Review Article

Genomics-based Approach and Prognostic Stratification Significance of Gene Mutations in Intermediate-risk Acute Myeloid Leukemia

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Abstract

Objective: Intermediate-risk acute myeloid leukemia (IR-AML), which accounts for a substantial number of AML cases, is highly heterogeneous. We systematically summarize the latest research progress on the significance of gene mutations for prognostic stratification of IR-AML.

Data Sources: We conducted a systemic search from the PubMed database up to October, 2014 using various search terms and their combinations including IR-AML, gene mutations, mutational analysis, prognosis, risk stratification, next generation sequencing (NGS).

Study Selection: Clinical or basic research articles on NGS and the prognosis of gene mutations in IR-AML were included.

Results: The advent of the era of whole-genome sequencing has led to the discovery of an increasing number of molecular genetics aberrations that involved in leukemogenesis, and some of them have been used for prognostic risk stratification. Several studies have consistently identified that some gene mutations have prognostic relevance, however, there are still many controversies for some genes because of lacking sufficient evidence. In addition, tumor cells harbor hundreds of mutated genes and multiple mutations often coexist, therefore, single mutational analysis is not sufficient to make accurate prognostic predictions. The comprehensive analysis of multiple mutations based on sophisticated genomic technologies has raised increasing interest in recent years.

Conclusions: NGS represents a pioneering and helpful approach to prognostic risk stratification of IR-AML patients. Further large-scale studies for comprehensive molecular analysis are needed to provide guidance and a theoretical basis for IR-AML prognostic stratification and clinical management.

Key words: Gene Mutations; Intermediate-risk Acute Myeloid Leukemia; Next Generation Sequencing; Prognostic Analysis

Introduction

Acute myeloid leukemia (AML) is the most common type of acute leukemia, with an estimated annual incidence of 3-4 cases per 100,000 people.[1] Its pathogenesis is characterized by the accumulation of somatically acquired genetic changes in hematopoietic progenitor cells that alter the normal mechanisms of self-renewal, proliferation, and differentiation. In recent years, the progress of genomic and molecular technologies has led to the rapid uncovering of the molecular pathogenesis of AML and has revealed AML as a highly heterogeneous disease, thus improving the predictive value of the cytogenetic-based risk groups. However, nearly half of AML patients have normal cytogenetics and are consequently ascribed to the intermediate-risk (IR) category despite this significant heterogeneity. It is clear that molecular mutational analysis has the potential to improve prognostic stratification systems. Fms-related tyrosine kinase 3 internal tandem duplications (*FLT3-ITD*), nucleophosmin 1 (*NPM1*), and CCAAT/enhancer-binding protein alpha (*CEBPA*) mutations have been incorporated into the *National Comprehensive Cancer Network Guidelines* and have

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changed the prognostic stratification of patients. In addition, increasing access to whole-genome or exome mutational analysis techniques is allowing the discovery of a bewildering array of novel mutations associated with AML. Many other mutations in several genes with potential prognostic significance have been identified by next generation sequencing (NGS) and single nucleotide polymorphism (SNP) array analysis,[2] enabling researchers to explore a vast diversity among cytogenetically defined subsets of AML, especially among IR-AML patients, which account for nearly 60% of all AML patients.

A variety of gene mutations often occur concomitantly. A recent study from The Cancer Genome Atlas, analyzing the genomes of 200 adult AML patients, demonstrated that multiple gene mutations coexist in a single patient, and each mutation can affect other mutations, thus suggesting the existence of a strong link between mutations in different categories of genes.[3] Therefore, identification of a single mutation is not sufficient to predict clinical prognosis. Such results highlight the value of a comprehensive molecular genetic screening to improve the risk stratification of IR-AML patients. With the advent of the era of whole-genome sequencing, an increasing number of mutations and mutation sites have been found, and we now have a list of recurrently mutated genes in IR-AML [Table 1]. In addition, high-throughput sequencing based on microarray technology can identify multiple different mutations at the same time, allowing for a comprehensive analysis of mutations to predict prognosis, and consequently has raised interest in recent years.

In the present study, the importance of the genomics-based approach and sophisticated genomic technologies (i.e., NGS) of leukemogenesis and clonal evolution are discussed. Also, the prognostic significance of single and comprehensive mutational analysis in IR-AML patients is reviewed.

