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Molecular prognostic factors in cytogenetically normal acute myeloid leukemia

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Abstract

Chromosomal abnormalities are detected in 50–60% of patients with acute myeloid leukemia (AML) and are important predictors of prognosis and risk of relapse. The remaining patients, those with cytogenetically normal AML, are a seemingly homogeneous group that in fact consists of subsets of patients with distinct clinical outcomes. This heterogeneity is likely related to acquired gene mutations, as well as altered miRNA and gene-expression profiles, which occur within the group. The identification of recurrent molecular abnormalities has improved prognostication and provided insight into mechanisms of leukemogenesis for patients with cytogenetically normal AML, as well as led to the discovery of novel therapeutic targets. As the number of mutations continues to expand, bioinformatic algorithms that allow for integration of multiple markers will be necessary to provide optimal care for patients with this disease.

Keywords

acute myeloid leukemia; mutational analysis; normal karyotype; prognostic markers

Acute myeloid leukemia (AML) is a biologically heterogeneous disease of the hematopoietic system characterized by clonal accumulation and expansion of immature myeloid cells within the bone marrow [1]. For the most part, AML is a disease of older patients, occurring in 13–15 per 100,000 adults above the age of 65 in the USA, with a median age at diagnosis of 67 years [1]. A number of clinical factors such as age, performance status or a history of prior chemotherapy have been associated with outcome, but the most important factor in predicting risk of relapse has been chromosomal abnormalities detected at diagnosis [2,3]. The nonrandom chromosomal abnormalities identified in 50–60% of patients are highly prognostic of response to initial therapy and are used to direct treatment strategies, including allogeneic transplantation in first complete remission (CR).

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While cytogenetic analysis is an important part of the diagnostic evaluation in all patients, outcome risk has been difficult to define for patients presenting without chromosome aberrations. However, recently, the authors and other researchers have discovered several molecular markers that allow for the definition of outcome risk, even in patients with cytogenetically normal AML (CN-AML): a group that comprises 40–50% of patients with newly diagnosed disease. Thus, the presence or absence of somatically acquired genetic alterations in both CN-AML and other cytogenetic groups is now an important consideration in risk stratification of these patients. As a result of the identification of these mutations, patients with CN-AML are recognized as a diverse group with distinct clinical outcomes. Indeed, both the WHO and the European LeukemiaNet (ELN) classifications include recurrent molecular abnormalities as a complement to cytogenetics [4,5].

Aside from risk stratification, the identification of these molecular markers has led to new insights into mechanisms of leukemogenesis and the development of novel targeted therapies. In this article, the authors review these genetic markers and discuss their clinical and therapeutic implications for patients with CN-AML.

FLT3 mutations

Aberrant activation of signaling pathways that promote proliferation and survival of leukemic blasts is considered a key mechanism in myeloid leukemogenesis. FMS-like tyrosine kinase 3 (FLT3), a member of the class III receptor tyrosine kinases, is normally expressed on the surface of hematopoietic progenitor cells and plays a role in the regulation of cellular differentiation and proliferation [6]. When mutated, the gene that encodes for the FLT3 receptor tyrosine kinase confers a proliferation and survival advantage to leukemic blasts [7]. The most common form of the *FLT3* mutation is an internal tandem duplication (ITD) in exons 14 and 15 that maps to the juxtamembrane domain (JMD) and results in ligand-independent FLT3 receptor dimerization and constitutive activation of the kinase domains via autophosphorylation [7]. The second type of *FLT3* mutation is due to missense point mutations involving the D835/I836 residues of the second tyrosine kinase domain (TKD) [8-10].

In initial studies of the mutation in patients with newly diagnosed non-FAB M3 AML, 23% had a *FLT3* gene mutation [11]. While the presence of the mutation did not affect the CR rate, it did appear to predict for relapse and an adverse overall survival (OS) [11]. Subsequent larger studies of patients have provided additional information about patients with *FLT3* mutations [11-16]. First, the overall frequency of the mutation is higher in *de novo* AML (23–27%) as compared with secondary (15%) or therapy-related AML (11%), and occurs in 25–35% of CN-AML patients. With regard to other cytogenetic groups, *FLT3*-ITD mutations are rare in core-binding factor (CBF) AML (i.e., AML harboring t(8;21) or inv(16), or corresponding respective fusion genes *RUNX1/RUNX1T1* and *CBFB/MYH11*) or AML with complex karyotype, but do occur in 30–35% of patients with acute promyelocytic leukemia (APL), although this does not appear to impact the rate of relapse or OS of these patients [14-16]. Clinically, patients with *FLT3*-ITD mutations tend to present with higher total white blood cell (WBC) counts, and as mentioned previously, while the rate of achievement of CR is not different between patients with mutated or wild-type *FLT3*, the presence of this mutation confers an increased risk for relapse and death. However, in cases where the allelic ratio (of mutant to wild-type *FLT3*) is low, patients appear to have an outcome similar to those without an *FLT3*-ITD mutation, while an increased allelic ratio predicts for worse survival [17,18]. The prognostic significance of *FLT3*-ITD insertion sites within the extracellular domain of the FLT3 receptor has also been evaluated [19]. In a cohort of 241 patients, of whom 86.3% had CN-AML, ITD integration at the β1-sheet of the TKD1 was associated with both an inferior CR rate and OS as compared with patients with

insertion sites in the JMD, JMD hinge region or other TKD1 sites. The presence of a *FLT3*-ITD mutation at the time of relapse has also been shown to be associated with a lower rate of achieving a second CR and shorter OS [20].

