis. In a different study, *IDH1/IDH2* mutations were found to be suitable targets for the detection of minimal residual disease in AML and for predicting relapse, while mutant *DNMT3A* persisted in remission reflecting clonal hematopoiesis⁶².

Further, in a study by the UK National Cancer Research Institute Working Group, of 2,569 samples obtained from 346 intensively treated patients with *NPM1*-mutated AML, and with targeted sequencing of 51 genes performed in 223 diagnostic samples and 49 relapse samples, MRD as assessed by quantitation of *NPM1* transcripts by reverse transcriptase PCR provided powerful prognostic information independent of other risk factors⁶³. In this study, persistence of *NPM1*-mutated transcripts in blood after the second cycle of chemotherapy was associated with a greater (82%) risk of relapse after 3 years of follow-up, as compared to the risk (30%) in patients without MRD. Also, the presence of MRD was the only independent prognostic factor for death in multivariate analysis, and relapse was reliably predicted by rising levels of *NPM1*-mutated transcripts in these patients⁶³.

Therapeutic targeting of mutations in AML

The detection of subclinical leukemic cells is significant due to developed therapeutic agents targeting specific genetic mutations in AML. Several such pharmaceutical agents are in clinical trials, with promising early results, for incorporating these novel agents in combination or with chemotherapy, with therapies that may make it to single agent therapy in clinical practice in the next five years described in a recent review⁶⁴. The potential for therapeutic targeting of the most common mutations in CN-AML, with aberrations of the *FLT3*, *NPM1*, *DNMT3A*, *IDH1*, *IDH2* and *TET2* genes, was reviewed in another recent article⁶⁵.

Therapeutic targeting of *FLT3* mutations is desirable since *FLT3* mutations confer a poor prognosis in AML, and several agents have been developed targeting both the *FLT3*-ITD and the point mutation in codon 835 of the second tyrosine kinase domain of the *FLT3* gene⁶⁶. A landmark global, prospective study presented at the annual ASH meeting in December 2015 showed the favorable results of a clinical trial using midostaurin, an agent that targets *FLT*-ITD and *FLT3*-TKD in AML patients⁶⁷, finally indicating that *FLT3* inhibitors (and *FLT3* testing) will soon become mandatory for standard of care in AML with *FLT3* mutations. In this randomized, placebo-controlled, double-blind trial from 17 countries, 3,279 previously untreated AML patients, age 18-60 (excluding acute promyelocytic leukemia) in 225 sites/17 countries were screened for *FLT3* mutations (*FLT3*-ITD low or high mutant allelic fraction, and *FLT3*-TKD) at one of 7 academic laboratories subject to extensive assay cross-validation⁶⁷. Of critical importance, this effort demonstrated that a prospective trial in a pre-therapy genetically defined subgroup of AML patients was feasible and that the addition of the multi-kinase inhibitor midostaurin to standard chemotherapy and for one year of maintenance therapy significantly improved event-free survival and overall survival in patients whose leukemic blasts had a TKD or ITD (low or high *FLT3* mutation burden)⁶⁷.

Molecular and clinically distinct classes of AML defined by modeling of genomic structure, towards a personalized approach for every patient with AML

Significantly, a complete analysis of the genomic structure of 1,540 AML patients enrolled in clinical trials of the German-Austrian AML Study Group was presented at the ASH 2015 meeting⁶⁸. In this study, in addition to cytogenetic analysis, 5,234 pathogenic lesions across 77 genomic loci were

mapped, and ≥ 1 driver mutation in 96% patients and ≥ 2 in 85% were identified. The investigators subdivided AML into at least 11 molecular and clinically distinct classes defined by t(15;17), t(8;21), inv(16)/t(16;16), t(6;9), inv(3)/t(3;3), AML defined by *MLL*-rearrangements, *CEBPA* biallelic mutations, *NPM1* mutations, *TP53*/complex karyotype, AML with chromatin/splicing factor mutations, and provisionally AML with < 3 aneuploidies. Approximately 87% of patients in their study with acquired mutations were molecularly classified, and each class was defined by a distinct subset of genetic lesions, with evidence of preferred order in mutation acquisition⁶⁸. Of clinical significance, based on comprehensive genomic profiling, the study investigators were able to show that the genetic heterogeneity in AML is not random, and instead, informs towards a personalized approach for every patient for risk stratification, MRD monitoring and combination therapy protocols⁶⁸.