Leukemogenesis and Clonal Evolution

Mutation categories and interaction of genetic alterations

Several lines of evidence support a "double-hit" model of leukemogenesis in which Class I mutations confer a proliferative advantage without an effect on differentiation, whereas Class II mutations impair hematopoietic differentiation and subsequent apoptosis. Due to recent NGS studies, many new mutations occurring in genes involved in epigenetic regulation have been identified, suggesting that a third complementation group of mutations should be added to the two-hit model. Mutations in AML are classified into "driver" mutations, which provide a selective advantage, and "passenger" mutations, which were present in the original transformed cell before it started its clonal expansion.[18] It is becoming increasingly apparent that a single driver mutation is not enough to initiate leukemia, but the accumulation of several diver mutations (Class I, Class II and/or epigenetic mutations) and their concerted action is required for the clonal expansion of leukemia cells. There is evidence that as few as two highly complementary mutations can be sufficient to generate AML. In a knock-in mouse model, the combination of *NPM1/FLT3-ITD* mutations caused AML, with all mice becoming moribund in 31–68 days. In contrast, no case of AML was observed in the *NPM1* or *FLT3-ITD* single-mutant groups. However, the probability that additional mutations are rapidly acquired cannot be ruled out as in that model most AMLs showed acquired loss of heterozygosity of *FLT3-ITD*.[19] AML is a molecularly and clinically heterogeneous disease, and studies have revealed that this heterogeneity depends largely on specific combinations of somatic driver mutations. Therefore, it is important to identify the precise combinations of overlap mutations that are associated with leukemogenesis and impact on its distinguishing features, including response to treatment and prognosis.[6]

To date, several combinations of different categories of mutations have been identified, and it has been shown that some certain driver mutations are dependent on each other, and presumably synergize. For example, cytogenetically normal AML (CN-AML) with biallelic *CEBPA* (*bi-CEBPA*) mutations has very specific zinc finger 1 mutations in the transcription factor GATA binding protein 2 (*GATA2*).[11] In addition, *NPM1* and *FLT3-ITD* mutations have been identified as the two most commonly co-occurring AML mutations. In addition, mutations in epigenetic modifiers frequently overlap with Class I and/or Class II mutations.[6,20] Particularly, isocitrate dehydrogenase 1/2 (*IDH1/2*), DNA (cytosine-5-)-methyltransferase 3 alpha (*DNMT3A*), and tet methylcytosine dioxygenase 2 (*TET2*) mutations are frequently associated with *NPM1* and *FLT3-ITD* mutations. In contrast, additional sex combs like 1 (*ASXL1*) and mixed-lineage leukemia (*MLL*) mutations are exclusively found in combination with Class I mutations, with the only exception of the frequent overlap with *MLL* partial tandem duplication and *FLT3-ITD*. In addition, these two mutations mainly affecting the histone methylation status, also overlap with different patterns of Class II mutations, such as *CEBPA* and runt-related transcription factor 1 (*RUNX1*), and epigenetic mutations such as *IDH1/2* or *TET2* mutations.[15] Interestingly, genes that are functionally overlapped are often mutually exclusive, which is evidenced by the fact that *IDH1/2* and *TET2* mutations are not detected in the same patient.[21] Likewise, mutations in *DNMT3A*, *ASXL1*, and *MLL*, which are all epigenetic modifiers, are also exclusive in AML cells.[20] In addition, mutations in the cohesin complex genes such as *RAD21* homolog (*Schizosaccharomyces pombe*), structural maintenance of chromosomes 1A (*SMC1A*), *SMC3*, Stromal Antigen 2, and spliceosome complex genes such as splicing factor 3B subunit 1, serine/arginine-rich splicing factor 2, and U2 small nuclear RNA auxiliary factor 1 have been discovered recently and reported to be mutually exclusive, further indicating the redundant potential for leukemogenesis.[7,18]

