Introduction

Acute myeloid leukemia (AML) is a neoplastic disease of the hematopoietic stem cell defined by impaired differentiation capacity, clonal expansion and accumulation of immature cells with suppression of polyclonal residual hematopoiesis. The resulting peripheral cytopenias accounts for the most frequent clinical symptoms at diagnosis (fatigue, dyspnea, fever and infections, hemorrhage) and the leukemic cells “tropism” for tissues outside blood and bone marrow justifies the other symptoms characterizing AML clinical course. Since the first attempt of classification, proposed in 1976 by the French-American-British (FAB) Group, the diagnosis is based on the morphological identification and quantification of “blast cells” in bone marrow smears.

However, over the past 40 years, it has become evident that the initial FAB criteria and their subsequent modifications are far to capture the real clinical and biological variability of the disease and, even less, to predict prognosis. The identification of recurrent cytogenetic abnormalities not only provided the first hint in understanding leukemia biology but soon became one of the most powerful tool to define AML risk, driving clinician decision-making (1-6). Unfortunately, despite the improvement of cytogenetic techniques, the conventional G-banding karyotyping fails to identify structural and/or numerical chromosomal alterations in about 45% of patients at diagnosis. These patients are arbitrarily included in the intermediate risk group, even if outcomes are largely heterogeneous and the optimal therapeutic approach is not clear. Advances
in molecular genetics, initially focused on explaining the clinical heterogeneity of patients with cytogenetically normal (CN) AML, led to the identification of various somatic mutations, often with prognostic impact, that have eventually been included in the recently revised WHO and European LeukemiaNet (ELN) classification of myeloid neoplasms (4,7). Moreover, the diffusion of the parallel sequencing diagnostic platforms, permitted the evaluation of mutational profiling in the whole-exome or in the whole-genome, identifying driving and co-occurring mutations in more than 95% of patients, thus paving the way to a genome-based AML grouping, each with distinct clinical phenotype and outcome (8,9).

Herein we aimed to overview the most relevant molecular markers in AML with a focus on their prognostic significance and on their potential use to develop new therapies with molecular targets.

Molecular mutations resulting in chromosomal rearrangements

PML-RARA

The fusion protein PML-RARA results from the balanced chromosomal translocation t(15;17)(q24;q21) and is the hallmark of acute promyelocytic leukemia (APL). The PML-RARA rearrangement is present in >98% of APL cases and defines a diagnosis of APL regardless of blast percentage (De Braekeleer et al., Exp Rev Hematol 2014). The fusion protein releases the cell from the transcriptional control of retinoic acid (RA), thus repressing the transcription of genes involved in physiological myeloid differentiation. This pathological block can be overcome by greatly increasing the level of RA; so, the addition of all-trans retinoic acid (ATRA) to conventional chemotherapy or, more recently, arsenic trioxide has transformed APL from one of the most heinous to the most curable acute leukemia (Kayser et al., Leukemia 2018).

RUNX1-RUNX1T1 (formerly AML1-ETO) and CBFB-MYH11

AML carrying one of the two reciprocal chromosomal translocations t(8;21)(q22;q22) and inv(16)(p13q22)/t(16;16) (p13;q22), resulting in the gene rearrangements RUNX1-RUNX1T1 (previously known as AML1-ETO) and CBFB-MYH11 respectively, are collectively defined as core binding factor (CBF) leukemias (Sohl et al., Am J Hematol 2014). These abnormalities involve two distinct subunits of the CBF transcriptional protein, that is pivotal for normal hematopoiesis. RUNX1, formerly known as acute myeloid leukemia 1 (AML1), gene encodes for the CBFA2 subunit, that regulates DNA binding with CBFB. Both subunits are necessary for the correct functioning of the transcriptional complex, and translocations involving either of them result in a reduced transcription of different targets such as GM-CSF, MPO and IL3 and epigenetic silencing of others. Though the two CBF AML are generally considered together, there is a significant heterogeneity, mainly in the molecular signature, within this group of patients, resulting in different outcome (Sinha et al., Semin Hematol 2015).

Mixed lineage leukemia (MLL)

MLL gene, located on chromosome 11q23, is expressed in hematopoietic cells and encodes for a histone methyltransferase involved in epigenetic regulation of transcription (Muntean AG & Hess JL, Annu Rev Pathol 2012). MLL is involved in translocations with over a hundred partner genes, that has been described in adult and pediatric cases of AML, acute lymphoblastic leukemia (ALL) and myelodysplastic syndromes (MDS) (Meyer C et al., Leukemia 2013); overall, an MLL translocation is found in about 10% of acute leukemias. The fusion with partner genes transforms MLL in a leukemogenic effector by deregulating transcriptional control. Despite the wide number of partner genes, MLL translocations are generally associated with adverse outcomes.

