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## Gene Mutations in Acute Myeloid Leukemia — Incidence, Prognostic Influence, and Association with Other Molecular Markers

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Olga Blau

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### Abstract

Acute myeloid leukemia (AML) is a clonal disorder affecting pluripotent stem cells and is characterized by ineffective hematopoiesis. Most AML patients harbor cytogenetic and molecular defects that identify entities with peculiar biologic and clinical data and distinct therapeutic responses. Approximately 50%–60% of de novo AML and 80%–95% of secondary AML patients display chromosomal aberrations. Structural chromosomal rearrangements are the most common cytogenetic abnormalities in de novo AML, with an incidence of 40%. Last years, large collaborative studies have demonstrated the importance of cytogenetic aberrations for the prognosis of AML patients.

The large group of patients with cytogenetically normal (CN) AML refers to the intermediate risk category. It is known that this group of patients is very heterogeneous with respect to prognosis. The recent large-scale sequencing of AML genomes is now providing opportunities for patient stratification and personalized approaches to treatments that are based on individual mutation profiles. Genes recurrently mutated in AML belong to distinct functional groups or pathways. A few recurring gene mutations with prognostic relevance in AML have been identified and have become incorporated into current prognostication models. For patients with CN AML, prognosis can be specified by mutational status of the genes *NPM1*, *FLT3*, and *CEBPA*. CN AML patients with *NPM1* mutation, but no *FLT3*-ITD, or with *CEBPA* mutation, have a favorable prognosis. In contrast, CN AML patients with *FLT3*-ITD mutation have a poor prognosis.

Recently a new class of mutations affecting genes for DNA methylation and post-translational histone modification was identified in AML. These mutations frequently occur in the DNA nucleotide methyltransferase 3A gene (*DNMT3A*) and isocitrate dehydrogenase 1/2 gene (*IDH1/2*). Different studies have shown a negative impact of *DNMT3A* mutations on outcomes in patients with AML. The prognostic effect is known to depend on certain biological factors as well as a combination of cytogenetics and other mutations such as those in *FLT3* and *NPM1*. In contrast, the impact of *IDH1/2* mutations on prognosis is not completely understood. It appears that prognosis may depend on specific patient populations and a combination with *NPM1* mutations.

Moreover, a growing number of recurrent mutations in additional genes have recently been identified. Increasing evidence suggests that AML develops throughout the process of branching evolution.

**Keywords:** AML, mutations, prognosis, *FLT3*, *NPM1*, *DNMT3A*, *IDH1/2*

## 1. Introduction

Acute myeloid leukemia (AML) is the most common type of leukemia among hematologic malignancies in adults. In the last years, progresses in molecular technologies have led to identify AML as a highly heterogeneous disorder. AML is a clonal hematopoietic disease that arises from multiple acquired genetic lesions accumulating in hematopoietic progenitors. The mutations give rise to a malignant clone [1]. In the initial development, Knudson's two-hit hypothesis has provided important insights into the pathogenesis of leukemia. Later studies using mouse models have confirmed that genetic abnormalities in leukemia could be divided into two classes. Class I mutations confer a proliferative or survival advantage to blast cells, while class II mutations block myeloid differentiation and give self-renewability [2-4]. Recently, next-generation sequencing methods provided more complete insight in oncogenic events. Early mutations may be present many years before disease develops [5]. Evidence from many murine models has confirmed that early mutations lead to clonal expansions by progenitor cells. Later, cooperating mutations would arise in cells that already contain initiating early mutations [6].

Karyotype analysis allows detecting genetic changes on a chromosomal level by visual assessment of chromosomal banding. Recurrent chromosomal abnormalities are found in about 55% of adults with AML. Some, not all, of the chromosomal aberrations are strong independent predictors of outcome and are the mainstay of the World Health Organization (WHO) classification of AML risk groups [7]. In AML patients with cytogenetically normal karyotype (CN AML) who have an intermediate-risk cytogenetics, clinical outcomes vary greatly. Identification of recurrent mutations in AML improved the understanding of the molecular pathogenesis. Later studies revealed recurrent genetic markers in more than 85% of CN AML patients [8]. Some of the mutations add important prognostic information and

also indicate potential therapeutic targets. The more detailed insight into the genetic architecture of AML is challenging the established classification and prognostication systems [8]. Particular mutations have already been included in the latest WHO classification that was established in 2008 and in subsequent recommendations for diagnosis of AML by an international expert panel [9].