Genes commonly mutated in AML with intermediate risk, normal karyotype (CN-AML)

NPM1

The official name for *NPM1* is "nucleophosmin (nucleolar phosphoprotein B23, numatrin)", with the gene located on chromosome 5q35.1, from base pair 171,387,648 to base pair 171,410,884. The gene encodes for a nucleolar protein named "nucleophosmin" that shuttles between the nucleus and the cytoplasm, and is thought to play a part in many cellular functions including processes involved in protein formation, DNA replication and the cell cycle⁶⁹. Much before its role in AML was discovered in 2005^{12,70-72}, *NPM1* was well-known to have a pathogenetic role in human non-Hodgkin's lymphomas⁷³, as the most commonly involved partner gene in the *NPM1*-*ALK* fusion in cases of *ALK*-positive anaplastic large cell lymphomas, which are described separately in the WHO 2008 classification⁷⁴. The translocations and mutations involving the *NPM1* gene in lymphomas and leukemias were reviewed in detail elsewhere⁷⁵. In AML, *NPM1* mutations are prognostically favorable in the absence of *FLT3*-ITD mutations^{12, 70-72}. It has been suggested that the altered nucleophosmin protein is unable to allow the normal tumor suppressor role of the Arf nucleolar protein, and the subsequent reduction in the tumor suppressor function leads to the leukemic proliferation in AML⁷⁶.

FLT3

The official name for the gene is "fms-related tyrosine kinase 3", with the gene located on chromosome 13q12. The gene

encodes for a Class III receptor tyrosine kinase with important roles in hematopoietic stem/progenitor cell survival and proliferation⁷⁷. Mutations that result in constitutive activation of this receptor lead to acute leukemia. Mutations of the *FLT3* gene are present in about one-third of AML patients⁷⁷. There are two major types of *FLT3* mutations that lead to constitutive activation of the *FLT3* receptor: internal tandem duplication (ITD) mutations of 3-400 base pairs that map to the juxtamembrane region, which are present in ~23% of AML patients, and point mutations that most frequently involve the kinase domain that occur less frequently than the ITD mutations⁷⁷. In pediatric patients with AML, the overall incidence of ITD mutations is lower than in adults with AML. Notably, the prognostic impact of *FLT3* mutations in AML depends on the co-occurrence of other gene mutations. A large study of 1,425 young adult patients with AML showed *FLT3*-ITD mutations to have a dismal prognosis in the absence of *NPM1* mutations, a better prognosis if *NPM1* mutations were present without *FLT3* mutations, and a prognosis intermediate between the two situations if both *FLT3* and *NPM1* mutations were present¹⁴. In contrast, *FLT3* tyrosine kinase mutations (TKD) showed a significantly more favorable prognosis than *FLT3*-ITD mutations in AML⁷⁸. In another large study of 3,082 consecutive AML patients from the German cooperative group trials, *FLT3*-TKD mutations were shown to have an unfavorable effect if present with an MLL mutation or a t(15;17) translocation, but a favorable prognosis when paired with an *NPM1* mutation⁷⁹.

DNMT3A

The official name for *DNMT3A* is "DNA (cytosine-5)-methyltransferase 3 alpha", with the gene located on chromosome 2p23, from base pair 25,232,961 to base pair 25,342,590⁸⁰. In humans, DNA methylation occurs in cytosines that precede guanines, called dinucleotide CpGs, which occur in CpG-rich regulatory regions. DNA methylation occurs at the 5' position of the cytosine ring and leads to silencing of genes and noncoding genomic regions, thereby providing a means for epigenetic regulation⁸¹. The *DNMT3A* gene encodes for a DNA methyltransferase enzyme, DNMT3A, which is required for genome-wide *de novo* methylation and is essential for the establishment of DNA methylation patterns during development, genomic imprinting and X-chromosome inactivation. DNMT3A is critical for the epigenetic silencing of hematopoietic stem cell regulatory genes, and is thus essential for hematopoietic stem cell differentiation⁸². Somatic mutations of *DNMT3A* were described in AML^{83,84}, and subsequently also in MDS, MPNs

and T-acute lymphoblastic leukemias⁸⁵⁻⁸⁷.