Somatic mutations in AML

Nucleophosmin (NPM1)

Nucleophosmin is a highly conserved, ubiquitous nucleolar phosphoprotein with a molecular weight of 37 kDa, coded by a gene containing 12 exons on chromosome 5q35. By continuously shuttling between cellular compartments, NPM1 act as a chaperon protein, involved in many processes critical for cellular homeostasis, such as ribosome biogenesis and transport, apoptotic response to stress stimuli, maintenance of genomic stability by control of cellular ploidy, DNA repair, chromatin condensation/decondensation (10). NPM1 knockout mice show abnormal organogenesis and lethal defects in primitive hematopoiesis. Heterozygous NPM1 knockout mice develop a myelodysplastic-like syndrome and have increased...
susceptibility of development of myeloid and lymphoid malignancies (11), supporting the hypothesis of a direct role of NPM1 in tumorigenesis which would act both as proto-oncogene and tumor suppressor gene (12). In solid tumors NPM1 function is increased through protein overexpression and its increase level has been associated with tumor stage or disease progression through increased Myc-dependent hyperproliferation (13-19). In hematologic disease NPM1 function is mostly perturbed by mutations in the C-terminal region. Haplo-insufficiency of the gene results in the reduction of the protein level in the nucleolus with a loss of function phenotype, and, through dimerization, in the cytoplasmic mis-localization of wild type and mutated protein. The cytoplasmic sequestration of the protein (c+NPM1), is associated with a gain of function phenotype due to its chaperone interaction with a number of tumor suppressor molecules, ultimately leading to the inhibition of p53 activity (20). NPM1 mutations are found in about 50–60% of cases with normal cytogenetics and in 30% of all cases, making them the most frequent genetic lesions in AML (21). Since the first description by Falini and colleagues in 2005 (22), the recognition of biological importance of NPM1 mutations as founder genetic lesions in leukemogenesis and the clinical impact of NPM1 mutations led to inclusion of NPM1 mutated AML as a distinct leukemic entity in the 2016 WHO classification. NPM1 mutations are usually restricted to exon 12 and are heterozygous with a retained wild type allele (23). To date, more than 50 molecular variants have been identified (24), named alphabetically from A to F in order to their discovery. The type A mutation, consisting of a “TCTG tetranucleotide tandem duplication” is the most common, accounting for 75-80% of cases. Mutations from B to D (15%) carry tetranucleotide insertions, the other rare mutations have variable size insertions. Despite their diversity, NPM1 gene variants produce all the same biological effect, i.e., they alter the tryptophan residues crucial for nucleolar localization and create of a new nuclear export motif at the C-terminus with consequent cytoplasmic accumulation of the aberrant protein (25). NPM1 mutations are highly stable in disease history (26-28) and the very rare loss of NPM1 mutations seems to be associated with the acquisition of new cytogenetic abnormalities (29).

NPM1 mutations are more frequent in “de novo “leukemia and usually detectable in all leukemic cells. They precede associated mutations and are mutually exclusive with other recurrent genetic abnormalities. Moreover, leukemic cells have a distinct gene, microRNA and methylation expression signature (30-32). Disease onset is characterized by hypercellular bone marrow and multilineage involvement, high peripheral white blood cell/blast cell counts and high incidence of gingival and lymph node invasion. c+NPM1 blast cells did not have specific morphology, but cytotype is more often M4 or M5 and immunophenotype is mostly CD34 negative with strong CD33 expression (33-35). NPM1 mutations have been associated with good response to induction therapy (22) and, in CN-AML, NPM1 mutations seem to overcome the negative prognostic impact of multilineage dysplasia (36). In a meta-analysis of nine studies including 4,509 subjects, Liu and colleagues confirmed the favorable effect of NPM1 mutations on complete remission (CR) rates, disease free survival (DFS) and overall survival (OS) (37). However, many studies have demonstrated that positive prognostic impact of NPM1 mutations is largely dependent from the absence of concomitant Flt3-ITD mutation (35,38-40) or from Flt3-ITD with low allelic burden (41-43), so that this category has been added to favorable risk group in the ELN 2017 recommendations (4). Less defined is the prognostic role of the different mutation sub-groups. Koh and colleagues, in a small cohort of 18 patients observed a trend to better outcome in patients with group A mutations (44); Pastore and colleagues did not found prognostic differences among mutations subgroups (45). Alpermann and colleagues demonstrated a negative impact on event free survival (EFS) by type A mutations and found an additional negative effect in presence of concurrent mutations, such as Flt3-ITD and DNMT3a (46).

With this background, qualitative detection of different type NPM1 mutation is mandatory at AML diagnosis. Moreover, since the stability of mutations, peripheral blood quantitative PCR has been demonstrated a highly sensitive method for minimal residual disease (MRD) monitoring and personalized patient management (47,48).

The high frequency of NPM1 mutation and the better knowledge of wt- and c+NPM structure and functions have increased the efforts to identification NPM1 targeted drugs. Different target strategies are, at present, under investigation.

**Interference with NPM1 oligomerization**

NCS34884 is a small molecule inhibitor able to disrupt the hydrophobic region required for NPM1 oligomerization inducing the apoptosis of leukemic cells (49). At low doses NCS34884 seems to have higher sensitivity for haploinsufficient NPM1-mutated compared to NPM1-
wt leukemic cells and ATRA and doxorubicin show a synergistic effect (50,51).

**Targeting nucleolar assembly**

_CIGB-300_ is a synthetic peptide able to bind NPM1 preventing its phosphorylation by casein kinase-2. Inhibition of CK-2 mediated NPM1 activation results in damage to the nucleolar architecture and to apoptosis induction (52).

**Selective inhibition of nuclear export**

_Selinexor_ (KPT-330) is an oral selective inhibitor of exportin 1 (XPO1), protein involved in cytoplasmic NPM1-mutated mis-localization. Exportin 1 inhibition by selinexor increases apoptosis and induces myeloid differentiation through p53 and CEPBA upregulation (53,54). The major limitation of selinexor is its lack of specificity for leukemic cells and its side effects. A second generation of XPO1 inhibitor, KPT-8602 has been recently demonstrated higher activity and reduced toxicity in preclinical models (55) and is under investigation in a phase 1/2 trial (NCT02649790).