## 2. Nucleophosmin 1 (*NPM1*) mutations

The nucleophosmin/nucleoplasmin (NPM) family of chaperones has diverse functions in the cell. The *NPM1* gene maps to chromosome 5q35 and encodes a phosphoprotein that moves between the nucleus and the cytoplasm. The gene product involves a number of cellular processes such as chromatin remodeling, genome stability, ribosome biogenesis, DNA duplication, and transcriptional regulation. *NPM1* also interacts with a number of proteins at the mitotic spindle and in the nucleolus and includes in regulation of the ARF/p53 pathway [10, 11]. *NPM1* is clearly having both growth promoting and tumor suppressive functions [11, 12].

In AML, there are some chromosomal translocations involving *NPM1* gene. These genetic alterations usually disturb the cellular transport of *NPM1* [13]. In AML carrying the t(3;5) (q25;q35) translocation, leukemic cells display fusion protein *NPM1-MLF1* (myelodysplasia/myeloid leukemia factor 1) and show aberrant *NPM1* expression in cytoplasm [14]. In rare cases of acute promyelocytic leukemia carrying the translocation t(5;17), the *NPM1-RAR $\alpha$*  fusion protein was detected [15]. The transforming role of partner genes in these cases is well established [13]. Nevertheless, *NPM1* moiety seems to be not only provides a dimerization substrate for the C-terminal onco-protein. *In vivo* studies using mouse model have shown that *NPM1* is a haploinsufficient tumor suppressor gene [16]. Therefore, loss of *NPM1* could also contribute to the pathogenesis of AML [17, 18].

Mutations in the *NPM1* gene represent one of the most common gene mutations in AML [19]. Approximately 25%–30% of AML patients and about 60%–85% of CN AML patients display *NPM1* mutation [13, 20]. *NPM1* mutations are heterogeneous; more than 50 different variants of mutations are identified. A more common variant of mutations is the insertion of four nucleotides at position 288–290 at exon 12. Mutations type A with insertion of “TCTG” at position 288 is the most frequent aberration (75%–80% of cases) [14, 19, 21]. In most cases (about 95%), three mutations types (A, B, and D) are found [13]. *NPM1* mutations result in common changes at the C-terminus end of the *NPM1* protein, that is, changes of tryptophans and insertion of a new nuclear export signal motif. These changes cause aberrant cytoplasmic accumulation of *NPM1* mutants, thus preventing or decreasing *NPM1* binding to the nucleolus. Aberrant *NPM1* expression in cytoplasm is easily detectable by immunohistochemistry [21, 22]. *NPM1* mutations often combined with other AML-associated mutations, especially with *FLT3* (fms-related tyrosine kinase 3), *DNMT3A* (DNA (cytosine-5-methyltransferase 3 alpha), *IDH1* and *IDH2* (isocitrate dehydrogenase 1 and 2 (NADP+)), *NRAS* (neuroblastoma RAS viral (v-ras) oncogene homolog), and others. Most likely, these mutations do not accu-

mutate in a random order but instead could be allocated to early and late events in the transformation process [5, 23].

The prognostic status of *NPM1* depends on the presence of other concurrent genetic alterations. In the absence of *FLT3*-internal tandem duplication (ITD) mutations, *NPM1* mutations are associated with improved outcome for CN AML patients, even in those older than 60 years [9]. Current European Leukaemia Net (ELN) recommendations for diagnosis and treatment of AML determines CN AML with *NPM1* mutation without *FLT3* mutation as a favorable risk and does not recommend allogeneic stem cell transplantation (alloSCT) in first complete remission (CR) [9]. Recently, a beneficial prognostic effect of *NPM1* mutations was reported in AML patients with simultaneous mutations in *IDH1* or *IDH2* [8], whereas a worse prognosis of CN AML without *FLT3*-ITD but with mutations in *IDH1* or *IDH2* has also been described [24]. *NPM1* mutations inform treatment decisions also in elderly patients because it identifies those who might benefit from intensive chemotherapy.