In a study of 511 hematologic malignancies (194 *de novo* AML, 115 MDS, 103 chronic myelomonocytic leukemia and 99 T-acute lymphoblastic leukemias), *DNMT3A* mutations showed the highest frequency in AML (36% of AML), also with strong association with *NPM1*, *FLT3*-ITD, and *IDH1* mutations⁸⁷. The majority of *DNMT3A* mutations are missense, affecting the arginine codon 882, and are predominantly heterozygous^{84,87}. In a different study of *de novo* adult AML, the clinical significance of *DNMT3A* mutations was found to be age-dependent, with R882 mutations associated with an adverse prognosis in patients aged >60 years, and non-R882 *DNMT3A* mutations associated with an adverse prognosis in adult patients <60 years⁸⁸. Interestingly, *DNMT3A* mutations were rare events in a study of pediatric AML⁸⁹, and this observation, in conjunction with the identification of *DNMT3A* mutations in normal individuals with increasing age, suggests that *DNMT3A* mutations may occur in AML primarily due to increasing age, and other mutations that co-occur subsequently lead to the development of AML.

IDH1 and IDH2

The official name for *IDH1* is "isocitrate dehydrogenase 1 (NADP+)", with the gene located on chromosome 2q33.3, from base pair 208,236,227 to base pair 208,255,143⁹⁰. The official name for *IDH2* is "isocitrate dehydrogenase 2 (NADP+), mitochondrial", with the gene located on chromosome 15q26.1, from base pair 90,083,978 to base pair 90,102,554⁹¹. The *IDH1* gene encodes for the isocitrate dehydrogenase 1 enzyme, which is present in the cell cytoplasm and peroxisomes, while the *IDH2* gene encodes for the isocitrate dehydrogenase 2 enzyme in mitochondria. Both enzymes convert isocitrate to 2-ketoglutarate to produce NADPH needed for cell metabolism. Somatic gain of function mutations in *IDH1* and *IDH2* that occur in CN-AML alter the enzymes, which now instead produce a different compound, 2-hydroxyglutarate (an oncometabolite) that interferes with the process of cell fate determination such that immature blood cells with mutant *IDH1* divide uncontrollably, instead of maturing to normal cells, and lead to CN-AML. Mutations in *IDH* genes, therefore, lead to markedly altered cellular respiration, and lead to the accumulation of the abnormally produced metabolite, which can serve as a biomarker in cancers with mutant *IDH* genes. Among human cancers, mutations of *IDH1* and *IDH2* genes occur predominantly in gliomas and AML, with such mutations found only rarely in other cancer types despite exhaustive sequencing efforts⁹². Similar to gliomas, human

AML samples with *IDH* mutations also show specific DNA methylation profiles⁹². In contrast with gliomas, however, where *IDH1* and *IDH2* mutations have a favorable prognosis, *IDH* mutations in AML have been either shown to confer a worse prognosis⁹³⁻⁹⁵, or be favorable for overall survival in intermediate-risk AML⁴⁴.

CEBPA

The official name for *CEBPA* is "CCAAT/enhancer binding protein (C/EBP), alpha", with the gene location on chromosome 19q13.1, from base pair 33,299,934 to base pair 33,302,564⁹⁶. The gene belongs to a family of genes called basic leucine zipper proteins, and encodes for a transcription factor protein, which is involved in the maturation of hematopoietic cells and is also believed to act as a tumor suppressor. In individuals with an inherited germline mutation in *CEBPA*, the occurrence of an additional somatic mutation in *CEBPA* leads to an AML with biallelic *CEBPA* mutation, which is the entity proposed to be included as a distinct entity in the upcoming WHO 2016 classification⁴.