Table 1: Recurrently mutated genes in IR-AML

Author, years	Functional groups or pathways	Gene names	Mutation categories	Clinical relevance
Levis, 2013[4]	Tyrosine kinase	*FLT3-ITD*	Class I mutations	Poor prognosis
Janke *et al.*, 2014[5]	Tyrosine kinase	*FLT3-TKD*	Class I mutations	Controversial data
Patel *et al.*, 2012[6]	Tyrosine kinase	*KIT*	Class I mutations	Poor prognosis
Naoe and Kiyoi, 2013[7]	RAS pathway	*NRAS*	Class I mutations	No prognostic relevance
Naoe and Kiyoi, 2013[7]	RAS pathway	*KRAS*	Class I mutations	No prognostic relevance
Schlenk and Döhner, 2013[1]	Protein phosphatase	*PTPN11*	Class I mutations	No clear data
Becker *et al.*, 2010[8]	Nuclear-shutting protein	*NPM1*	Class II mutations	Good prognosis
Dufour *et al.*, 2010[9]	Transcription factor	*CEBPA*	Class II mutations	bi-mut: Good prognosis
Mendler *et al.*, 2012[10]	Transcription factor	*RUXN1*	Class II mutations	Poor prognosis
Greif *et al.*, 2012[11]	Transcription factor	*GATA2*	Class II mutations	Good prognosis
Ley *et al.*, 2010[12]	DNA methylation	*DNMT3A*	Epigenetic mutations	Controversial data
Abbas *et al.*, 2010[13]	DNA hydroxymethylation	*IDH1/2*	Epigenetic mutations	Controversial data
Chou *et al.*, 2011[14]	DNA hydroxymethylation	*TET2*	Epigenetic mutations	Controversial data
Metzeler *et al.*, 2011[15]	Chromatin modifier	*ASXL1*	Epigenetic mutations	Poor prognosis
Schlenk and Döhner, 2013[1]	Chromatin modifier	*MLL*	Epigenetic mutations	Poor prognosis
Naoe and Kiyoi *et al.*, 2013[7]	Chromatin modifier	*EZH2*	Epigenetic mutations	No clear data
Grossmann *et al.*, 2012[16]	Tumor suppressor	*TP53*	Tumor suppressors	Poor prognosis
Gaidzik *et al.*, 2009[17]	Tumor suppressor	*WT1*	Tumor suppressors	Poor prognosis
Patel *et al.*, 2012[6]	Tumor suppressor	*PHF6*	Tumor suppressors	Poor prognosis
Schlenk and Döhner, 2013[1]	Spliceosome complex	*SF3B1*	Spliceosome complex	No clear data
	Spliceosome complex	*SRSF2*	Spliceosome complex	No clear data
	Spliceosome complex	*U2AF1*	Spliceosome complex	Poor prognosis
Welch *et al.*, 2012[18]	Cohesin complex	*RAD21*	Cohesin complex	No clear data
	Cohesin complex	*SMC1A*	Cohesin complex	No clear data
	Cohesin complex	*SMC3*	Cohesin complex	No clear data
	Cohesin complex	*STAG2*	Cohesin complex	No clear data

IR-AML: Intermediate-risk acute myeloid leukemia; *FLT3-ITD*: Fms-related tyrosine kinase 3 internal tandem duplications; *NPM1*: Nucleophosmin 1; *CEBPA*: CCAAT/enhancer-binding protein alpha; *GATA2*: GATA binding protein 2; *DNMT3A*: DNA (cytosine-5-)-methyltransferase 3 alpha; *IDH1/2*: Isocitrate dehydrogenase 1/2; *TET2*: Tet methylcytosine dioxygenase 2; *ASXL1*: Additional sex combs like 1; *MLL*: Mixed-lineage leukemia; *WT1*: Wilms tumor 1; *SF3B1*: Splicing factor 3B subunit 1; *SRSF2*: Serine/arginine-rich splicing factor 2; *U2AF1*: U2 small nuclear RNA auxiliary factor 1; *SMC1A*: Structural maintenance of chromosomes 1A; *SMC3*: Structural maintenance of chromosomes 3; *STAG2*: Stromal antigen 2; *RAD21*: RAD21 homolog (*Schizosaccharomyces pombe*).

Clonal evolution and order of mutational acquisition

AML results from the accumulation of multiple mutations and is subject to an evolutionary process. There are two major clonal evolution patterns found in AML relapse, reflecting either the evolution from the founding to the relapsing clone through acquisition of further mutations, or the expansion of a subclone of the founding clone with gain of additional mutations.[22] As described above, several patterns of overlap mutations and their interactions have been apparent to date. However, the order of acquisition and stability of genetic mutations during the disease process is not fully clarified. Defining whether a mutation is an initiating or a cooperating event has been challenging, because the preleukemic hematopoietic stem cells (HSCs) cells are clinically silent and are outcompeted by their malignant descendants.[23] In general, our knowledge of initiating mutations is limited as it only derives from studies of stable mutations throughout the disease process, from mechanistic studies on the properties of specific mutations, and from patterns of co-occurrence between mutations in residual HSCs or leukemia cells.[24,25] In general, it is thought that Class I mutations with proliferative advantage are later events that cooperate with initiating mutations to cause leukemogenesis, and the initiating mutations are thought to be Class II mutations. However, comparable analyses of mutation status in paired diagnosis and relapse samples revealed that there are not set rules or strict order of acquisition of mutations in AML. For example, *NPM1* mutations have ever been considered as initiating mutations in AML because of their mutual exclusivity with chromosomal translocations. However, in a study of 53 paired *NMP1*-mutant AML samples, *DNMT3A* mutations were persistent in five relapsed patients who lost *NPM1* mutations, with a single relapsed case in which *NPM1* mutations were maintained but *DNMT3A* was lost, implying that the mutation order is not strict.[26]