**Nucleus translocation of c-NPM1**

_Oridonin_ is a natural product isolated from Rabdosia rubescens commonly used in a Chinese traditional medicine for its anti-carcinogenesis and anti-inflammatory properties. In vitro leukemia models have demonstrated its ability to revert the cytoplasmic mis-localization of mutated and wt NPM1. As a consequence, the stabilization of p14arf and p53 increases apoptosis (56). _Avrainvillamide_ is a small molecule inhibitor isolated from a marine fungal strain of Aspergillus sp. able to bind the C-terminal DNA binding domain of c-NPM1, and to re-localize the mutated protein in the nucleoli, with a mechanism currently unknown (57).

**Selective C+NPM1 destruction**

_All-trans retinoic acid_ (ATRA) and _arsenic trioxide_ (ATO), currently used for the treatment of acute promyelocytic leukemia, have been recently demonstrated high activity also in NPM1 mutated acute leukemia. In cell lines, ATO seem induce apoptosis especially in those carrying type A mutation and ATRA have a synergistic effect. They selectively trigger c-NPM1 degradation, probably for its lower resistance to oxidative stress induced by ATO, while the levels of NPM1-wt remain unchanged (58).

**Proteasomal c-NPM1 degradation**

_EAPB0503_ is an analog of imiquimod, known to have very high activity on melanoma cells. It has recently proposed as target drug in NPM1 mutated acute leukemia for its ability to promote c-NPM1 degradation and to correct the wtNPM1 mis-localization, stabilizing p53 and inhibiting leukemia cell growth (59).

**FLT3**

FLT3 is a membrane-bound receptor with tyrosine kinase activity, involved in regulation of proliferation and differentiation of early hematopoietic progenitors. It belongs to the class III tyrosine kinase (TK) group and shows a high sequence and structural homology with platelet-derived growth factor receptor, c-kit and c-fms. The molecule contains an extra cellular immunoglobulin-like domain binding the cytokine Fms-like TK 3 ligand (FL), a transmembrane domain, and an intracellular domain that consist of a juxtamembrane dimerization segment, a TK domain interrupted by a short kinase insert and a C-terminal tail. Upon interaction with its ligand, the unphosphorylated monomeric receptor undergoes dimerization and the activation of various downstream effectors ultimately regulates the hematopoietic homeostasis. In hematopoietic malignancies overexpression of wild type protein was commonly found and it was hypothesized that very high level of FLT3-wt may promote constitutive activation of the receptor in malignant cells and may negatively affect prognosis (60,61). However, mutations of FLT3 are among the most common genetic abnormalities observed in AML, affecting either the juxtamembrane or the TK domain of the receptor.

**FLT3-internal tandem duplication (FLT3-ITD)**

Mutations in the juxtamembrane region consist of the “head to tail” duplication of small sequences, variable from 3 to more than 400 base pairs, resulting in the transcription and translation of a receptor with an elongated juxtamembrane domain. The consequence is the disruption of a regulatory segment preventing the adoption of active configuration in absence of ligand binding, leading to cytokine-independent phosphorylation, constitutive dimerization of the receptor and increased cellular proliferation (62-64). In addition to intracellular pathways activated by FLT3-wt, such as Ras/MEK/Erk and Akt/PI3K, the mutated receptor promotes STAT5 phosphorylation. So, different STAT5 target genes, not expressed upon physiological FL binding, are involved. They includes the activation of gene regulating cell-cycle, accounting for the proliferative advantage of FLT3-ITD.
mutated cells, but also the suppression of gene coding for myeloid differentiation transcription factors, such as PU.1 and CEBPa (65-67) and an increased production of reactive oxygen species and DNA breaks favoring genomic instability (68). Moreover, FLT3-ITD harboring cells acquire survival advantage through the suppression of the pro-apoptotic regulator transcription factor FOXO3a (69). Many studies in transgenic mouse models demonstrated that FLT3-ITD promotes the development of a myeloproliferative disease with splenomegaly, expansion of myeloid compartment, decrease of B cell compartment and exhaustion of HSC as a result of the their increased cell-cycle entry (70-73).

In adult AML, FLT3-ITD occurs in 15–20% of all patients and in 28–35% of those with normal cytogenetics (74) and is strongly associated with poor prognosis. Adult FLT3-ITD AML has lower CR rate, higher relapse risk and worse survival (43,75-80).

FLT3-ITD seems to affect outcome also after stem cell transplantation, a procedure that, due to the relevant relapse risk and dismal OS attained with standard chemotherapy, is generally recommended in FLT3-ITD positive patients in first CR (81).

In elderly patients the incidence and the prognostic role of FLT3-ITD is less defined. In a study of Southwest Oncology Group (SWOG) including 140 AML patients aged >55 years the incidence of FLT3-ITD was similar to that of younger patients, and was associated to disease resistance but did not impact on OS (82). Similar results were found by Daver and colleagues in 388 elderly patients, even if in this series FLT3-ITD was detected only in 12%. The authors explained the lack of prognostic power of FLT3-ITD with the presence, in these elderly patients, of many other negative prognostic factors that may overcome the power of FLT3 mutations (83). Conversely, subgroup analysis from other studies demonstrated an association between FLT3-ITD and poor prognosis in elderly AML (84,85).

There is increasing evidence that, besides the mere presence of ITD, outcome is influenced by FLT3-ITD allelic variation (i.e., mutant to wt allelic ratio) (75,77,86-88). Schlenk and colleagues reported that only patients with a high allelic ratio (≥0.51) took advantage from allogeneic stem cell transplantation (42). According with these observations, ELN 2017 recommendations have included FLT3-ITD with low allelic burden and concomitant NPM1 mutation in the favorable risk group (4). Variability of allelic ratio may be in part explained by loss of heterozygosity (LOH) of wild type allele, involving the entire 13q region telomeric to FLT3, in part by the co-existence in the same disease of leukemic sub-clones with different FLT3 mutation status (89).