*NPM1* mutation provides a sensitive marker for minimal residual disease (MRD) detection by qPCR. Because of their stability in the course of disease and relative homogeneity of mutation pattern, *NPM1* represent a useful target for MRD monitoring, in particular in CN AML. The applicability of an RNA- or DNA-based q-PCR assay for *NPM1* mutation monitoring has been shown by several groups [25-28]. Many authors have shown that *NPM1* mutation as MRD marker is a relevant factor for the identification of patients at high and low risk of relapse [28].

### 3. *FLT3* mutations in AML

The *FLT3* gene in chromosome band 13q12 encodes a protein known as fms-like tyrosine kinase 3, which belongs to the family of receptor tyrosine kinases (RTK). RTKs transmit signals from the cell surface into the cell through a signal transduction. RTK3 family members are characterized by an extracellular domain comprised of 5 immunoglobulin-like domains and by a cytoplasmic domain with a split tyrosine kinase motif [29, 30]. The *FLT3* protein is located in the membrane of certain cell types where *FLT3* ligand binds it. *FLT3* is highly expressed in CD34+ hematopoietic progenitor cells and variable express in the more mature monocytic lineage. *FLT3* expression has been described in lymphohematopoietic organs such as the liver, spleen, thymus, and placenta [30, 31].

The binding with ligand activates the *FLT3* protein, which subsequently activates a series of proteins inside the cell that are part of multiple signaling pathways and leads to receptor oligomerization and transphosphorylation of specific tyrosine residues, which activates the downstream signaling pathways including STAT5, RAS/mitogen-activated protein kinase, and phosphatidylinositol 3-kinase/AKT. The signaling pathways stimulated by the *FLT3* protein control many important cellular processes such as the growth, proliferation, and survival of cells, particularly of hematopoietic progenitor cells [32, 33].

The *FLT3* receptor consists of an extracellular domain composed of a transmembrane region, a cytoplasmic juxtamembrane domain (JMD), and 2 cytoplasmic tyrosine kinase domains



(TKD; TKD1 and TKD2) interrupted by a short kinase insert. The JMD can be subdivided into 3 distinct parts: the binding motif, which is implicated in activation and in stabilizing the inactive kinase conformation; the switch motif, which consists of 2 phosphorylation sites and contains the STAT5-binding motif; and the linker/zipper peptide segment, which can undergo large amplitude rotations by pivoting about its attachment point [34].

Two predominant types of *FLT3*-activating mutations have been described in association with AML. The first involves ITD mutations that are found in about 25% of adults and 15% of pediatric AML cases [9, 35-37]. The *FLT3* mutations lead to constitutive activation by auto-phosphorylation of the *FLT3* RTK that could activate multiple signaling pathways and lead to cell proliferation [38, 39]. ITDs are located in exons 14 and 15 of the *FLT3* gene and show a broad variation in the position of insertion site, as well as in the number and sizes of the duplicated fragments. The length of the duplicated JMD region varies from 3 to 400 nucleotides but, despite this heterogeneity, the resultant transcripts are always in-frame [40]. These mutations are mostly located in the JMD. Localization outside the JMD is present in about 25% of the cases [41-43].

*FLT3*-ITD mutations have a significantly adverse impact on prognosis due to a high relapse rate, which translates into an inferior overall survival (OS) [23, 36, 44-46]. The effect on prognosis is modulated by the mutated to wild-type allele ratio, with inferior outcome in the presence of a higher load of ITDs in *FLT3*. The high *FLT3*-ITD/*FLT3*-WT ratio predicts for low CR rate and OS [42-44]. Localization outside the JMD was associated with inferior outcome [42]. Many groups studied the role of alloSCT to overcome the negative impact of *FLT3*-ITD in AML patients. Some data suggest that *FLT3*-ITD positivity also outweighs other conventional prognostic markers in predicting relapse [47]. Recently published data from the German-Austrian AML Study Group showed that the high allelic ratio is a predictive factor for the beneficial effect of alloSCT [43].