Proposed 2016 WHO classification for acute myeloid leukemia

In the session presented by the WHO leading authors for the proposed 2016 classification for hematopoietic neoplasms, at the March, 2015 United States and Canadian Academy of Pathology (USCAP) conference⁴, Dr. Daniel A. Arber from Stanford University had presented the proposed revision for classification of acute leukemias. Dr. Arber's presentation at the website⁴ is the source for the information provided in this review about the currently proposed revision that could undergo changes before being published in 2016. Both entities of AML with mutated genes (AML with mutated *NPM1*, and AML with mutated *CEBPA*) that were included as provisional in the 2008 WHO classification were proposed to now be included as distinct clinicopathologic entities. In addition, AML with mutated *RUNX1*, and AML with *BCR-ABL* were proposed as distinct entities. Other gene mutations present in AML are considered important for prognosis and are recommended for analysis, but AML with these other gene mutations do not represent distinct entities.

A diagnostic algorithm for acute leukemias and classification of AML was initially presented by Dr. Daniel Arber after the WHO 2008 classification, incorporating clinical history, morphology, immunophenotyping, cytogenetics and mutations of *NPM1* and *CEBPA*. At the 2015 USCAP meeting, Dr. Arber presented a revised algorithm for the proposed 2016 classification of AML, now

incorporating mutations in several genes⁴. A major change, as compared to the 2008 WHO classification, is that in addition to cytogenetic analysis, which is still necessary upfront, molecular analysis for mutations is now also required upfront in the pathologic diagnostic work-up of an acute leukemia. Similar to the 2008 WHO classification, clinical history and cytogenetics are required for precise classification: if a newly diagnosed AML patient has Down syndrome, or a previous history of cytotoxic or leukemogenic therapy, the classification would be "myeloid proliferation of Down syndrome" or "therapy-related AML" respectively, and, if with cytogenetics, a recurrent genetic abnormality is found, then the classification would be an "AML with recurrent genetic abnormality." However, if *NPM1* is mutant, or *CEBPA* is biallelic mutant, or *RUNX1* is mutant, then regardless of any morphologic dysplasia that may be present, the diagnosis would be "AML with mutated *NPM1*" or "AML with mutated *CEBPA*," or "AML with mutated *RUNX1*," respectively, under the category of AML with recurrent genetic abnormalities, in the absence of a history of MDS or MPN, and in the absence of cytogenetic abnormalities specific for MDS, as described⁹⁷, since these cytogenetic abnormalities are sufficient to diagnose AML with MDS-related changes when >20% peripheral blood or bone marrow blasts are present. For any of the three AML entities with specific mutated genes (*NPM1*, *CEBPA* or *RUNX1*), if there is a history of previous MDS or MPN, the classification would be an "AML with MDS-related changes", and if there is a history of Down syndrome or previous leukemogenic therapy, the classification would be related to the clinical condition (Down syndrome or therapy-related), and not an AML with recurrent genetic abnormality. Presence of an MDS-related cytogenetic abnormality, as described in the WHO 2008 classification⁹⁷, excluding del(9q) if *NPM1* is mutant, would also classify as an AML with MDS-related changes, instead of AML with mutated *NPM1*, or AML with mutated *CEBPA*, or AML with mutated *RUNX1*⁴.