Prospective separation of residual HSCs from leukemia cells coupled with exome sequencing allow the investigation of preleukemia subclones and the identification of the order of mutations in AML.[27-29] In one study of six *de novo* CN-AML patients with *FLT3-ITD* mutations, by screening residual HSCs with tumor exome sequencing, mutations in *NPM1*, *TET2*, *DNMT3A*, and *SMC1A* were detected and thought to be early events, but not *FLT3-ITD* and *IDH1* mutations, indicating that these were likely to be late events.[25] In addition, one recent research identified *DNMT3A* mutations preceding *NPM1* mutations in

preleukemic HSCs from double *DNMT3A/NPM1* mutant AML patients, and present in stem/progenitor cells at diagnosis and remission, suggesting that *DNMT3A*, however not *NPM1*, is the leukemia-initiating mutation.[30] In addition, the mutation patterns during different clinical stages are variable. For example, *FLT3-ITD* is relatively unstable as its mutation patterns are often discordant between diagnosis and relapse.[31] This was further confirmed by one study of 137 AML patients with normal karyotype, in which 46 with *NPM1* mutations at the time of new diagnosis, and 30 patients had available *NPM1* status at the time of complete remission (CR).[32] Among the patients with mutated *NPM1* at diagnosis, 23% relapsed. *NPM1* status was available for 6 patients at the time of relapse, and 5/6 patients had mutated *NPM1* whereas 1/6 patients remained *NPM1* wild-type. In contrast, among the 91 patients with wild-type *NPM1* at diagnosis, none acquired a mutated *NPM1* clone, either at CR or at relapse.

The Prognosis of Single Mutational Analysis

As previously mentioned, although showing strong clinical heterogeneity, AML patients are currently assigned to the IR cytogenetics prognostic group. Recently, using novel technologies such as sequencing whole genomes or whole exomes, mutations in a variety of genes such as *FLT3*, *NPM1*, *CEBPA*, *IDH1*/2, and others have been described, and the evaluation of their prognostic impact has led to improvements in risk-stratification strategies. The first step in the development of a novel prognostic model was to evaluate the prognostic impact of single markers.

Fms-related tyrosine kinase 3 mutations

FLT3 is a member of the Type III receptor tyrosine kinase family, which plays an important role in proliferation, survival, and differentiation of hematopoietic progenitor cells. Mutations in *FLT3* can be divided into two categories:[4] (1) The most common alterations, *ITD* in or near the juxtamembrane domain of the *FLT3* receptor, which occurs in exons 14 and 15; (2) Point mutations resulting in single amino acid substitutions occurring within the activation loop of the tyrosine kinase domain (*TKD*), primarily involving codons 835 and 836. The most common mutation type with important clinical prognostic significance is *FLT3-ITD*, found in approximately 28–34% of CN-AML patients; this mutation has been shown to be an independent negative prognostic factor.

FLT3-ITD mutations are generally associated with significant leukocytosis and poor response to chemotherapy; although both *ITD* and *TKD* mutations can activate the *FLT3* receptor causing an uncontrolled proliferation of leukemic blasts, they result in distinct gene expression profiles as well as signal transduction patterns. A recent multi-center clinical trial on 672 CN-AML patients revealed that *FLT3-ITD* mutations are associated with decreased relapse-free survival (RFS) and overall survival (OS) compared with nonmutants in a multivariate analysis.[5] Studies investigating additional downstream signaling pathways showed that *FLT3-ITD* can induce phosphorylation of signal transducer and activator of transcription 5A on tyrosine 591 whereas *FLT3-TKD* are associated with the activation of AKT and mitogen-activated protein kinase. These findings explain the difference between the two categories of mutations in clinical outcomes and provide a theoretical basis for future targeted therapies.