The second type of *FLT3* mutation is point mutations, which most frequently occur in the activation loop of the TKD. *FLT3*-TKD mutations occur in 10% of both adult and pediatric AML patients [48]. These mutations also lead to constitutive tyrosine kinase activation. The most common TKD mutation occurs at codon 835, converting aspartic acid to tyrosine (D835Y). Also seen are mutations D835V, D835E, and D835H, converting aspartic acid to valine, glutamic acid, and histidine at residue 835, respectively. The rare mutations convert glycine to glutamic acid at residue 831 (G831E) and arginine to glutamine at residue 834 (R834Q), as well as the deletion of isoleucine at residue 836 [36, 45, 48]. TKD mutations differ from ITD in *FLT3* in their biologically transforming potency. Prognostic impact of *FLT3*-TKD mutations remains controversial [49, 50].

Specific gene expression signatures have been reported for CN AML with both *FLT3*-ITD and *FLT3*-TKD1 mutations. The *FLT3*-ITD signature predicts a less favorable outcome analogous to the *FLT3*-ITD mutation. High expression of wild-type *FLT3* also seems to adversely affect prognosis [51].

Sequencing studies show that *FLT3* mutations frequently occur together with mutations and alterations of other genes, especially *DNMT3A* (13.3%), *NPM1* (6.8%), Wilms tumor 1 (*WT1*,

5%), runt-related transcription factor 1 (*RUNX1*, 3.5%), mixed-lineage leukemia (*MLL*, 2.5%), CCAAT/enhancer binding protein alpha (*C/EBPα*, 1.5%), and core-binding factor (1.5%) [8, 52].

In addition, *FLT3*-ITD mutation status is different approximately in 30% of AML patients at the time of diagnosis and at relapse. *FLT3*-ITD mutations may be present in only a subset of leukemic blasts, consistent with a role in disease progression. This data suggest that *FLT3*-ITD may contribute as the initial transforming event in relapse of AML and it can reflect the selection and outgrowth of a mutant clone or evolution of a new clone harboring this mutation [53, 54].

Recent studies also show that both the *FLT3* mutations, as well as the collaborating mutations, can have prognostic significance. Recently published data submit that *FLT3*-ITD retains its negative prognostic impact in intermediate-risk AML, even in the context of other genetic abnormalities, such as *NPM1*, *DNMT3A*, and *TET2* [8, 55].

The prevalence and prognostic implications of *FLT3* mutations make them a promising therapeutic target in AML. A number of tyrosine kinase inhibitors (TKI) against *FLT3* are currently in clinical trials, with varying degrees of clinical responses, but even those patients who respond develop resistance to monotherapy [56]. One of the mechanisms of acquired resistance to several *FLT3* TKIs is the selection for *FLT3*-TKD mutations documented in relapsed patients [57]. Moreover, some data reported that *FLT3*-TKD AML blasts do not confer increased sensitivity to tyrosine kinase inhibition [58].

#### 4. CCAAT/Enhancer-Binding Protein α (*C/EBPα*) mutations

The *C/EBPα* gene is localized on chromosome 19q13.1. This gene is intronless and it encodes a transcription factor that contains two transactivation domains: a dimerization leucine zipper region and a DNA-binding domain. It recognizes the CCAAT motif in the promoters of target genes [59]. Activity of this protein can modulate the expression of genes involved in cell cycle regulation. *C/EBPα* directly interacts with cyclin-dependent kinase 2 and 4 and arrests cell proliferation by blocking the association of these kinase with cyclins [60]. *C/EBPα* is involved in lineage specification as a transcription factor, it is crucial for the development of myeloid progenitors to the neutrophils. It is exclusively expressed in myelomonocytic cells. *C/EBPα* is specifically upregulated during granulocytic differentiation, and conditional expression of *C/EBPα* alone is sufficient to trigger neutrophil differentiation in bipotential precursors. In addition, *C/EBPα* is capable of arrest cell proliferation [61, 62]. *C/EBPα* regulates the expression of many myeloid genes, including genes encoding growth factor receptors (granulocyte-, macrophage-, and granulocyte-macrophage colony-stimulating factor) and the secondary granule proteins [59, 61]. Numerous studies suggest that *C/EBPα* is a general inhibitor of cell proliferation and a tumor suppressor [63, 64].