Whereas the prognostic relevance of *FLT3*-ITD mutations is clear, that of *FLT3*-TKD mutations, which occur in 5–10% of all AML cases, remains controversial. In the assessment of the impact of *FLT3*-TKD mutations (in the absence of *FLT3*-ITD) in patients less than 60 years of age with CN-AML, the authors found that *FLT3*-TKD mutations were associated with a worse disease-free survival (DFS) relative to *FLT3* wild-type patients [9]. Conversely, in a subset analysis of patients with CN-AML in a report by the Medical Research Council, a trend for an improved OS was observed in patients with *FLT3*-TKD mutations, although this analysis included patients with *FLT3*-ITD mutations and those who had received an allogeneic stem cell transplant (SCT) as postremission therapy, factors that may have led to differences in the outcomes that were observed [8].

Although clinical trials with tyrosine kinase inhibitors as either single agents or in combination with chemotherapy have yet to show an improvement in the risk of relapse or OS for patients with *FLT3* mutations, more potent and specific inhibitors of FLT3 are in development that may have greater clinical benefit [21]. Given the adverse prognostic risk associated with the *FLT3*-ITD mutation, allogeneic SCT has been recommended in these patients if not entered into clinical trials [22].

NPM1 mutations

The *NPM1* gene encodes for a protein that functions as a molecular chaperone that shuttles between the nucleus and cytoplasm to regulate processes within the cell, such as transport of preribosomal particles, responses to stress stimuli and DNA repair, and the activity and stability of tumor suppressors such as p53 [23]. Mutations within exon 12 of the gene result in abnormal expression and localization of the protein within the cytoplasm. As the most common molecular marker in patients with CN-AML, *NPM1* mutations are found in 45–60% of patients and are associated with achievement of CR and an overall favorable outcome, especially in the absence of an *FLT3*-ITD mutation [22,24-26]. In the initial clinical description of this mutation, Falini *et al.* reported an aggregate mutational frequency of 35.2% in 591 patients with either primary or secondary AML [26]. Of those with CN-AML, 61.7% had the mutation, which in this group was associated with a response to induction chemotherapy as the CR rate was 71% in these patients. These initial findings have been validated in larger, more homogenous populations of patients, and additional clinical features of patients with the *NPM1* mutation have been reported, including an association with myelo-monocytic/monocytic leukemia, elevated WBC counts and the presence of extramedullary involvement.

In a group of younger patients (age 16–60 years) with CN-AML entered on to two consecutive multicenter trials of the AML Study Group for patients with primary or secondary AML, an interaction between *NPM1* and *FLT3* was observed. The highest remission rate occurred in those with *NPM1* mutated/*FLT3*-ITD negative disease, a group that also had a more favorable OS [25]. As allogeneic SCT was also a part of these trials, in a donor versus no donor study, patients who were *NPM1* mutated/*FLT3*-ITD negative did not benefit from transplantation, and in fact, their probability of survival was 60% at 5 years – on par with patients with CBF AML [25]. These results led to a proposal to reclassify patients with this molecular profile from the intermediate to favorable risk category, and indeed, both the WHO and ELN recognize this new classification [4,5].

Most initial studies primarily focused on the impact of this mutation in younger patients, defined as those aged younger than 60 years, and its interaction with other molecular

markers. The Cancer and Leukemia Group B (CALGB) has reported on the impact of *NPM1* as a single marker in older patients (defined as those greater than 60 years) with CN-AML treated with intensive chemotherapy [24]. Of the 148 patients tested, 83 patients (56%) had *NPM1* mutations. These patients were also noted to have similar clinical features to those observed in younger patients. Again, an improved CR rate was seen: 83 versus 48% in those without the *NPM1* mutation, as well as a longer survival at 3 years: 35 versus 8%. What was most interesting was that in patients ≥70 years, the presence of an *NPM1* mutation was associated with higher CR rates and a longer DFS and OS, where in patients aged 60–69 years, the presence of an *NPM1* mutation did not significantly affect DFS or OS.

Owing to the strong predictive nature of this marker, similar chemotherapeutic approaches are now recommended for both CN-AML with mutated *NPM1* without *FLT3*-ITD and CBF AML, reserving allogeneic SCT until the time of relapse [4]. All-*trans* retinoic acid (ATRA), an important chemotherapeutic agent utilized in the treatment of patients with APL, has previously been shown to improve the OS of older patients with non-APL AML after intensive chemotherapy, an effect now thought to be due to the presence of leukemic cells with mutant *NPM1*[27]. A recent report by the German–Austrian AML Study Group of younger patients (age <60 years) with *NPM1*-mutated AML randomized to receive ATRA in combination with intensive chemotherapy has also shown an improved OS as compared with *NPM1*-mutated patients who did not receive ATRA chemotherapy [28]. Additional clinical trials combining ATRA with intensive chemotherapy in patients with *NPM1* mutations are needed to further confirm these findings.