Nucleophosmin 1 mutations

The *NPM1* gene is mapped to chromosome 5q35, and its product, NPM, functions as a shuttle for other proteins between the nucleus and the cytoplasm. Mutations in exon 12 of the *NPM1* gene, consisting of a 4-nucleotide sequence insertion, lead to abnormal cytoplasmic localization of the protein caused by disruption of the N-terminal nucleolar localization signal.[33]

NPM1 mutations are found in 25–35% of adult AML cases; they are particularly frequent in CN-AML (45–64%)[34] and are now regarded as an independent favorable prognostic factor. Within the group of patients with a normal karyotype, studies have shown a statistical trend toward favorable outcomes with a CR rate >90%. Becker *et al*. investigated 148 patients and showed that, compared with the nonmutated group, the *NPM1* mutation group had a higher CR rate, longer event-free survival (EFS) and OS.[8] Recently, it has been reported that *NPM1* mutations are responsible for chemosensitivity; however, the molecular mechanism remains unclear. Cilloni *et al*. reported that mutated *NPM1* is able to bind to nuclear factor-kappa B, inactivating it via. cytoplasmic sequestration, further enhancing chemosensitivity.[35]

DNA (cytosine-5-)-methyltransferase 3 alpha mutations

The *DNMT3A* gene is located on chromosome 2. This gene is involved in adding methyl groups to the cytosine residue of CpG dinucleotides, and thus plays an important role in epigenetic regulation. Consequently, mutations in the *DNMT3A* gene cause abnormal methylation, leading to tumorigenesis.[36]

The *DNMT3A* mutation rate in AML patients is 22%, and up to 33.7% in CN-AML. Mutations in *DNMT3A* are more common in M4 and M5 patients older than 60 years of age, and missense mutation in exon 23 involing R882 is the most common type.[12,37]

Studies have demonstrated that *DNMT3A* mutations are independently associated with decreased OS. Ley *et al*. first showed that the *DNMT3A* mutation group had significantly shorter OS (12.3 months vs. 41.1 months, $P < 0.001$) than the nonmutation group.[12] Neither patient age nor mutation site affected the prognosis of *DNMT3A* mutation-positive AML patients. Marcucci *et al*. reported a different prognostic effect of *DNMT3A* mutations in older (>60 years old) versus younger (<60 years old) patients, according to the affected codon; older patients with *DNMT3A* mutations in exon 23 involving R882 had an inferior outcome, whereas younger patients with *DNMT3A* mutations affecting residues other than R882 had worse prognosis.[38]

Isocitrate dehydrogenase 1 and isocitrate dehydrogenase 2 mutations

IDH genes include *IDH1* and *IDH2* and their mutations result in a substrate shift from α-ketoglutarate with accumulation of 2-hydroxyglutarate, a putative oncometabolite, consequently leading to tumorigenesis.[39] *IDH1/IDH2* mutations are heterozygous, and their combined frequency is approximately 17% in unselected AML and 27% in CN-AML cases.[13,40] All known *IDH1/IDH2* mutations involve an arginine (R), in codon 132 for *IDH1* and codon 140/172 for *IDH2*.

The prognostic significance of *IDH1*/2 mutations is controversial. Increasing evidence has shown that the prognostic impact varies according to the specific mutation. A meta-analysis of 15 studies with 8121 AML cases showed that patients with *IDH1* mutations had inferior OS compared to patients without the mutations.[41] In addition, in CN-AML patients, those with *IDH1* mutations had a lower CR rate. Abbas *et al*. reported that CN-AML patients with $IDH1^{R132}$ mutations had lower 5-year EFS and OS than those without mutations whereas $IDH2^{R140}$ mutations had no significant effect on patient survival.[13] In contrast, recent studies by the Green *et al.* showed that $IDH2^{R140}$ mutations were an independent favorable prognostic factor for RFS and OS, and that $IDH2^{R172}$ mutations predict poor outcomes.[42,43]

Tet methylcytosine dioxygenase 2 mutations

The *TET2* protein is involved in epigenetic regulation and plays an important role in DNA demethylation.[44] *TET2* mutations cause loss of protein function, leading to abnormal proliferation and differentiation of HSC, and tumorigenesis. The mutation rate for AML patients is 7–23% and often occurs in exons 3–11.[45,46]

The prognostic significance of *TET2* mutations is still controversial. Abdel-Wahab *et al.* reported *TET2* mutations were associated with reduced OS.[45] Chou *et al.* found that in IR-AML patients, the *TET2* mutation group had significantly lower OS than the nonmutation group, especially when accompanied by other poor prognostic molecular markers.[14] However, there were no significant differences in CR rate and EFS between the two groups. Similarly, no prognostic impact of *TET2* mutations on survival was found in a study of 783 young patients with primary, secondary, and therapy-related AML by the AML study group (AMLSG).[47]