When *C/EBPα* gene is altered by mutations in AML, DNA-binding is altered or eliminated. *C/EBPα* mutation was first described by Pabst and colleagues in 2001 [65]. These mutations are detected in 10%–18% of CN AML patients and are predominantly found in M1 and M2

morphological subtypes of AML [65, 66]. Clinically, *C/EBPα* mutations are associated with lower leukocyte counts and lactate dehydrogenase levels and with aberrant expression of T-cell surface markers such as CD7 at presentation [67].

*C/EBPα* mutations can occur across the whole coding region with two main spots frequently involved, one of them affecting the N-terminus, another affecting the C-terminus. Mutations in the amino terminus truncate the full-length protein. N-terminal mutations are nonsense mutations leading to exert dominant-negative effects on the unmutated *C/EBPα* protein. As the mutant proteins block the binding of wild-type *C/EBPα* with DNA, occurs transactivation of granulocytes target genes and block of differentiation of myeloid progenitor cells. N-terminal *C/EBPα* mutations allow the development of committed myeloid progenitors, which represent templates for leukemia-initiating cells. C-terminal mutations are usually located between the basic region and the leucine zipper coding sequence resulting in disturbed DNA binding by the mutant protein as well as altered dimerization with its partner proteins [66]. C-terminus mutations increase the proliferation of premalignant stem cells and block myeloid lineage differentiation when homozygous. The majority of all mutations are homozygous mutations. Combination of both mutations is associated with accelerated disease development [59, 60, 66, 68]. The mechanism of *C/EBPα*-mutant leukemogenesis has been demonstrated in studies of *C/EBPα* knockout mice [69].

There is evidence that *C/EBPα* mutations are early events in the generation of leukemic clones. In contrast to *FLT3* mutation, in *C/EBPα* mutations, the majority of relapsed patients display the same mutations in both *C/EBPα* alleles [69]. Schin et al. demonstrated that 91% of *de novo* AML harboring *C/EBPα* mutations at diagnosis retained the identical mutant patterns but frequently changed in the allelic distribution at relapse [70].

Three different *C/EBPα* mutant patterns have been reported in AML patients. One half of patients carry single mutation on one allele (*C/EBPα*-sm), and these patients express wild type of *C/EBPα*. Second half of patients have double-mutated *C/EBPα* (*C/EBPα*-dm). In these cases, no wild-type *C/EBPα* protein is expressed. Some of *C/EBPα* mutated patients harbor bi-allelic mutations with an N-terminal frame-shift mutation on one allele and a C-terminal in-frame mutation on the other allele [71, 72]. Third variant of aberration is a homozygous *C/EBPα* mutation due to loss of heterozygosity, also no wild-type *C/EBPα* protein is expressed [73].

Expression profiling revealed that *C/EBPα* mutant cases cluster together, suggesting that they share similar gene expression signatures. Moreover, C-terminal *C/EBPα*-sm patients may be less distinct from *C/EBPα*-dm cases than N-terminal *C/EBPα*-sm patients [68, 71, 74]. Recent study suggest that homozygous *C/EBPα* mutations have a similar gene expression signature as *C/EBPα*-dm and thus may be considered as equivalent [75].