**Mutations in the TK domain (FLT3-TKD)**

FLT3-TKD mutations are missense point mutations in exon 20 of TK domain, occurring in about 5–10% of AML patients; in contrast to age-dependent increase of FLT3-ITD, the prevalence of point mutations is stable through all ages. The most common is a nucleotide substitution at codon 835 causing the change of an aspartic acid to tyrosine (D835Y). However, other point mutations, deletions, and insertion within or in the surrounding codons have been reported (75,90,91). As a consequence, the TK domain remains in an “open conformation” permitting the binding of ATP, and the ligand independent receptor phosphorylation. Moreover, despite both FLT3-ITD and FLT3-TKD mutations promote constitutive activation of the receptor, they differ in downstream activation pathways and in induced genetic programs (92-94). TKD mutations do not activate STAT5, and this fact not only impacts the lineage phenotype of the disease, but in mouse model results in a less aggressive malignancy (95-98). However, the prognostic relevance of FLT3-TKD is still controversial (86,99-101) and, in the era of molecular therapies FLT3-TKD mutations may represent a potential target for specific direct inhibition.

Based on the exiting success of tyrosine kinase inhibitors in chronic myeloid leukemia, over the past 10 years, many small molecules targeting FLT3-TK by competing for the ATP binding site have been developed and entered clinical trials. As monotherapy, they only gave a transient clearance of peripheral blood and bone marrow leukemic cells. Moreover, their initial efficacy was often lost due to the emergence of secondary mutations or the activation of alternative intracellular activation pathways. The actual advantage in combination with chemotherapy is still under investigation and for an exhaustive review of the current ongoing clinical trials we refer to the manuscript by Assi and Ravandi (102).

FLT3 inhibitors are commonly classified by their potency and by the specificity of inhibition. The first generation of inhibitors includes non-selective molecules, such as sunitinib, sorafenib, lestaurtinib and midostaurin, able to affect many other intracellular pathways (such as PDGFR, VEGFR, Kit, JAK2). Among them, sunitinib, approved for the treatment of renal and hepatocellular carcinomas, and
lestaurtinib have not been further investigated after early trials' results because of significant toxicity and low efficacy in AML, both as single agent or in combination with chemotherapy (103-106). Sorafenib has been employed in many phase I/II trials, demonstrating acceptable toxicity and better efficacy, also in combination with chemotherapy or hypomethylating agents, and in elderly patients (107-111). Midostaurin is, to date, the only FLT3 inhibitor approved in the US and Europe for the treatment of adult, newly diagnosed, FLT3 mutated AMLs in combination with standard chemotherapy. In a population of patients aged less than 60 years, positive for an FLT3 mutation, the registration trial (Ratify, NCT00651261) clearly demonstrated that the addition of midostaurin significantly prolonged OS (74.7 vs. 25.6 months in patients receiving placebo; P=0.009). Moreover, midostaurin provided an increase in EFS (8.3 vs. 3.0 months, P=0.002) and its benefit was confirmed in all three FLT3 subgroup (FLT3-TKD, FLT3-ITD low and FLT3-high allelic ratio) (112). Many other trials aimed to test the activity of midostaurin in prevent relapse after standard chemotherapy or SCT, or in elderly patients in combination with hypomethylating agents are ongoing (102).

The second generation of FLT3 inhibitors includes molecules with high potency and specificity against FLT3. Quizartinib demonstrated, in vitro, a 10-fold lower activity on other receptor TKs and a very high potency against FLT3 and, in vivo, a good tolerability profile (113). A phase III randomized trial exploring the efficacy of standard chemotherapy plus quizartinib or placebo in untreated FLT3-ITD mutated patients is ongoing (Quantum-First, NCT02668653). Crenolanib shows activity not only against ITD but also TKD mutations. Various trials evaluating crenolanib activity in combination with conventional chemotherapy or hypomethylating agents or as maintenance therapy after SCT are currently active (102). As crenolanib, gilteritinib have high potency and selectivity against FLT3-ITD and TKD mutations. Moreover, for its activity against AXL kinase, it appears a molecule potentially able to escape resistance (114).

To counteract the rapid emergence of resistance remains the major challenge in the use of FLT3 inhibitors. Despite the low prognostic significance of TKD mutations at diagnosis, the acquisition of additional point mutation under inhibitor pressure can induce resistance to many compounds of the same class. However, it is becoming evident that a major role in resistance is played by the activation of alternative intracellular pathways, so new strategies preventing the development of resistance or sensitizing leukemic cells to inhibitors are needed.