Runt-related transcription factor 1 mutations

RUNX1 (also called *AML1*) is a member of the *RUNX* transcription factor family and plays an essential role in normal hematopoiesis. *RUNX1* mutations can occur in all exons, including the N-terminal RUNT domain and the C-terminal transcriptional activation domain.[48] The incidence of *RUNX1* mutations in AML cases is 6–25%; the mutations are also found at a relatively high frequency (26.3%) in CN-AML cases.[10,49]

RUNX1 mutations have been proved to be an independent poor prognostic factor. Mendler *et al*. found that the incidence of *RUNX1* mutations in older patients with CN-AML is twice as high as that of younger ones, and in both age groups *RUNX1* mutations were associated with lower CR rate, shorter EFS and OS.[10] A study of 945 AML patients by the AMLSG also showed that patients with *RUNX1* mutations had unique genetic characteristics, and showed resistance to chemotherapy as well as inferior outcomes.[49] Further subgroup analysis revealed that compared with high-dose postremission therapy, allogeneic HSC transplantation had a favorable impact on RFS in patients with *RUNX1* mutations.

CCAAT/enhancer-binding protein alpha mutations

CEBPA is an important transcription factor that can induce differentiation and inhibition of proliferation of myeloid progenitor cells. The mutations are divided into two main categories:[50] (1) N-terminal frame shift mutations that result in the occurrence of a termination codon, leading to an increased production of a nonfunctional truncated protein of 30 kDa; (2) C-terminal in-frame mutations in the bZIP domain, leading to proteins with disrupted homo- and hetero-dimerization domains and impaired DNA binding activities.

CEBPA mutations are mainly found in 10-18% CN-AML cases.[51] There are two kinds of mutation: Monoallelic *CEBPA* (*mo-CEBPA*) and *bi-CEBPA*. In the majority of *bi-CEBPA* mutations, both alleles are mutated, typically consisting of an N-terminal mutation in one allele and a C-terminal mutation in the second allele. In recent years, multiple studies have identified that only *bi-CEBPA* mutations are an independent prognostic factor for favorable outcomes and have been included in the WHO classification as a provisional entity.[9,52] One study on 598 AML patients showed that the *bi-CEBPA* mutation group had longer OS and EFS than the *mo-CEBPA* mutation and wild-type group whereas both *mo-CEBPA* mutations and wild-type *CEBPA* (*wt-CEBPA)* had no influence on prognosis.[52]

Wilms tumor 1 mutations

Wilms tumor 1 (*WT1*) gene is mapped to chromosome 11p13 and encodes a transcription factor with an N-terminal transcriptional regulatory domain and 4 C-terminal zinc finger motif. *WT1* mutations occur in approximately 10% of AML cases and are most frequent in the CN-AML subgroup.[53]

The prognostic impact of *WT1* mutations remains somewhat inconclusive. Some studies showed that *WT1* mutations were an independent risk factor for poor prognosis and were associated with a high-risk of relapse and drug resistance.[54,55] However, the largest study of 617 CN-AML patients by the German-Austrian AMLSG showed that *WT1* mutations alone had no significant impact on prognosis.[17]

SNP rs16754 locates in the mutational hot spot of *WT1* in exon 7 and has been shown to be associated with favorable outcomes in patients with CN-AML.[56] However, the exact mechanism remains unclear and requires further studies in the future.

Comprehensive Analysis of Multiple Gene Mutations

As previously mentioned, one gene often has a number of different mutation sites, and multiple mutations often coexist in a single patient. Consequently, a single gene mutation is far from sufficient in predicting clinical outcomes, revealing the need for a comprehensive analysis of combined multiple gene mutations. The traditional generation sequencing (Sanger sequencing) identifies only one gene mutation and only one mutation site. Consequently, it is unable to analyze a large number of gene mutations and mutation sites. Sanger sequencing is a complicated, labor-intensive, and time-consuming technique. High-throughput sequencing based on microarray technology has become increasingly popular; in addition, a method has been developed combining chip sequence capture and high-throughput second-generation sequencing technology, to sequence exons. This new technique can identify multiple different mutation sites of numerous genes at the same time and makes comprehensive analysis of multiple mutations possible.

In recent years, prognostic classification models have aimed at combining different biomarkers and have had great success. Compared with the European Leukemia Net (ELN) model which used only 3 molecular markers, the model proposed by Patel *et al*. relies on 18 molecular markers and allows the separation of IR cytogenetics group into 3 distinct prognostic subgroups.[6] In addition, another study by Grossmann *et al*. proposed a novel hierarchical prognostic model of AML solely based on molecular mutations.[16] All these studies further illustrate the necessity and importance of comprehensive analysis.