Similar to the monitoring of minimal residual disease (MRD) in distinct AML subtypes, such as APL with t(15;17) (q22;q12) *PML-RARA, NPM1* mutations have been studied as a possible marker for MRD monitoring in patients with CN-AML [29]. In a recent study of 245 patients with *NPM1*-mutated AML, monitoring of *NPM1*-mutated transcripts by RNA-based real-time quantitative PCR identified patients at an increased risk of relapse [30]. In this study, while transcript levels at diagnosis did not affect DFS or OS, real-time quantitative PCR positivity after two induction cycles or at the completion of consolidation chemotherapy was associated with a higher cumulative incidence of relapse. In addition, serial monitoring of transcript levels thereafter allowed predication of relapse. Future studies are needed that incorporate treatment intensification based on MRD assessment, in order to determine whether this may improve outcome in these patients.

CEBPA mutations

CCAAT/enhancer binding protein α (*CEBPA*) is a member of the family of basic region leucine zipper transcription factors and is required for the control of not only metabolic processes, such as glucose metabolism, but also granulocytic differentiation [31,32]. As there are two AUG translation initiation sites within the same open-reading frame, *CEBPA* mRNA gives rise to two separate translation products, the shorter form known as p30 and the longer form known as p42. The transactivating domains are only present in the p42 isoform, while the domains necessary for interaction with other transcription factors are present in both. *CEBPA* mutations are found in 10–15% of patients with CN-AML and are typically biallelic, involving either frameshift mutations within the N-terminus, which creates a nonfunctional isoform that lacks the transactivating terminus, or in-frame insertions or deletions within the C-terminus, which disrupt the DNA binding domain [32]. Most *CEBPA* mutant AMLs exhibit absent wild-type expression of *CEBPA* due to either homozygous (same type of mutation present on each allele) or heterozygous (different types of mutations present on each allele) mutations.

The authors reported results of screening for *CEBPA* mutations, as well as gene and miRNA-expression profiling, in 175 young adults (age <60 years) with previously untreated AML. They identified 32 patients (18%) with *CEBPA* mutations who had significantly better 5-year event-free survival (EFS) rates of 53% compared with 30% in *CEBPA* wild-type patients [33]. Interestingly, 29 (91%) of the 32 patients with *CEBPA* mutations were in a CN-AML molecular high-risk subset (i.e., patients with *FLT3*-ITD and/or wild-type *NPM1*); however, the presence of the mutation predicted for improved DFS and OS, independent of other molecular markers (e.g., *FLT3, NPM1* etc). In addition, patients with one affected allele could not be distinguished from wild-type cases with regard to outcome, and they expressed a gene-expression profile that was different from those with double *CEBPA* mutations, who had a more favorable outcome [33]. The gene-expression signature associated with *CEBPA* mutations was also associated with upregulation of genes and miRNA involved in erythroid differentiation. A larger study of 1427 patients enrolled in UK Medical Research Council AML10 or AML12 trials identified 107 patients with *CEBPA* mutations, 48 with a single mutation and 59 patients with double *CEBPA* mutations [34]. As noted in previous studies, double *CEBPA* mutations were associated with a favorable outcome, while the OS of patients with a single *CEBPA* mutation was poorer. In this cohort, the presence of a single *CEBPA* mutation did not provide additional benefit for patients with an *NPM1* mutation; however, the presence of a *FLT3*-ITD mutation was associated with a worse OS regardless of *CEBPA* mutant status. The difference in outcome observed in this study in patients with *CEBPA* mutations and *FLT3*-ITD mutations requires further evaluation in larger studies.

Additional studies have shown that patients with CN-AML and a *CEBPA* mutation have an outcome similar to that which is observed in patients with low-molecular-risk (mutated *NPM1* without *FLT3*-ITD) AML and, as a result, are also classified in the favorable risk category of the ELN classification and treated similarly [4,33,35,36].

IDH1/IDH2 mutations

Isocitrate dehydrogenase (IDH), a member of the β-decarboxylating dehydrogenase family of enzymes, catalyzes the oxidative decarboxylation of 2,3-isocitrate to yield 2-oxoglutarate and carbon dioxide in the Krebs cycle [37]. Mutations within *IDH1* and *IDH2* encode for an isoform of the protein with a loss of function that both impairs this reaction and creates a gain of function in the reverse reaction that reduces α-ketoglutarate to an oncogenic molecule, 2-hydroxyglutarate [38]. First described in colorectal cancer, recurrent *IDH* mutations have been found in primary brain tumors including glioblastomas, astrocytomas and oligodendrogliomas, and now more recently, in AML [38-43]. First reported following whole-genome sequencing and validation analysis of a patient with CN-AML, a mutation within *IDH1* was subsequently found to be present in 13 out of 80 samples from patients with CN-AML [39]. In a larger group of 358 patients with *de novo* CN-AML reported by the CALGB, 118 patients (33%) harbored mutations in *IDH* genes: 49 patients in *IDH1* and 69 patients in *IDH2*[41]. While there was no difference in outcome when comparing all patients with *IDH1* mutations and *IDH1/IDH2*wt, in younger patients (age <60 years) with molecular low-risk disease (*NPM1* mutated/*FLT3* negative), *IDH1* mutations were associated with a shorter DFS. The outcome of patients with R172 *IDH2* mutations, a previously unreported *IDH2* mutation, was also not significantly different from the *IDH1/IDH2*wt; however, in older patients, where R172 was most common, the CR rate was lower in patients with the mutation: 20 versus 67%. Given that the presence of R172 *IDH2* mutations in gliomas predict for a favorable outcome, where in this subgroup of patients with AML it is an adverse risk factor, tumor biology as well as gene- and miRNA-expression patterns are likely to influence the phenotype that is observed.