**CEPBA**

CCAAT enhancer binding protein alpha is a transcription factor coded on an intronless gene on chromosome 9q13.1, involved in differentiation of many tissues (115) and, in hematopoietic system, in driving early myeloid precursors to granulocyte or monocyte maturation (116,117). Moreover, recent data demonstrated its role in proliferation control and self-renewal capacity of hematopoietic stem cells (116). In CEPBA deficient mice models, neutrophil maturation reaches only the myeloblast stage, resembling the clinical feature of leukemia patients. In AML, CEPBA mutations occur in 5–10% of “de novo” cases, with a higher incidence in CN-AML and in those with 9q deletion (21). Compared to other subtypes, CEPBA-mutated AML tends to have lower platelets counts, higher hemoglobin levels, higher peripheral blast percentages and rarer extra medullary involvement. Though lacking specific morphologic characteristics, CEPBA+ cases show frequent aberrant surface expression of CD7 (118). In acute leukemia two major CEPBA mutations have been described: at the N-terminal region, resulting in a nonfunctional truncated protein due to a premature termination of the synthesis, or at the C-terminal region, in which in-frame deletions or insertions impair its DNA binding and dimerization ability. About two-thirds of AML patients display two mutations (biallelic or double mutations), while the remaining third carry single allele mutation (119). Only biallelic mutation is associated with favorable prognosis, retained despite the presence of associated multilineage dysplasia (120-122). Patients with a single mutation show contradicting outcome (123). Furthermore, the positive effect of CEPBA double mutation on survival is confirmed also in patients who acquired mutation at relapse (124). Data emerging from gene expression profiling confirmed the distinct gene expression signature associated with biallelic CEPBA mutations, so this AML subgroup has been recognized as a distinct diagnostic entity by the 2016 WHO classification of myeloid neoplasms (7). In mouse models CEPBA-induced leukemogenesis demonstrated that N-terminal and C-terminal mutations have different impact on stem cell homeostasis. The maximum leukemogenic effect was obtained when a C-terminal mutation present in premalignant stem cells was combined with an N-terminal mutation. The first induced the expansion of stem cell
compartment, the second maintained the myeloid lineage commitment, as observed in double mutated patients (125).

**Runt-related transcription factor (RUNX1)**

*RUNX1* gene located on chromosome 21q22.12 and encodes for a transcription factor interacting with many cofactors and enhancers, thus playing an essential role in embryogenesis and hematopoiesis. The homozygous lack of *RUNX1* is lethal, with mid-gestation death of embryos due to hemorrhagic necrosis of central nervous system and block of definitive hematopoiesis (126). The disruption of *RUNX1* in adult models results in an increase of hematopoietic progenitors, defective megakaryocytic maturation and defective lymphocytic development. However, the functional changes induced by *RUNX1* loss result in overt leukemia only after acquisition of additional mutations (127). Besides the involvement of *RUNX1* in many recurrent chromosomal translocations, in AML also intragenic recurrent mutations in the functional domain and in the DNA binding domain of the protein have been found (128). *RUNX1* mutations occur in 5–18% of adult AML, are mutually exclusive with recurrent genetic abnormalities included in the WHO classification or with complex karyotype but are often associated with trisomy of chromosome 13 and with deletion of chromosome 7. Patients with *RUNX1*-mutated AML are mostly older male, with secondary disease arising from an antecedent myelodysplastic syndrome, minimally differentiated (M0) cytology. *RUNX1* mutations are associated with resistance to induction chemotherapy in about 30% of patients and with inferior EFS, DFS and OS (129,130). To date, allogeneic SCT appears the best option for increasing survival rate in patients harboring *RUNX1* mutations (129).

**KIT**

The stem cell factor receptor (*c-kit*, CD117), officially known as “KIT proto-oncogene receptor tyrosine kinase” is a 145 kDa protein coded on chromosome 4(q12) by a single copy gene of twenty-one exons (131). As FLT3, CSF-1R, PDGFRβ and PDGFRα, it belongs to type III receptor TK family. In the hematopoietic system, KIT is highly expressed in about 70% of CD34 positive cell, including lineage-restricted progenitor cells and immature cells capable of *in vitro* long-term hematopoiesis. Moreover, CD117 is expressed also on megakaryocytes. C-kit is downregulated in all lineages during maturation and, in mature cells, it is detectable only in mast-cells, in CD56+ natural killer cells and in activated platelets (132-136). SCF binding induces KIT dimerization, phosphorylation and activation of many downstream pathways, such as PI3K, MEK, RAS and RAF, involved in regulation of survival, proliferation and migration of hematopoietic cells.

In adult AML KIT mutations are found in 5% of patients, but higher frequencies are reported in the CBF subgroup (16–46%) (137,138). Most of them are point mutations in exon 17 (such as D816V) or in exon 8, with a gain of function leading to increased receptor activation upon SCF binding (139). However, other mutation on codon 816 or in different exons, insertion and deletion in exon 8, or internal tandem duplication of the juxtamembrane domain (exon 11), have been less commonly reported. The prognostic impact of KIT mutation is controversial. Many authors reported an increased relapse risk and reduced survival (140-142), while others did not find significant differences (143,144). In a meta-analysis including 2,933 patients with CBF AML, Chen and colleagues confirmed a negative effect of KIT mutations on relapse risk, but not on CR rate and OS; however, in the subgroup with t(8;21), a negative effect also on OS was observed (145). Besides, screening for KIT mutations can be useful for the possibility of targeting by TKIs, even though the results of a small phase II study of BCR/ABL1 inhibitor dasatinib as maintenance in CBF AML at high relapse risk suggest that efficacy of single agent dasatinib may be affected by the emergency of KIT-negative sub-clones resulting from spontaneous or dasatinib-driven clonal evolution (146).

**RAS**

RAS proto-oncogenes (NRAS, KRAS) encode for a membrane G protein of 21kDa associated with plasma and internal membranes of the cell through galectin 1 and galectin 3 anchor proteins. The engagement of receptors by growth factors determine a conformational change of RAS, followed by GTP hydrolysis and consequent activation of many downstream effectors involved in cell-cycle progression and proliferation, block of apoptosis and survival advantage, increased cell motility vesicles budding and transport (147).