Comprehensive analysis of nucleophosmin 1 mutations

Further analyses in the context of other molecular aberrations have shown that *NPM1* mutations are frequently (i.e., 40%) present in patients with *FLT3* mutations, and patients with a normal karyotype with *NPM1*-mut/*FLT3-ITD*-neg have a better prognosis. Studies have also revealed that the adverse prognosis of *FLT3* mutations overcome the beneficial effect associated with *NPM1* mutations, and that a high *FLT3-ITD* mutant/wild-type ratio results in lower CR rate, irrespective of the *NPM1* status.[57]

These results indicate that testing for *NPM1* mutations should always be performed together with *FLT3* analysis. This also emphasizes the value of comprehensive molecular genetic screening.

Comprehensive analysis of DNA (cytosine-5-) -methyltransferase 3 alpha mutations

Clinically, *DNMT3A* mutations are enriched in IR cytogenetics and often occur together with *FLT3-ITD*, *NPM1*, and *IDH1*/2 mutations.[58] Consequently, the prognostic significance of *DNMT3A* mutations requires analysis in combination with other mutations.

In the largest subgroup analysis so far published, including 1770 young AML patients,[59] *DNMT3A* mutations were found to be associated with unfavorable prognosis in the ELN molecular-unfavorable subgroup of CN-AML. Hou *et al.* studied 506 AML patients and also showed that *DNMT3A* mutations displayed significant adverse prognosis only in those patients with the accompanying favorable genotypes *NPM1*-mut/*FLT3-ITD*-mut, *NPM1*-neg/*FLT3-ITD*-mut, or *NPM1*-neg/*FLT3-ITD*-neg. The results were different when the accompanying favorable genotype was *NMP1*-mut/*FLT3-ITD*-neg.[60] An additional study investigating young adults (<60 years old) showed that, when *NPM1* mutations were positive, whether associated with *FLT-ITD*-mut or *FLT3-ITD*-neg, *DNMT3A* mutations had no significant predictive value.[61]

Comprehensive analysis of isocitrate dehydrogenase 1/2 mutations

There is increasing evidence that the prognostic impact of *IDH1/2* mutations depends on the context of concurrent mutations of other genes. As previously mentioned, $IDH1^{R132}$ and $IDH2^{R140}$ mutations are associated with IR cytogenetics, in particular among CN-AML patients with *NPM1* mutations. In contrast, $IDH2^{R172}$ mutations are rarely found together with other known recurrent gene mutations. Meanwhile, both *IDH1* and *IDH2* mutations have been shown to be less frequent in AML patients with *CEBPA* mutations. In view of this, the prognostic significance of *IDH1* and *IDH2* mutations should be analyzed in combination with other mutations. However, the results of the current research conclusion have been inconsistent.

In AML patients exhibiting the genotype *NPM1*-mut/*FLT3-ITD*-neg, two reports from cooperative study groups showed a negative impact of cooperating *IDH1/2* mutations on RFS and OS.[37,62] Paschka *et al*. also revealed that the *IDH* mutation group had reduced OS compared to the wild-type group in a study of 805 cases of AML with the genotype *NPM1*-mut/*FLT3-ITD*-neg.[62] In contrast, Patel *et al*. reported a favorable impact of the genotype *NPM1*-mut/*FLT3-ITD*-neg only if cooperating *IDH1/2* mutations were present.[6]

Such opposed effects of genotypes on outcomes highlight statistical shortcomings of retrospective molecular studies.

Comprehensive analysis of tet methylcytosine dioxygenase 2 mutations

Although a recent publication by Figueroa *et al.* showed that *TET2* mutations are mutually exclusive with mutations of *IDH1/2*,[21] the association between *TET2* mutations and other genetic alterations has not been fully addressed. As mentioned previously, the prognostic significance of single *TET2* gene mutations remains controversial, and the comprehensive analysis of *TET2* mutations on prognosis is also in dispute. Although one study showed positive association of *TET2* mutations with *NPM1* mutations in AML patients achieving CR,[63] other reports did not find such correlation.[45,64] In a study of 427 patients with CN-AML, Metzeler *et al.* demonstrated that in the molecular favorable risk group harboring *bi-CEBPA* mutations and/or

NPM1-mut/*FLT3-ITD*-neg, *TET2* mutations were associated with inferior EFS and disease free survival.[64] In addition, they found that *TET2* mutations were significantly more frequent in older than younger patients. Therefore, although multivariable analysis revealed an independent impact of *TET2* mutations, age may be an important confounding factor. This was further supported by Gaidzik *et al.* focusing on a large cohort of younger adults.[47] In their study, *TET2* mutations had no prognostic impact in the whole group or in any of the subgroups, including those with genotypes *NPM1*-mut/*FLT3-ITD*-neg and *bi-CEBPA* mutations.