A larger study of 805 patients aged 16–60 years reported a lower aggregate frequency of *IDH* mutations, 16% (7.6% *IDH1* and 8.7% *IDH2*), with most mutations occurring in patients with CN-AML [42]. Although this study did not perform a subset analysis based on age, the authors did note a negative prognostic impact of *IDH* mutations on patients with *NPM1*-mutated/*FLT3*-ITD-negative disease. The *IDH1* SNP rs11554137 ($IDH1^{105GGT}$), which is located in the same exon as the R132 mutation site, was found in 12% of CN-AML, and was an independent prognostic factor associated with an inferior OS in high-risk molecular subsets of CN-AML patients (either *NPM1* wild type or *FLT3*-ITD positive) [43].

Patel and colleagues recently reported on an integrated mutational analysis in patients with AML and found an improved 3-year OS rate in those with both *IDH1* or *IDH2* and *NPM1* mutations (89 vs 31%), as compared with those who had mutant *NPM1* and both wild-type *IDH1* and wild-type *IDH2*[44]. The difference in OS and favorable impact of *IDH1* or *IDH2* mutations observed in this study, as compared with previous studies, may be due to the higher dose of daunorubicin utilized, although additional studies are needed to confirm this finding. Nonetheless, *IDH1/IDH2* mutations help to further classify the favorable molecular risk group of *NPM1*-mutated patients.

DNMT3A mutations

Aberrant DNA methylation resulting in epigenetic silencing of structurally normal genes relevant to the regulation of cellular differentiation, proliferation and survival has been described in AML and is considered to play a role in disease pathogenesis [45]. Methylation of the cytosine residue of CpG dinucleotides is catalyzed by one of three isoforms of the enzyme DNA methyltransferase: DNMT1, DNMT3A or DNMT3B, all of which are overexpressed in malignant blasts as compared with normal bone marrow cells [46]. An amino acid substitution at position 882 of *DNMT3A* was first identified in genomic DNA from CD34-positive AML blasts by Yamashita *et al.*, and then later this mutation was studied by Ley *et al.* in a cohort of patients with *de novo* AML to determine whether it was recurrent or carried prognostic information [47,48]. Among the 282 patient samples analyzed, 62 patients (22.1%) had mutations in *DNMT3A* which were expected to affect translation of the enzyme, 166 patients were considered to have an intermediate-risk cytogenetic profile and 56 patients (33.7%) carried the mutation. Interestingly, while the presence of the *DNMT3A* mutation was associated with a worse OS as compared with those without the mutation (12.3 vs 41.1 monthsl; $p < 0.001$), both in patients with CN-AML and those with intermediate cytogenetic risk, *DNMT3A* mutations did not appear to alter global patterns of methylation or significantly alter gene expression [47].

In a larger study of 489 patients with *de novo* or secondary AML treated uniformly with intensive response-adapted double induction and consolidation therapy, the overall frequency of *DNMT3A* mutations was 17.8%, with the highest frequency occurring in patients with CN-AML at 27.2% [49]. A shorter OS was observed in patients with *DNMT3A* mutations as compared with patients with wild-type *DNMT3A* (1.73 vs 3.33 years) and remained a negative prognostic marker for OS, confirming earlier reports by Ley *et al*[49]. However, relapse-free survival was similar in both groups. In addition to having a shorter OS, *DNMT3A* mutations predicted for a lower CR rate in multivariate analysis of patients with CN-AML. The adverse effect of mutated *DNMT3A* on survival was most pronounced in patients with *NPM1* wild-type/*FLT3*-ITD AML, which also confirms previous reports. Additional reports on the prognostic impact of *DNMT3A* mutations confirm a worse OS and relapse-free survival and that *DNMT3A* mutations predicted for worse treatment response in patients with wild-type *NPM1, FLT3* or *CEBPA* mutations [50].

The authors have further defined the impact of *DNMT3A* mutations in patients with CN-AML with respect to age and type of *DNMT3A* mutation [51]. In a cohort of 415 patients with CN-AML who received cytarabine- and daunorubicin-based induction chemotherapy, *DNMT3A* mutations were found in 34.2% of patients, and similar to other reports, missense mutations affecting arginine codon 882 (R882-*DNMT3A*) were more common (62%) than other mutations (non-R882-*DNMT3A*). While there was no significant impact of R882-*DNMT3A* mutations on DFS or OS for younger patients (age <60 years), those with non-R882-*DNMT3A* mutations had both a shorter DFS (20 vs 49%; p = 0.007) and trend toward a shorter OS (29 vs 52%; p = 0.09) at 3 years as compared with *DNMT3A* wild-type patients. Conversely in older patients, R882-*DNMT3A* mutations were associated with both a significantly shorter DFS (3 vs 21%; p = 0.006) and OS (4 vs 24%; p = 0.01) at 3 years compared with *DNMT3A* wild-type, where non-R882-*DNMT3A* mutations did not have an effect. The basis for these observed differences in outcome based on mutation type are not clear, although the impact of the frequency of different molecular markers present within each of the age groups may be a possible explanation.

In the integrated mutational analysis reported by Patel *et al.* mentioned previously, an interesting association between treatment intensity and *DNMT3A* mutational status was observed [44]. Those patients with *DNMT3A* mutations randomized to receive a higher dose of daunorubicin, 90 mg/m^2, during induction chemotherapy had an improved rate of survival as compared with patients with wild-type *DNMT3A*. If confirmed in larger cohorts of patients, *DNMT3A* mutational status in younger patients may be used to direct anthracycline dose intensification during induction.