In AML, activating NRAS mutations are described in 8–13% of adults, while KRAS mutations are less common and found only in 2% of patients; mutations occur mostly in codons 12, 13 or 61. NRAS mutations are more frequent
in cases with t(3;5) and KRAS mutations in patients with inv(16) and younger than 60 years, but their prognostic impact remain still controversial. Despite small-size studies have hypothesized an adverse impact on outcome (148-150), the evaluation in larger number of patients did not confirm their negative effect (151,152). Since RAS activity depends on post-translational farnesylation, from the early 2000s many studies tried to target the RAS pathway with farnesyl-transferase inhibitor tipifarnib, with disappointing results (153-157).

**Tumor protein p53 (TP53)**

The TP53 gene codes for a DNA binding protein acting as a tumor suppressor. In response to cellular stress it promotes cell cycle arrest, apoptosis and DNA repair (158). Mutations of TP53 are more common in solid than in hematologic malignancies (159) and in AML they occur in 8 to 14% of all cases (8). A significant higher frequency is found in patients with complex karyotypes, where the incidence ranges from 69% to 73% (160). Moreover, in those patients TP53 mutations correlate with the total number of chromosomal abnormalities and with monosomal karyotype (161). Mutations are mostly single nucleotide changes and type of mutations and mutational allelic burden are similar in “de novo” and therapy-related AMLs (162), despite the frequency of mutations is higher in therapy-related disease. In general, TP53 alterations are associated with poorer prognosis, higher relapse rate and inferior EFS and OS. The negative prognostic significance of TP53 mutations appears independent from age, cytogenetics and all other co-occurring molecular alterations (162-164).

**Wilms tumor 1 (WT1)**

WT1 is a gene of 10 exomes located at chromosome 11p13, encoding for a transcription factor involved in cell growth and metabolism by modulating the expression of membrane receptors and growth factors, components of extracellular matrix, and genes affecting cell survival (165). In normal hematopoiesis WT1 expression is confined to the CD34 positive population, and acts as a suppressor gene regulating progenitor cell growth and maturation (166,167). In AML WT1 is overexpressed in most patients, and increased expression is associated with resistance to chemotherapy, high relapse rate and poor survival (168). However, the role of WT1 overexpression in leukemogenesis is not completely understood. Studies in murine models suggest that its pathogenic role may be context-specific and may depend from the temporal acquisition of co-occurring mutational events. Recurrent somatic loss-of-function mutations have been described in 6–13% of AML patients. These include deletions, insertion or base substitutions in exons 1, 7 and 9, resulting in the expression of a protein lacking the zinc-finger domain. As for gene overexpression, little is known about the leukemogenic role of mutated WT1. It has been recently hypothesized a role as epigenetic modifier, even if the pattern of genes deregulated by mutations remains under investigation (169). In adult AML WT1 mutations is associated with reduced survival and high relapse rate (170,171). The suggested perturbation of epigenome by WT1 mutations may open new therapeutic options employing epigenetic-targeted therapies.

**Plant Homeodomain Finger 6 (PHF6)**

The PHF6 gene consists of 11 exons coded on chromosome X, acting as a suppressor gene involved in neurogenesis and hematopoiesis. Recurrent mutations have been identified in 3–8% of AML patients and have been associated with adverse prognosis, especially in intermediate risk group (172). Van Vlierberghe and colleagues reported a higher frequency in males and in less mature subtypes (FAB M0-M2) and frequent association with other cooperative mutations (FAB M0-M2) and frequent association with other cooperative mutations (173).

**DNA methyltransferase 3a (DNMT3A) mutations**

The DNA methyltransferase family catalyze the formation of 5-methylcitosine by adding a methyl group to cytosine in CpG dinucleotides. The increased methylation of CpG islands results in transcriptional silencing of downstream genes (174). DNAMT3A mutations occur in 12–22% of adult AML, with higher frequency in patients with normal cytogenetics (175). DNAMT3A mutations are associated with older age and higher WBC count compared to the wild type counterpart. They result in the production of a truncated protein, with reduced methyltransferase activity through a missense mutation causing in most of cases an arginine to histidine substitution at codon R882. Even if the precise mechanism by which they contribute to leukemogenesis is not completely understood, it is well known that DNAMT3 mutations are an early event in leukemic transformation, may be present in pre-leukemic stem cells and can persist also after CR achievement (176,177). Regarding their clinical role, two
recent meta-analysis including more than 10,000 patients confirmed a poor prognostic impact on OS and RFS in de novo adult patients (178,179). Nonetheless, improved outcome has been reported in intermediate cytogenetics patients receiving induction chemotherapy with high dose anthracyclines (180), suggesting their potential role as therapeutic marker. On the other hand, patients harboring DNMT3A mutation show high response rates and superior OS by employing the DNA methyltransferase inhibitor decitabine (181,182) suggesting their usefulness in the therapeutic decision-making. Clinical trials evaluating the efficacy of DNA methyltransferase inhibitors decitabine and guadecitabine are ongoing (183-185).

