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REVIEW

Mutational landscape of AML with normal cytogenetics: Biological and clinical implications

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ABSTRACT

Acute myeloid leukemia (AML) is a molecularly heterogeneous disease. Based on cytogenetics and FISH, AML patients are stratified into three major risk categories: favourable, intermediate and unfavourable. However, prognostic stratification and treatment decision for the intermediate risk category, that mostly comprises AML patients with normal cytogenetics (CN-AML), has been difficult due to the clinical heterogeneity and scarce knowledge of the molecular alterations underlying this large AML subgroup. During the past decade, the identification of several mutations associated with CN-AML has resulted into important advances in the AML field. In this review, we address the biological features of the main mutations associated with CN-AML and the impact of next generation sequencing studies in expanding our knowledge of the molecular landscape of CN-AML. In addition, we outline the prognostic value of mutations for risk stratification of CN-AML patients and discuss the potential of mutations discovery process for developing new molecular targeted therapies.

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1. Introduction

Acute myeloid leukemia (AML) is a molecularly heterogeneous group of malignancies. Cytogenetics and FISH have been traditionally used to stratify AML patients into three major risk-based categories: favourable, intermediate and unfavourable.[1] This prognostic categorization has an important impact in treatment decision. In general, there has been agreement that AML patients with favourable recurrent cytogenetic alterations, e.g. inv(16) and t(8,21), should be treated with conventional therapy whilst patients belonging to the poor risk group (e.g. carrying a monosomic karyotype) should undergo an allogeneic hematopoietic stem cell transplantation (HSCT). However, treatment decision for patients belonging to the intermediate risk category that mostly comprise AML with normal cytogenetics (CN-AML) has been difficult, due to the high clinical and molecular heterogeneity of this group (accounting for 40-50% of all adult AML).

More recently, the discovery of several gene mutations associated with CN-AML has resulted into three important advances in the AML field. First, an improvement in the molecular definition of "AML with recurrent genetic abnormalities" of the World Health Classification (WHO). Indeed, in 2008, this category was expanded to include, as provisional entities, *NPM1*-mutated AML and *CEBPA*-mutated AML,[2] thus allowing to categorize more than 50% of AML on the basis of the underlying genetic lesion. Second, the identification of molecularly defined subsets of patients with different prognosis within CN-AML. Third, the capability to use certain mutations that are very common and stable (i.e. *NPM1* mutations) to monitor minimal residual disease in about 60% of CN-AML. Hopefully, understanding the role that mutations underlying CN-AML play in leukemogenesis may help to develop new molecular targeted therapies.

In this review we address the biological features of the main gene mutations occurring in association with CN-AML and their impact in clinical practice.

2. Mutations with widely recognized clinical impact

2.1. FLT3 mutations

The fms-like tyrosine kinase 3 (*FLT3*) gene encodes for a protein belonging to the so- called type III receptor tyrosine kinase, that also comprises KIT and PDGFR.[3] FLT3 plays a key role in the proliferation, survival and differentiation of early hemopoietic progenitors. Internal tandem duplication (ITD) mutations of the *FLT3* gene were first identified in 1996 by Nakao et al..[4] Using reverse transcriptase-polymerase chain reaction (RT-PCR) to investigate mRNA expression of the *FLT3* gene in leukemias they found a few patients with AML showing unexpectedly longer transcripts. Further investigation of these cases demonstrated that partial sequences of the gene were tandemly duplicated.

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FLT3 mutations affect primarily two main regulatory regions of the protein: the juxtamembrane (JM) region and the activation loop of the tyrosine kinase domain (TKD).[5] In about two-third of cases ITD mutations occur in the JM region whereas in the remaining cases ITDs insert in the first tyrosine kinase (TK1) domain. The TKD domain of FLT3 is affected by point mutations, small insertions or deletions mainly involving codons 835 and 836.

FLT3-ITD mutations are detected in about 20% of unselected cases of AML and approximately 30% of CN-AML.[6] Unlike *NPM1* mutations,[7] they are not mutually exclusive of AML with recurrent cytogenetic abnormalities, being detectable in a significant percentage of cases carrying the t(15,17) and t(6,9).[8,9] TKD mutations have been found in about 10% of all AML, mostly clustering with CN-AML[10]and inv(16).[11]

Both ITD and TKD mutations lead to the constitutive activation of the FLT3 receptor thus inducing the uncontrolled proliferation of the leukemic blasts. However, they markedly differ in their gene expression profiles and also in their signal transduction properties, e.g. effects on the STAT5 pathway that may explain differences in clinical phenotypes.[12,13] *FLT3* mutations appear to play a critical role in leukemogenesis by cooperating with other mutations, especially those affecting the *NPM1* and *DNMT3A* genes.[14,15]

FLT3-ITD mutant AML is usually associated with significant leukocytosis and early relapse. There is broad consensus that *FLT3*-ITD mutations are generally associated with an inferior outcome in AML.[6] However, *FLT3*-ITD mutations are characterized by a high heterogeneity with respect to mutation load, size and localization[16,17]and these features may impact on prognosis. The mutation load is usually expressed as *FLT3* allelic ratio (*FLT3*-mutations/wild-type ratio). With a few exceptions,[18] most studies have shown that AML patients with a high *FLT3* mutant-to-wild-type ratio have a less favourable outcome that those with lower ratios.[17,19] Loss of the *FLT3*wt allele in *FLT3*-ITD mutated cases usually occurs through mitotic recombination that leads to partial uniparental disomy of chromosome 13q and it is associated with a particularly poor outcome.[20] This negative prognostic effect is likely due to the fact that the wild-type FLT3 interferes with and blocks the aberrant signalling of the ITD-mutant receptor allele.[21] It is unclear whether the site and length of *FLT3*-ITD mutation is prognostically relevant. In fact, patients carrying ITD mutations that extend to the TK1 region showed a particularly poor outcome in one study[22]but not in another.[16] The prognostic relevance of the *FLT3*-TKD mutations also remains controversial. In fact, they have been associated with no, negative or positive impact on prognosis.[23]