AML with mutated *NPM1*

This entity was described as one of the two provisional entities of AML with mutated genes, in the 2008 WHO classification²². In AML, the mutations in *NPM1* lead to an altered nucleophosmin protein due to heterozygous mutations that occur almost entirely in exon 12 of the *NPM1* gene, as first described in 2005⁷⁰. At that time, and with subsequent studies, the prognostic impact of *NPM1* mutations in AML was noted to depend on co-existing *FLT3-ITD* mutations. While the presence of *FLT3-ITD* mutations

led to a worse prognosis in CN-AML regardless of whether *NPM1* mutations were present or absent, mutant *NPM1* conferred a favorable prognosis in young adult patients with CN-AML if *FLT3*-ITD mutations were absent^{12,14,71,72}, and a better prognosis even when *FLT3*-ITD mutations were present, as compared with only *FLT3*-ITD mutations without mutant *NPM1*¹⁴. Although the majority of AML with mutated *NPM1* have a normal karyotype, 14.7% (93 of 631) patients were found to have an abnormal karyotype, with the most frequent abnormalities being +8, +4, -Y, del(9q) and +21⁹⁸. The presence or absence of an abnormal karyotype did not alter the favorable prognosis of the *NPM1* mutant and absent *FLT3*-ITD category of AML⁹⁸. Accordingly, in the European LeukemiaNet recommendations published in 2010, *NPM1* mutant AML with no *FLT3*-ITD mutations and normal cytogenetics lowered the risk from intermediate to favorable, while *FLT3*-ITD with wild type *NPM1* or with mutant *NPM1* and with normal cytogenetics, had an intermediate risk¹⁷. The favorable prognostic significance of *NPM1* mutant and absent *FLT3*-ITD is present in AML patients with age 55-65 years, but not in older AML patients > 65 years of age⁹⁹.

Quantitative detection of mutant *NPM1* genes or mRNA transcripts is useful in clinical monitoring of AML^{63,100,101}. The molecular subtypes of *NPM1* mutations also have varying clinical outcomes in intermediate risk AML, as recently shown¹⁰². In that study from the Munich Leukemia Laboratory, 877 (~31%) of 2,859 adult newly diagnosed AML patients showed *NPM1* mutations, and 806 of these 877 samples were *de novo* AML with intermediate risk karyotypes¹⁰². Type A *NPM1* mutations [insertion of TCTG (thymine, cytosine, thymine and guanine)] were most frequently found in 458 (69%) cases, Type B mutations (insertion of CATG) in 72 (11%) and Type D (insertion of CCTG) in 51 (8%). All other *NPM1* mutation types were rarely found in their study. *DNMT3A* was found to be most frequently co-mutated ($n=337$, 55%) with *NPM1*, followed by *FLT3*-ITD ($n=270$, 41%), and while *FLT3*-ITD showed a worse prognosis only with Type A *NPM1* co-mutations, *DNMT3A* mutations conferred a worse prognosis with both Types A and D *NPM1* mutations, suggesting that in addition to co-existing *FLT3*-ITD mutations, *DNMT3A* mutations should also be examined for predicting prognosis in AML with mutated *NPM1*¹⁰².

AML with mutated *CEBPA*

This entity was included as provisional in the 2008 WHO classification²². AML with biallelic mutant *CEBPA* occurs in

individuals who have an initial germline monoallelic mutation in *CEBPA*, followed by a subsequent acquired, somatic mutation in the second *CEBPA* allele. This AML entity may occur sporadically or in familial form, with the first family harboring a germline *CEBPA* mutation and showing autosomal dominant of AML reported in 2004¹⁰³. The germline *CEBPA* mutation typically occurs in the N-terminal region of the gene, while the somatic, acquired mutation occurs in the C-terminal region. The molecular events underlying disease progression and evolution in familial AML with biallelic mutated *CEBPA* were described in a recent multi-institutional study of ten families having 24 members with AML¹⁰⁴. Germline *CEBPA* mutations appeared to be highly penetrant, with the majority presenting with AML at an early median (range) age of 24.5 (1.75-46) years. All AML cases were *de novo* in the absence of preceding cytopenias. Germline mutations, universally located in the N-terminal region of the gene, caused a frameshift preceding the internal p30 start codon, while the somatic mutations were located in the C-terminus in all 18 examined patients, with the majority representing inframe insertions or deletions within the DNA binding and leucine zipper domains¹⁰⁴. Interestingly, in contrast with sporadic AML, in these familial AML cases, somatic (acquired) mutations identified at relapse were not the same as the somatic mutations present at diagnosis, and were also confirmed to be absent as a subclone in the initial diagnostic AML, indicating that in familial cases, disease recurrence may be initiated by a completely new clonal leukemic occurrence¹⁰⁴. Their study highlighted the critical importance of evaluation of family history, for continued long-term clinical follow-up, and for germline DNA assessment in patients <50 years presenting with AML with biallelic mutated *CEBPA*¹⁰⁴.