Most patients with *C/EBPα* mutations had a normal karyotype. Importantly, *C/EBPα* mutations have not been observed in patients with a favorable karyotype [76]. The association of deletion 9q and *C/EBPα* loss-of-function mutations could suggest that loss of a critical segment of 9q and disruption of *C/EBPα* function possibly cooperate in the pathogenesis of del(9q) AML [77]. Concurrent mutations are significantly less frequent in *C/EBPα*-dm compared with *C/EBPα*-sm AML. It is correct for *FLT3*-ITD and in particular for *NPM1*, which are essentially not



present among *C/EBPα*-dm cases [68, 78, 79]. Recently, the mutation in transcription factor *GATA2* was found to have a strong association with *C/EBPα*-dm mutation [75].

The prognostic impact of *C/EBPα* mutations seems to be favorable. The most significant effect of mutation on clinical outcome is its association with better relapse-free survival or OS [80-84]. Recent data show that a favorable outcome is limited to double, not to single, *C/EBPα* mutations [71, 78]. These data suggest that only the *C/EBPα*-dm AML should be definitely designated as AML with the favorable risk of molecular abnormalities [84]. These results have important implications for the application of risk-stratified therapy and require confirmation. Given the evidence of the prognostic value of *C/EBPα* mutations, analysis of a possible interaction between *FLT3*-ITD and *C/EBPα* mutations is of particular interest. There are contradictory studies whether coexisting *FLT3*-ITD adversely affects the favorable prognosis of *C/EBPα* mutations. Some studies showed significantly worsened prognostic outcome in patients with *FLT3*-ITD and *C/EBPα* mutations [82, 85]. In contrast, in other study negative prognostic influence among patients with *C/EBPα* mutations were not found [81]. Obviously, further studies of numerous concurrent mutations analysis are necessary to determine the relationship between these molecular markers.

## 5. *RUNX1* mutations

The runt-related transcription factor 1 (*RUNX1*) gene is located on chromosome 21q22 and it consists of 10 exons. *RUNX* family proteins were found to have an essential role in the regulation of gene expression by temporal transcriptional repression and epigenetic silencing via chromatin alterations, especially in the context of chromosomal translocations. The protein encoded by *RUNX1* gene represents the alpha subunit of the core binding factor (CBF) and is found to be involved in the development of normal hematopoiesis. CBF is a heterodimeric transcription factor that binds to the core element of many enhancers and promoters [86]. *RUNX1* protein consists of runt homology domain, transcription activation domain, and repression domain. The runt homology domain is a highly conserved protein motif, it is responsible for both DNA binding and heterodimerization with the beta-subunit of CBF. The transcription activation domain is responsible for the interaction with a transcription coactivator of *RUNX1* [87]. The *RUNX1* gene is part of the t(8;21) fusion gene in CBF AML and is also affected by recurrent gene mutations in AML. The *RUNX1* gene is one of the most frequently deregulated genes in leukemia.

The reported incidence of *RUNX1* mutation in AML varied from 3% to 46% depending on the patient population selected, the regions of *RUNX1* screened, and the methods used [88-90]. The role of *RUNX1* mutation in the leukemogenesis of AML remains to be defined. *RUNX1* mutation, a class II mutation, has been implicated as the initiating event to block differentiation of hematopoietic cells, and the subsequent class I gene mutation would synergistically provide growth advantages of these cells and lead to the development of AML [88]. Most of *RUNX1*-mutated patients concurrently had other gene mutations and the majority simultaneously showed class I mutations, most commonly *FLT3*/ITD, *FLT3*/TKD, and *N-RAS*, which might

result in hyperactivation of the receptor tyrosine kinase-RAS signaling pathways [88]. Interestingly, Tang et al. reported high coincidence of *RUNX1* mutations with *MLL/PTD*, and both *FLT3/ITD* and *FLT3/TKD* mutations [88].

*RUNX1* mutations in AML are associated with poor outcomes, which contrast with the favorable prognostic effect of gene fusions involving *RUNX1* [88, 90]. Differential prognostic value of chromosomal damage and mutation in *RUNX1* consequences the importance of a complete assessment of genetic factors in the pathogenesis of AML.