Isocitrate dehydrogenase mutations (IDH1/2)

*IDH1* and *IDH2* genes code for two enzymes of the acid citric cycle, both catalyzing the oxidative decarboxylation of isocitrate to alpha-ketoglutarate (α-KG), to produce NADPH either in peroxisomes (IDH1) or in mitochondria (IDH2). NADPH has key functions in detoxification processes and α-KG affects the activity of many dioxygenases involved in different cellular processes and in epigenetic control of gene expression (186). Somatic mutations of *IDH1* and *IDH2* genes are among the most common mutations recurring in AML, with a prevalence of 4–9% and 8–19%, respectively; they are particularly frequent in CN-AML patients (172,187), often occur with trisomy 8, while co-occurrence of both mutation is very rare (188). IDH mutations detected at diagnosis tend to be stable during disease progression and, in general, patients do not acquire mutations during follow up (189). However, differences in detection limits or expansion of the mutant clone could uncover, at relapse, only apparently new mutations, complicating their use as a marker of minimal residual disease. All IDH1/2 mutations are heterozygous and consist in single amino-acid substitution at codon 132 in exon 4 of the *IDH1* gene and codons 140 or 172 in exon 4 of *IDH2*. The mutated IDH (mIDH) leads to the formation and accumulation of the R enantiomer of 2-hydroxyglutarate (R-2-HG) that acts as an “oncometabolite” by inducing a dysregulation of epigenetic methylation processes and inhibition of α-KG enzymes, including the ten-eleven-translation (TET) family. The consequent “hypermethylation phenotype” causes the transcriptional silencing of genes crucial for differentiation of hematopoietic progenitors, ultimately inducing a maturation arrest as well as an increase of progenitor cell numbers with high proliferative potential (190). Besides, hematopoietic cells harboring IDH mutations show a dramatic decrease of ataxia-telangiectasia mutated (ATM) protein, that represent the first trigger to recruit repair factors in response to DNA damage. The impaired DNA repair promotes the acquisition of additional mutations and clonal expansion of IDH mutated stem cells and favors the evasion of p53-mediated tumor suppression upon oncogenic stress. From a clinical point of view, patients carrying IDH mutations tend to be older and to have higher blast cell count and platelets count at diagnosis (191). To date, the prognostic impact of IDH1/2 mutations in AML remains unclear (192). A meta-analysis including 8121 patients concluded that those with mIDH1 have inferior OS, and in the subgroup with normal cytogenetics mIDH1 seemed to confer resistance to induction therapy resulting in a lower CR rate (193). Likewise, studies on prognostic significance of mIDH2 are inconsistent regarding all outcome variables. The different results among studies may depend from study methodology (i.e., many of them combine IDH1/IDH2 for the analysis) as well as from mutational context. However, regardless of prognostic role, considering their frequency and the oncogenic function, mIDH1/2 represents an excellent target for therapeutic purposes. Many selective mIDH inhibitors are in in preclinical development and many other in various stages of clinical investigation (194). They are able to bind the catalytic site of mIDH hindering the conformational changes causing R-2-HG production by reduction of α-KG (193) or binding a remote site which inactivate mIDH1 (196). Results of the phase I trial on the IDH2 inhibitor, AG221 (enasidenib), in relapsed/refractory patients demonstrated a mild, mostly gastrointestinal, toxicity. Six percent of patients developed an IDH-associated differentiation syndrome resembling that observed in acute promyelocytic leukemia during treatment with retinoic acid. Clinical efficacy was good with an overall response rate of 38.5%. CR was achieved in of 20.2% of treated patients (197) irrespective to the arginine mutation (R140 or R172). Amatangelo and colleagues, analyzing samples from patients included in that trial demonstrated that, in responsive patients enasidenib reduced intracellular R-2-HG and restored differentiation generating fully functional neutrophils (198). On the basis of these promising results, in August 2017 FDA approved the use of enasidenib for IDH2 mutated relapsed/refractory AMLs (199). A phase 3 trial evaluating enasidenib vs. conventional care in relapsed/refractory patients ≥60 years and a phase I/II trial assessing safety and tolerability of enasidenib in association
with standard induction therapy are still ongoing (200). The recently reported results of phase I trial on the mIDH1 inhibitor ivosidenib (AG120), demonstrated similar efficacy and safety profile. Overall response rate was 41.6% and complete remission or complete remission with partial hematological recovery was obtained in 21.6%, with a median time to CR of 2.8 months. If appropriately managed, ivosidenib-associated adverse events (i.e., QT interval prolongation, differentiation syndrome and leukocytosis) did not require permanent discontinuation of the drug (201). Trails assessing safety, efficacy and tolerability of ivosidenib in combination with standard chemotherapy or with azacytidine in untreated patients harboring IDH1 mutation are currently recruiting (200).

**Ten eleven translocation family member 2 (TET2) mutations**

TET2 protein, coded on chromosome 4q24, converts 5-methyl- to 5-hydorxymethylcitostosine with α-ketoglutarate as cofactor, playing a role in epigenetic regulation of cellular processes.

TET2 mutations have been found in 8–28% of AML cases, mostly in the exons 3–12, and are associated with reduction of the catalytic function or with impaired DNA targeting (202). The “hypermethylation status” resulting from TET2 mutations overlaps that observed in IDH1/2 mutated AML, suggesting the involvement of a common leukemogenic pathway. However, TET2 mutations are mutually exclusive with IDH mutations, supporting the hypothesis of a distinct leukemogenic mechanism (190). In humans TET2 inactivation is present in pre-leukemic stem cells and is associated with clonal expansion (203). Experimental models have demonstrated that the TET2 mutations-induced hypermethylation especially affects enhancer regions regulating tumor suppressor genes so facilitating leukemogenesis (204). The prognostic impact of TET2 mutations is still under debate. Some studies failed to find an association between TET2 mutation and outcome (205,206), while others demonstrated an inferior EFS in presence of TET2 mutations, either considering the whole population or in specific subgroups (such as CN-AML, age less than 65 years and ELN favorable risk) (172,207-209). Liu and colleagues performed a meta-analysis including 395 AML patients with TET2 mutations concluding that it could be considered an adverse prognostic factor in patients with normal karyotype (210).