Mutations in *CEBPA* may be monoallelic, or biallelic, or there may be polymorphisms detected in the gene, in cases of AML¹⁰⁵. In large cohorts of patients with CN-AML, mutations of *CEBPA* were found in 12.8% and 10.6% cases, with biallelic and monoallelic mutations comprising about 60% and 40%, respectively^{105,106}. It is important to remember that whether sporadic or familial, the favorable prognosis of this AML entity is present only with biallelic mutations of *CEBPA*, and is absent with monoallelic *CEBPA* gene mutations in AML¹⁰⁵⁻¹¹⁰. Also, the favorable significance of biallelic mutant *CEBPA* is lost in the presence of a *FLT3*-ITD mutation, while *NPM1* mutations are unlikely to co-occur in AML with biallelic mutated *CEBPA*¹¹⁰. Mutations in *GATA2* may be present in AML patients with *CEBPA* mutations, but they do not affect the favorable prognosis of the biallelic mutant AML¹¹¹.

Familial predisposition to AML

Hematologic malignancies are usually considered to be sporadic, with very few recognized familial cases. With increasing evaluation of molecular genetics in myeloid neoplasms, there is now increasing appreciation of the need for clinical testing for assessing familial predisposition to MDS/AML. Accordingly, the proposed WHO 2016 classification will include a section on familial myeloid neoplasms⁴. Currently, clinical testing is available for several familial MDS/AML disorders, including the autosomal dominant familial platelet disorder with germ line *RUNX1* mutations, with propensity to develop AML¹¹², and for familial AML with biallelic mutated *CEBPA*. Disorders with inherited predisposition to AML have been reviewed elsewhere^{113,114}. Guidelines have been proposed for clinical management of patients and families with predisposition to familial AML, with recommendations including that all carriers of such predisposing genetic mutations undergo a baseline bone marrow biopsy and twice annual physical examination and complete blood counts with differential counts¹¹³. While 100% of the mutated *CEBPA* carriers develop AML, albeit with long latency periods, the development of AML in the familial disorder with *RUNX1* mutation is variable (20%-60%). Interestingly, in a recent genomic study of familial MDS/AML cases, mutations in genes that are recurrently mutated in *de novo* AML were underrepresented in familial MDS/AML cases¹¹⁵. Also, clonal hematopoiesis was detected in young, asymptomatic carriers of *RUNX1* mutations, providing a possible biomarker in these families with high risk for development of AML¹¹⁵.

Future directions

The WHO classification from 2008 for hematopoietic neoplasms, which is currently proposed to be revised in 2016, will include a revision of AML categories due to the significant advances in the molecular genetic landscape of AML. Since the classification recognizes only clinicopathological entities that have accumulated sufficient evidence to be classified as distinct, the continuing major advances in the molecular genetic aberrations underlying AML will likely lead to more frequent classification updates in the future. Increased use of molecular profiling at the time of diagnosis, remission and follow-up is expected to become standard of care, with NGS used for multi-gene profiling, instead of single gene tests. With the development of pharmaceutical agents against molecular targets, use of combination chemotherapy for AML is expected to become

more frequent. Finally, the rapid pace of our continuously increasing knowledge of AML pathogenesis, which forms the basis of diagnostic classification, risk stratification and clinical management, is currently far greater than that of concurrent advances in the clinical therapy for AML. Nonetheless, these major advances are now actually beginning to seem promising, significantly in contrast with what would have been considered a dream even less than a decade ago, for individually personalized therapy for patients with a malignant disease category characterized by extreme molecular heterogeneity such as AML.

Conflict of interest statement

No potential conflicts of interest are disclosed.

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