The authors have also evaluated the impact of *DNMT3A* mutations and response to therapy in mostly older patients who had been treated with the hypomethylating agent decitabine, either as a single agent at a dose of 20 mg/m^2 per day for 10 days or in combination with bortezomib [52]. While this was a small, cytogenetically heterogeneous group of patients with an overall CR rate of 41%, six out of the eight patients with *DNMT3A* mutations (75%) achieved a CR as compared with 13 out of 38 *DNMT3A* wild-type patients (34%; p = 0.05). Also, the median OS of patients with *DNMT3A* mutations was 16.8 versus 11.0 months in patients with *DNMT3A* wild-type, although this difference was not statistically significant. While the small sample size and heterogeneous patient population limit the interpretation of these results, should these findings be confirmed in a larger homogeneous cohort of patients, *DNMT3A* mutations may used to stratify older patients to decitabine-based therapy.

RUNX1 mutations

Chromosomal translocations involving the runt-related transcription factor 1 (*RUNX1*) gene are well documented and have been observed in AML. As a transcription factor, RUNX1 is involved in the regulation of normal hematopoietic differentiation [53]. Chromosomal translocations or mutations within the gene result in deregulation of its function. In a study of 470 patients with untreated *de novo* non-M3 AML, 62 patients (13.2%) had distinct *RUNX1* mutations and were characterized as having an inferior response to standard chemotherapy with a worse DFS and OS [54]. In a larger study of 945 younger adults with AML (aged 18–60 years), *RUNX1* mutations were identified in 6.3% of patients with CN-AML, 5.6% of patients overall, and the authors reported a direct association with the presence of *MLL*-partial tandem duplication (PTD) and *IDH1/IDH2* mutations and an inverse association with *NPM1* and *CEBPA* mutations [55]. Confirming previous reports, *RUNX1* mutations were associated with an inferior response to standard chemotherapy with a lower CR rate, higher frequency of relapsed disease and shorter EFS and OS. The difference in aggregate frequency of the mutation within the two separate trials has been

attributed to the different racial background of the patients enrolled on the trials (Asian vs non-Asian).

In the largest cohort of CN-AML patients treated with cytarabine- and anthracycline-based chemotherapy, Mendler *et al.* evaluated 392 patients and identified *RUNX1* mutations (*RUNX1*-mut) in 12.5% of patients (8% aged <60 years and 16% aged >60 years) [56]. *RUNX1*-mut patients had lower CR rates and shorter DFS and OS than *RUNX1* wild-type patients and also harbored *NPM1* and *CEBPA* mutations less frequently than *RUNX1* wild-type patients. Interestingly, genes normally expressed in hematopoietic stem cells and early progenitor cells were found to be upregulated in *RUNX1*-mut patients, as were genes that have been implicated in chemoresistance, both of which would have a negative impact on response to therapy.

WT1 mutations

The Wilms' Tumor 1 (*WT1*) gene, located at chromosomal band 11p13, encodes for a transcriptional regulator with tissue-specific expression and key roles in cellular growth and development [57]. Structurally, the N-terminal domain is composed of proline-glutamine-rich sequences necessary for both RNA and protein interactions, while the C-terminal domain is composed of four zinc fingers, which bind directly to DNA sequences to enhance or repress target gene expression [57]. While not completely understood, the WT1 protein is able to act as both a tumor suppressor and an oncogene. This dual functionality may be due to specific protein–protein interactions, post-translational modifications to the molecule or differential activity of the four isoforms of the protein [58,59]. In AML, *WT1* mutations occur in 7–12% of patients with CN-AML and their impact on outcome is controversial [60-63].

A large series of more than 600 patients with CN-AML reported that while *WT1* mutations were significantly associated with the presence of *FLT3*-ITD and *CEBPA* mutations, there were no differences in rates of CR, refractory disease or OS between patients with mutated or unmutated *WT1* disease. However, patients with both a *WT1* mutation and *FLT3*-ITD mutation did have a lower rate of CR and an increased rate of refractory disease [61]. However, in two separate series, also of patients with CN-AML, contrary results have been reported with a shorter OS and higher relapse rate in patients with mutated *WT1*[62,63]. Indeed, in the report published by Paschka *et al.*, the 3-year DFS was 13% in mutated *WT1* versus 50% in patients with unmutated *WT1*[62]. This group also further defined the prognostic impact of the *WT1* mutation in patients with CN-AML over the age of 60 and found that while the DFS was similar among patients with mutated and unmutated *WT1*, there was a trend toward a decreased CR and OS rate, although the impact was unclear given the overall poor outcome of the group [60].

More recently, an SNP in a mutational hotspot of *WT1*, SNP rs16754, was identified in 25.7% of CN-AML patients' blasts and germline DNA and in 36% of healthy volunteers. While reported to be a favorable independent predictor of relapse-free survival and OS, given the presence of this polymorphism in normal controls, this finding may reflect drug sensitivity or metabolism rather than a predisposition to disease development [64].