**Cohesin complex mutations**

Cohesin complex consists of four protein subunits (SMC1A, SMC3, RAD21, STAG1/STAG2) involved in sister chromatid cohesion (211). By holding chromatin strands within the ring-like structure resulting from the four components assembly, it maintains the polarity of sister chromatids during mitosis. Besides, cohesin complex is involved gene expression regulation and in DNA repair (212). Mutations in the different subunits have been described in adult and in children with AML, resulting in a loss of function impairing chromatin accessibility. In vitro studies seem to suggest that cohesion mutation enforce stem cell programs by increasing serial “replating” ability and stemness-associated gene expression in more immature populations but may not be leukemogenic. The pre-leukemic phenotype would require a second hit to drive transformation, proposing cohesin mutations as potential targets for new therapies designed to change leukemic evolution. Despite the improved knowledge in cohesion function, their clinical impact on outcome is still unclear (213-215). Recently, Tsai and colleagues have first reported an association between cohesin mutations and superior OS and DFS in a series of 391 de novo adult AMLs (216).

**Chromatin remodeling genes mutations**

**Additional sex comb like 1 (ASXL1) mutations**

The gene encodes for a chromatin binding protein acting as activator or repressor of transcription in localized areas, leading to DNA and or histone modifications (217). Mutations occur in 3–5% of AML patients, but the frequency is higher in intermediate risk (11–17%), in patients with age ≥60 years and in secondary AML (218-220). In adults ASXL1 mutations have been associated with poor prognosis in intermediate risk AML (218) and in elderly patients with reduced CR and shorter survival (221).

**BCL6 corepressor (BCOR) mutations**

BCOR gene is located on chromosome X and the BCOR protein acts as transcriptional repressor of BCL6, interacting with histone deacetylases (222). Its loss of function impairs proliferation and differentiation of myeloid cells (223). Mutations are detected in about 4% of adult AML, and in 17% of those with normal cytogenetics, with a negative prognostic role (224).

**Lysine (K) methyltransferase 2A (KMT2A) mutations**

Previously termed mixed lineage leukemia (MLL) gene,
located at chromosome 11q23 and encodes for a protein with histone methyltransferase activity involved in regulation of gene expression through histone modification. Partial tandem duplication of KMT2A gene, occurring between exons 2 and 8, has been reported in 4–14% of AML cases and is associated with an inferior outcome especially in CN-AML (164,225).

Enhancer of Zeste Homologue 2 (EZH2) mutations

EZH2 is a histone methyltransferase involved in balancing between cell differentiation and renewal in hematopoietic progenitors (226). Mutations have been described in about 2% of adult AML, are more frequent in megakaryoblastic leukemia and in leukemia associated with Down syndrome and was correlated with inferior survival (227,228).

Spliceosomal machinery mutations

The spliceosome complex consists of five small nuclear ribonucleoproteins (snRNPs) and of their associated protein factors responsible for the removal of noncoding regions from pre-messenger RNAs (229). Alternative splicing is important in regulation of hematopoiesis and the different isoforms co-occur at different maturity stages of progenitor cells. An imbalance in the splicing machinery resulting in the prevalence of one splicing variant can activate alternative maturation programs and contribute to development of leukemia. Recurrent mutations of splicing factors involving SF3B1, U2AF1, SRSF2, and ZRSR2 are common in MDS and are, in general, associated with better clinical outcome. On the contrary, their frequency is low in de novo AML (4% for SF3B1, 4.9% for SRSF2, 6.5% for U2AF1, and <1% for ZRSR2), are associated with older age and male gender and predict lower CR rates, shorter DFS and OS (230).

Conclusions

Over the last two decades the combination of conventional cytogenetics, PCR-based techniques and Sanger sequencing have already changed the diagnostic and prognostic approach to AML. In the last 5 years the more extensive use of next generation sequencing (NGS) has further refined the molecular landscape of AML, allowing the identification of recurrent mutations in most of patients, also within previously defined subgroups, yielding to an updating of AML classification. The wider use of NGS has also provided new insights in understanding leukemogenesis, by identifying the leukemia-initiating mutations and analyzing clonal evolution at relapse. On the other hand, the huge data outputs within few years has led clinicians to face the dilemma of how to translate this diagnostic information into clinical care, integrating the results of emerging technologies with more traditional laboratory features, and how to reduce the complexity of generated data into groups sharing cellular pathways potentially targetable by new drugs.

Efforts should be focused on the development of standardized diagnostic algorithms, able to identify the molecular markers permitting alternative therapeutic approaches and at the same time useful for minimal residual disease monitoring. In our opinion, only this way will eventually translate the massive amount of new biological data in significant advance in survival for patients with AML that, sadly, to date remains a dreadful disease.

Acknowledgements

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

References

5. Grimwade D, Walker H, Harrison G, et al. The predictive value of hierarchical cytogenetic classification in older...


122. Bereshchenko O, Mancini E, Moore S, et al. Hematopoietic stem cell expansion precedes the


221. Alpermann T, Haferlach C, Eder C, et al. AML with gain of chromosome 8 as the sole chromosomal abnormality (+8sole) is associated with a specific molecular mutation pattern including ASXL1 mutations in 46.8% of the patients. Leuk Res 2015;39:265-72.


doi: 10.21037/jlpm.2018.09.08

Cite this article as: Damiani D, Tiribelli M. Molecular landscape in adult acute myeloid leukemia: where we are where we going? J Lab Precis Med 2019;4:17.