Treatment of AML patients harbouring *FLT3*-ITD mutations is problematic since they usually respond poorly to standard chemotherapy regimens.[6] Allogeneic HSCT may be of benefit and this procedure is recommended for this subset of patients.[24–27] However, *FLT3*-ITD positivity remains a poor prognostic factor even after allogeneic HSCT since patients are at high risk of early relapse,[28] with a 100-day cumulative risk of 45% (95% CI, 33–57).[28]

Molecular targeted therapy directed to the genetic lesion is under investigation. Several FLT3 inhibitors have been developed, including first generation (midostaurin, lestaurtinib, sunitinib, sorafenib) and second generation (quizartinib) compounds.[29] Unfortunately, results with early FLT3 inhibitors used as single agents have been disappointing since they showed only a limited clinical activity, mainly manifesting as transient reduction in the count of circulating blasts. The major limitation to the use of these compounds for the treatment of AML has been their relative lack of selectivity or potency against FLT3 and suboptimal pharmacokinetics. More encouraging results have been reported with the second generation, more selective and potent anti-FLT3 agent AC220 (quizartinib).[30] This small molecule exhibits excellent pharmacokinetics properties and has shown significant activity in a phase 1 study.[29] Other expected obstacles to the development of an effective therapy with FLT3 inhibitors include the levels of FLT3 ligand[31] and the emergence of FLT3 kinase domain mutants resistant to FLT3 inhibitors.[32] Moreover, some *FLT3*-ITD AML may not be addicted to FLT3 signaling and response of AML to FLT3 inhibitors may be conditioned by the *FLT3*-mutant allelic burden.[33]

2.2. NPM1 mutations

The *NPM1* gene encodes for a multifunctional nucleolar protein that shuttles between the nucleus and the cytoplasm and is involved in translocations and mutations in various hematological malignancies.[34] Discovery of *NPM1* mutations in AML originated from the simple observation at the microscope that bone marrow biopsies from about one-third of AML showed ectopic expression of nucleophosmin in the cytoplasm of leukemic cells.[35] This immunohistochemical finding led in turn to sequence the *NPM1* gene and to discover mutations occurring at exon-12.[35]

Subsequent studies have reported more than 50 different types of *NPM1* mutations,[14] including a unique case occurring at exon-11.[36] Notably, all these mutation variants result into common changes at the C-terminus of the native NPM1 protein, i.e. the disruption of the nucleolar localization signal and the generation of a new additional nuclear export signal motif.[37,38] These changes interfere with the normal nucleo-cytoplasmic traffic of the protein, leading to the aberrant accumulation of nucleophosmin in the cytoplasm of leukemic cells carrying *NPM1* mutations.[37,38] Cytoplasmic nucleophosmin is easily detectable by immunohistochemistry in routinely fixed paraffin-embedded samples.[39] This technique can be used as surrogate for molecular analysis[39] especially useful for the diagnosis of *NPM1*-mutated myeloid sarcomas.[40]

NPM1 mutations are the most common single gene abnormality so far identified in adult AML, accounting for about 30% of all AML and 50-60% of CN-AML.[41] Their frequency (as well as that of *FLT3*-ITD mutations) seems to decrease with age in adult CN-AML.[42] Several evidences point to *NPM1* mutations as a founder genetic alteration in AML[14] (Table 1). Unlike other mutations, those affecting the *NPM1* gene appear specific for AML,[43] and usually occur in patients with de novo disease.[44] *NPM1* mutations in AML are highly stable during the course of the disease, being detected at relapse even many years after the initial diagnosis, in patients with more than one relapse and even in relapses that occur in extramedullary sites.[14] Loss of *NPM1* mutations has been very rarely reported in *NPM1*-mutated AML but the nature of these cases remains controversial since no in-depth studies were carried out to exclude that they represented a secondary, clonally unrelated AML. As expected for a founder genetic lesion, *NPM1* mutations are mutually exclusive of other AML recurrent genetic abnormalities,[7] including double *CEBPA* mutations.[14] In addition, *NPM1*-mutated AML is associated with a distinct gene expression profile (including down-regulation of *CD34* and up-regulation of *HOX* genes)[45] and a unique microRNA signature (up-regulation of miR-10a and miR-10b).[46] *NPM1* mutations appear dominant over other secondary AML features, such as chromosomal abnormalities[47] or multilineage dysplasia,[48] that are present in about 15% and 23% of *NPM1*-mutated cases, respectively. Finally, CD34+ leukemic stem cells from *NPM1*-mutated AML samples carry *NPM1* mutations[49] and *NPM1* mutations has been detected in a compartment of pre-leukemic hemopoietic stem cells.[50] Because of these unique features, *NPM1*-mutated AML has been included as provisional entity in the current WHO classification of myeloid neoplasms.[2]

The role played by the *NPM1* mutations in AML development is still partially understood. The normal NPM1 protein is involved in a variety of functions, including ribosome biogenesis, control of centrosome duplication and stabilization of ARF protein in the nucleoli.[51] More recent findings suggest that NPM1 may also play a role in regulating transcription[52] and apoptosis.[53] Whether mutations contribute to AML by interfering with one or more of these functions remains to be established. However, it is clear that the leukemogenic effect of NPM1 mutants is strictly dependent on the perturbation of the traffic of nucleophosmin.[38] In fact, all mutations act finalistically to get the

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