MLL mutations

PTDs of the mixed lineage leukemia gene, *MLL* (*MLL*-PTDs), were the first molecular marker described in patients with CN-AML [65]. While *MLL* is known to be involved in recurrent translocations in both AML and acute lymphoblastic leukemia, *MLL*-PTD results from a duplication of a genomic region containing either *MLL* exons 5 through 11 or 5 through 12, which is then inserted into intron 4 of the full-length *MLL* gene [66]. The initial

description of 98 patients with CN-AML (aged 18–84) reported an aggregate frequency of the mutation of 11% and a shorter duration of CR as compared to those without *MLL*-PTD rearrangements [67]. Subsequent studies have confirmed the frequency of the mutation in CN-AML and trisomy 13, as well as the short remission duration [53,68]. In a larger study of 238 patients with CN-AML, Whitman *et al.* found that after a median follow-up of 4.7 years, there were no differences in DFS or OS of patients with *MLL*-PTD rearrangements as compared to those without. The better outcome of this group was attributed to intensive consolidation with autologous SCT [69]. In Patel *et al.*'s more recent study, *MLL*-PTD was associated with a reduced OS; however, the rate of OS was improved in patients who received high-dose daunorubicin as compared with standard-dose daunorubicin [44].

Interestingly, the *MLL*-PTD protein retains its normal function and is able to contribute to DNA hypermethylation and epigenetic silencing of tumor suppressor genes. *In vitro*, this can be reversed following hypomethylating and histone deacetylase inhibitor treatment, and a clinical trial with this combination of therapeutic agents is ongoing in patients with relapsed or refractory AML [70].

Other gene mutations

Tet oncogene family member 2 (*TET2*) gene mutations are known to occur in myeloid malignancies including myelodysplastic syndromes and myeloproliferative neoplasms and were first reported to have an adverse impact on patients with ELN favorable-risk AML (mutated *NPM1* without *FLT3*-ITD or who have mutated *CEBPA*) [71-73]. Metzeler *et al.* reported an analysis of 427 patients with previously untreated CN-AML who received cytarabine/daunorubicin therapy and noted an aggregate frequency of the mutation of 23%, with an association with older age and higher pretreatment WBC count as compared with wild-type *TET2*[73]. Most interesting was the impact of the presence of this mutation on patients with favorable risk CN-AML. Among this group of patients, those with *TET2* mutations had a lower CR rate and a shorter EFS and OS than those with wild-type *TET2*, where there was no impact of *TET2* mutations in the ELN intermediate-I-risk group (defined as *CEBPA*-wt and *FLT3*-ITD and/or *NPM1* wt). More recently, Gaidzik and colleagues reported a lack of impact of *TET2* mutations in patients with CN-AML, and in fact there was no difference in the cumulative incidence of relapse, relapse-free survival or OS overall, or those with ELN favorable or intermediate-I-risk disease [74]. Of note, this study was restricted to patients aged 18–60 years and included more intensive induction and consolidation than the patients in the CALGB study reported by Metzeler *et al.* received. These factors may have contributed to the differences in the outcomes observed. The impact of *TET2* in Patel *et al.*'s integrated mutation analysis confirmed an association with adverse risk, where *FLT3*-negative patients with *TET2* mutations had an OS rate of 6.3% that was not improved with anthracycline dose intensification [44].

Similar to *TET2*, mutations in the additional sex combs like-1 (*ASXL1*) gene have also been identified in patients with AML, myelodysplastic syndromes and myeloproliferative neoplasms [75-77]. In a cohort of more than 400 patients with molecularly well-characterized CN-AML who received cytarabine- and daunorubicin-based induction chemotherapy, the impact of *ASXL1* mutations on outcome and their association with the disease features were analyzed [78]. Notably, *ASXL1* mutations were five times more common in patients 60 years of age or older (16.2%) than in younger patients (3.2%), and while nonsense and frameshift mutations in *ASXL1* were observed, duplication of the guanine nucleotide at position 1934 (c.2934dupgG) accounted for almost half of the mutations. While *ASXL1* mutations rarely occurred concurrently with *NPM1* or *FLT3*-ITD mutations, *CEBPA* mutations were found in 26% of *ASXL1* mutated (ASXL1-mut) as opposed to 9% of *ASXL1* wild-type (ASXL1-wt) patients ($p = 0.01$). Overall, *ASXL1*-mut

patients had a lower CR rate and shorter DFS and OS when compared with *ASXL1*-wt patients. Interestingly, in patients with CN-AML and ELN favorable genetic risk, *ASXL1* mutations had a negative impact on achievement of CR (50 vs 82% in *ASXL1*-wt p = 0.02). In addition, among those patients in this favorable-risk group who did achieve a CR, all *ASXL1*-mut patients relapsed within 13 months and died within 18 months from diagnosis, where 27% of *ASXL1*-wt remained disease free at 3 years and had an OS of 34%. There were no significant differences in CR, DFS or OS in ELN intermediate-I genetic risk patients. Therefore, similar to *TET2* mutations, *ASXL1* mutations aid in the identification of a high-risk subgroup of older CN-AML patients and may have a greater impact on prognosis than *TET2* mutations. Indeed, in this cohort of patients with ELN favorable CN-AML, those with *ASXL1* mutations had a significantly worse OS as compared with *ASXL1*-wt/*TET2*-wt, regardless of *TET2* status.

Other gene mutations such as *TP53* have been identified in patients with AML, albeit in a lesser frequency than other mutations, which has made it difficult to discern their impact on risk stratification of patients with AML [79,80]. Please see Table 1 for a summary of the prognostic impact of the gene mutations that have been discussed.

miRNA

miRNAs are noncoding RNAs of 19–25 nucleotides that effect protein expression by modulating post-transcriptional activity of mRNA [81]. Deregulation of miRNAs contributes to disease pathogenesis in AML by interfering with the expression of proteins necessary for normal differentiation, proliferation and apoptosis of hematopoietic cells. miRNA-expression profiling is able to distinguish between myeloid and lymphoid leukemia, as well as distinct cytogenetic subtypes of AML based on upregulation or downregulation of specific miRNAs [33,82-86].