Therefore, the prognostic value of *TET2* mutations, both alone and in comprehensive analysis, is limited, at least in younger patients; in older patients, a further study to confirm the results from Metzeler *et al.* is needed.

Comprehensive analysis of *CEPBA* mutations

As mentioned previously, *bi-CEBPA* mutations can be an independent favorable prognostic factor. However, growing amount of studies have revealed that the favorable prognosis of *bi-CEBPA* mutations in AML was influenced by the presence of other additional gene mutations.[65,66] Green *et al.* found that, when accompanied by *FLT3-ITD* mutations, this good survival advantage would be lost.[67] Similarly, the positive prognosis associated with *bi-CEBPA* presence was reversed by the existence of *TET2* mutations; conversely, the simultaneous presence of *GATA2* mutations resulted in a significant better OS. In addition, another recent study concluded that there was additional favorable benefit for *mo-CEBPA* mutations in the presence of *NPM1* mutations: When *mo-CEBPA* mutations were accompanied by *NPM1* mutations, OS and EFS were significantly improved compared with *wt-CEBPA* accompanied by *NPM1* mutation.[68]

Therefore, the evaluation of the prognostic impact of *CEBPA* mutations, analyzed in all IR-AML patients, should consider the concomitant presence of additional mutations.

Comprehensive analysis of Wilms tumor 1 mutations

Research found that *WT1* mutations were associated with *FLT3-ITD* mutations and were not found in favorable cyogenetics.[54] As mentioned previously, the data on the prognostic significance of *WT1* mutations alone are inconsistent. Gaidzik *et al*. showed that *WT1* mutations alone had no prognostic significance, whereas they have a possible negative impact in the case of concurrent *FLT3-ITD* mutations.[17]

Conclusion and Perspectives

Cytogenetics alone has not been sufficient to predict the clinical prognosis of AML, particularly in IR-AML patients, which show considerable heterogeneity. With the application of whole-genome and exome sequencing technologies in human malignancies, the study of gene mutation has gained increased attention with great progress made, and many gene mutations have been confirmed to be associated with AML prognosis.

However, it is important to note that the current review has several limitations and that numerous challenges still lie ahead. First, the multi-gene analysis performed by NGS techniques has revealed that tumor cells harbor multiple mutated genes, and most of these are passenger mutations with only a limited number of driver mutations (causing the tumor). So far, driver mutations identified in AML patients are estimated to be 1–30%, further studies are required to identify higher number of such mutations.[69] Second, the occurrence of AML is a polygenic and multi-step process, and consequently a single gene cannot be used to explain its pathogenesis and to analyze the impact on the survival outcomes of patients with AML. Therefore, high-throughput sequencing based on microarray technology is urgently needed. Currently, studies based on the traditional generation sequencing can only identify a finite number of mutations and are therefore unable to independently predict the outcomes in AML patients, as it is not possible to consider the entire set of known mutations in parallel. Therefore, such studies cannot, capture the genome complexity of leukemia cells, in which multiple mutations have different prognostic significance compared to a single mutation. Third, although some mutations have been found to be associated with prognosis, a clear prognostic significance remains controversial as, the interaction between different mutations and their relationship with prognosis of AML patients remains unclear. In addition, the prognostic impact of gene mutations were evaluated in retrospective studies, even if based on a large cohort of AML patients; because of the low prevalence of specific mutations or the combination of multiple aberrations, the conclusions may be misleading. Finally, complete understanding of the role that mutations involved in IR-AML play in leukemogenesis and clonal evolution is required and may assist in the development of new molecular targeted therapies. The fact that preleukemic HSCs clones persist at remission also suggests that they might constitute a reservoir from which relapse arises and should be targeted for durable remission. Novel markers are needed to improve the prognosis and therapeutic response of AML patients in the future.

In conclusion, massive mutational screening by next-generation high-throughput sequencing represents a pioneering and helpful approach to prognostic risk stratification that will improve the clinical management of IR-AML patients. Further large-scale studies utilizing genomics-based approach are needed to confirm the prognostic significance of gene mutations, and a comprehensive molecular analysis would provide guidance and a theoretical basis for IR-AML prognostic stratification and clinical management.

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Conflicts of interest

There are no conflicts of interest.

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