In patients with CN-AML, distinct miRNA-expression profiles have been correlated with certain molecular markers. The authors described the gene-expression signature of a series of older patients with CN-AML and *NPM1* mutations that featured upregulation of homeobox genes accompanied by higher expression levels of *miR-10a, miR-10b, miR-196a* and *miR-196b*[24]. Upregulation of genes involved in erythroid differentiation is a distinct feature of the gene-expression signature seen in patients with CN-AML and *CEBPA* mutations, and miRNA involved in erythroid lineage differentiation, members of the *miR-181* family, are also upregulated in this group suggesting a functional relationship between miRNA and gene expression [33]. Patients with *FLT3*-ITD-mutated AML also show aberrant miRNA expression with a two- to threefold increase in *miR-155*, as well as *miR-10a* and *miR-10b*.

The discovery of aberrant miRNA expression in AML has led to further insight into mechanisms of disease pathogenesis but has also provided additional prognostic information regarding OS and risk of relapse. For example, increased expression of *miR-181a* and *miR-181b* in high-risk CN-AML (*NPM1* wild type/*FLT3*-ITD mutated) is associated with a lower risk of relapse or failing to achieve a CR, which contrasts with the overall poor outcome of this group [87]. Across all cytogenetic subgroups, overexpression of *miR-20a, miR25, miR-191, miR-199a* and *miR-199b* adversely affect OS [83].

In addition to their prognostic impact, miRNAs have also been shown to be predictive of response to therapy. Blum *et al*. reported results of a Phase II trial of patients ≥60 years of age with newly diagnosed AML who were treated with a 10-day schedule of the hypomethylating agent, decitabine [88]. In addition to a CR rate of 47%, pretreatment levels of *miR-29b* were notably higher in responders as opposed to nonresponders (p = 0.02).

While assessed in a limited number of patients, this information supports further investigation of miRNA as potentially targetable entities, similar to *FLT3*.

Prognostic impact of gene expression

Variations in the expression of certain genes have also been shown to impact the prognosis of specific subsets of patients with AML, similar to what is seen with both chromosomal abnormalities and molecular markers in CN-AML. *ERG, EVI1, BAALC, MN1, MLL5* and *ID1* are six genes whose expression and prognostic impact have been studied in AML.

ETS-related gene (*ERG*) is a transcription factor involved in early lymphocyte differentiation and angiogenesis and plays a role in cellular growth and apoptosis [89]. Patients with CN-AML and high levels of *ERG* expression have a lower CR rate and worse EFS as compared with low expressers, and in patients with molecular low-risk disease (*NPM1* mutated/*FLT3* negative), high *ERG* expression was the only factor predicting shorter EFS [90]. Abnormal expression of *EVI1* is seen in patients with chromosomal translocations that involve chromosome 3q26, but *EVI1* is also highly expressed in the absence of this abnormality and is an independent negative prognostic indicator of survival [91]. Specifically, *EVI1* predicts for low CR rates, adverse relapse-free survival and EFS, effects that are most pronounced in the intermediate cytogenetic group, including those with CN-AML, and consideration for allograft may be advised for patients in first remission [91,92]. The brain and acute leukemia, cytoplasmic (*BAALC*) gene has a wide range of expression in CN-AML; however, higher expression levels predict lower CR rates and overall outcome with decreased DFS and OS [93,94]. The meningioma 1 gene (*MN1*) encodes for a nuclear protein that participates in cotranscriptional complexes with RAR-RXR or the vitamin D receptor [95,96]. In 119 patients with untreated CN-AML, upregulation of *MN1* expression was associated with a lower CR rate and shorter DFS [97,98]. The human mixed lineage leukemia 5 (*MLL5*) gene is important for normal myeloid differentiation, and the prognostic impact of *MLL5* was evaluated in a cohort of 509 patients with *de novo*, secondary or treatment-related AML [99,100]. Although a cytogenetically heterogenous group, in patients with CN-AML, high *MLL5* expression was a favorable prognostic marker for OS. Inhibitors of differentiation proteins are dominant inhibitors of helix-loop-helix DNA proteins and positively regulate cell growth [101]. Overexpression of *Id1* in younger patients (<60 years) was associated with a lower CR rate and shorter DFS and OS [102].

Differences in gene expression have also been used to refine risk stratification in patients with CN-AML. Metzeler *et al.* analyzed *ERG, BAALC* and *MN1* expression in 210 patients with previously untreated CN-AML who received intensive chemotherapy according to the AMLCG-1999 multicenter trial [103]. High *ERG* expression independently predicted for a lower likelihood of attaining a CR, while neither *BAALC* nor *MN1* expression levels exhibited independent prognostic value [103]. Please see Box 1 for a summary of the prognostic impact of genes and miRNA that have been discussed.

Box 1

Prognostic genes and miRNA in acute myeloid leukemia

- *ERG*: increased expression is unfavorable
- *EVI*: increased expression is unfavorable
- *BAALC*: increased expression is unfavorable
- *MN1*: increased expression is unfavorable

- *MLL5*: increased expression is favorable
- *ID1*: increased expression is unfavorable
- *miR-181a*: increased expression is favorable

Integration of gene mutation & gene-expression markers

It is likely that the number of mutations that may be found to be recurrent in AML will increase in the years to come. Researchers are exploring ways to best incorporate the presence or absence of each molecular marker, as well as the level of expression of each gene of interest and type of treatment, in such a way so as to account for their interrelationships as well as each marker's independent prognostic importance [104]. While such a model will be a welcome complement to cytogenetics and patient specific characteristics, validation in large studies of similarly treated patients will be necessary before this can become a standard part of the care of a patient with newly diagnosed AML. In an attempt to approach such an important issue in the treatment and management of patients with this disease, Patel and colleagues have described results of a mutational analysis of 18 genes in a cohort of 398 younger patients (age <60 years) and identified the previously described associations between treatment intensity, mutational status and outcome [44]. While these data suggest that dose intensity may be of benefit in specific genetic risk groups, larger groups of patients who are similarly treated will need to be studied to confirm these findings and to determine whether indeed the type of therapy given is able to influence or perhaps overcome the prognostic impact of specific molecular markers.

Expert commentary

For the practicing clinician, where is one to begin when prioritizing which molecular markers are to be tested to ensure a thorough and complete assessment of every patient? At this point, the majority of the mutations mentioned, as well as the miRNA and gene expression levels, are only available in a research setting. However, *NPM1, FLT3* and *CEBPA* are routinely available and should be assessed in all patients, regardless of age.

The importance of enrollment in the success of clinical trials cannot be overstated, as none of the information that has been presented would be known without individual patient participation and agreement to provide additional samples for future analyses. Future discoveries and overall progress in this disease will be dependent upon this. As we study each mutation's role in disease pathogenesis, future clinical trials will likely include novel agents that either target unfavorable mutations or attempt to pharmacologically create a genetic/molecular environment that is more favorable, such that standard cytotoxic chemotherapy is more effective. Currently, rapid molecular screening tests allow for identification and accrual of patients with either *FLT3* mutations or CBF AML to clinical trials combining tyrosine kinase inhibitors with anthracycline- and cytarabine-based chemotherapy, and prove the feasibility of this type of approach (e.g., CALGB 11001 and CALGB 10801).

Five-year view

Despite our ability to risk-stratify patients to appropriate treatments, in part owing to the prognostic information provided by the mutations mentioned, current therapies achieve a long-term remission in approximately 40% of younger patients (age <60 years), while older patients are more likely to die of relapsed disease [1,105]. The 'one fits all' approach to treatment for patients with AML is no longer acceptable and if it persists, the long-term

survival rates of patients with this disease will remain poor. Whole genome sequencing and other sophisticated molecular and genetic analyses will continue to identify recurrent molecular markers in AML, which will refine clinical and prognostic classification of AML (i.e., ELN classification). Furthermore, as whole-genome sequencing for individual patients is on the horizon, the hope is that in the future, for each new patient that is diagnosed with AML, the treating physician will be able to input each marker into a complex bioinformatic algorithm which will ultimately guide which therapeutic clinical trial is most appropriate for that specific individual.

As this area of research within AML continues to evolve, it will only be with rational drug development and clinical trial design that improvements in the overall outcome for these patients will occur.

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Key issues

- Patients with cytogenetically normal acute myeloid leukemia (CN-AML) are in fact a heterogeneous population of patients with distinct clinical outcomes.
- Recurrent molecular abnormalities such as *FLT3, NPM1* and *CEBPA* improve risk stratification and help guide treatment recommendations for patients with CN-AML.
- The identification of molecular markers has not only provided further insight into mechanisms of leukemogenesis but also identified potential targets for novel therapies.
- In the future, whole genome-sequencing and bioinformatic analyses that incorporate molecular markers will likely guide therapeutic decisions for patients with CN-AML.
- Participation in clinical trials and the collection of samples for correlative science analysis is recommended for all patients with newly diagnosed AML.

Table 1

Prognostic mutations in acute myeloid leukemia.

Mutation	Chromosome	Prognostic impact	Frequency in CN-AML (%)
FLT3-ITD	13q12	Unfavorable	25–35
FLT3-TKD		Controversial	5–10
NPM1	5q35	Favorable in younger patients with mutated *NPM1* without *FLT3*-ITD Favorable in older patients regardless of *FLT3*-ITD	45–60
IDH1	2q33	Unfavorable in younger *NPM1* wild-type CN-AML; controversial	7.6–13.6
IDH2	15q26	Unfavorable in older patients; controversial	8.7–19
DNMT3A	2p23.3	Age- and mutation-type dependent Unfavorable in younger patients with non-R882 mutations Unfavorable in older patients with R882 mutations	27.2–33.7
CEBPA	19q13.1	Favorable, especially if both alleles mutated	10–15
RUNX1	8q22	Unfavorable; however, evaluation of prognostic significance is ongoing	6.3–13.2
WT1	11p13	Unfavorable; controversial	10–13
MLL-PTD	11q23	Unfavorable if not treated with high-dose chemotherapy and HSCT	11
TET2	4q24	Unfavorable in the favorable risk category of ELN classification; controversial	23
ASXL1	20q11	Unfavorable in the favorable risk category of ELN classification	10.4

AML: Acute myeloid leukemia; CN-AML: Cytogenetically normal acute myeloid leukemia; ELN: European LeukemiaNet; HSCT: Hematopoietic stem cell transplantation.