*RUNX1* mutations are less frequent in cytogenetic high-risk AML and rarely occur in CBF-AML and APL. Among intermediate-risk AML, *RUNX1* mutations are mostly associated with normal karyotype, with trisomy 8, and with trisomy 13 [90, 91]. With regard to the correlation with other molecular markers, in some studies, a higher frequency of coincidence of *FLT3* mutation and *RUNX1* mutations was reported [88, 92]. But data from Dicker et al. did not confirm that [89, 91]. Significant correlation of *RUNX1* mutations with *MLL-PTD* and *IDH* mutations and an inverse correlation with *NPM1* and *CEBPA* mutations was observed in a large cohort of AML patients [90]. Recently, rare coexistence of *RUNX1* and *NPM1* mutations in *de novo* intermediate risk karyotype AML was reported [93, 94]. In the first study, it was found that *RUNX1* mutations in these cases were structurally unusual when compared to *RUNX1* mutations observed in *NPM1* wild-type cases and located outside the *RUNX1* homology domain and were also present in the germline. However, later study did not confirm structurally unusual *RUNX1* mutations in *NPM1* mutated cases. These data could suggest that *FLT3*, *RUNX1*, *MLL-PTD*, and *IDH* mutations contribute to leukemogenesis by other mechanisms than do *NPM1* and *C/EBP $\alpha$*  mutations.

## 6. RAS mutations

The *RAS* oncogene family was the first human oncogene discovered in human cancer and has been extensively studied over the last 3 decades. *RAS* gene is named for “rat sarcoma.” The *RAS* gene family is comprised of three homologues, *HRAS* (11p15.5), *KRAS* (12p12.1), and *NRAS* (1p13.2). The members of the *RAS* family are tyrosine kinase receptors that are important participants of many signaling pathways connected with functional control of a large variety of cellular effects including cell cycle progression, growth, migration, cytoskeletal changes, apoptosis, and senescence. The crosstalk between these multiple signaling pathways and others controlled by different sets of signaling molecules creates molecular networks whose balance is crucial to determine the final outcome of cellular responses in the cell [95, 96]. *RAS* proteins function as a conduit for signals received from RTK on the cell surface through downstream cell signaling partners to nuclear transcription factors regulating cell growth and cell-cycling proteins [97]. Under physiologic conditions, *RAS* activation is initiated by binding with ligand that induces RTK autophosphorylation, dimerization, and activation [98].

Mutations in *RAS* genes are frequent in AML and exemplify mutation Class I, initiating key downstream hyperproliferative signal transduction pathways. *NRAS* mutations are the most common. *NRAS* and *KRAS* mutations are present in about 25% and 15% of AML patients [52].

Constitutive activation of RAS originates from mutations in *RAS* itself or from mutation or overexpression of related RTK such as *FLT3* or *KIT*. Activated *NRAS* signals get through the several pathways to mediate oncogenic effects, especially the MAPK, PI3K–AKT, and Ral–GDS pathways [97, 98]. In contrast to other gene mutations frequently involved in AML, *NRAS* mutations are present much more often in patients with myelodysplastic syndromes (MDS) and secondary AML (sAML) arising from MDS. An analysis of samples from MDS patients and sAML identified only a modest increase in the frequency of *NRAS* mutations in the sAML cohort compared with the MDS group, suggesting that *NRAS* mutations may be an early event in MDS [89].

Despite being initially described almost 30 years ago, the prognostic implications of *RAS* mutations remain controversial. Several studies indicate that *RAS* mutation did not impact prognosis in CN AML patients [99, 100]. In contrast, *RAS* mutations have been linked to an inferior outcome in AML by some researchers [101]. In childhood AML, activating *NRAS* mutations commonly in cooperation with *NPM1* mutations occur frequently in the favorable risk population [102].

No association *NRAS* mutations with cytogenetic alterations have been identified. *NRAS* are similarly distributed among the major cytogenetic groups [102]. *NRAS* has been previously found to correlate with abnormalities of chromosomes 3 and 16 [100, 103]. However, this was not confirmed in the next study [102]. *RAS* mutations tend to occur together with *NPM1* mutations, while coexistence of *RAS* mutations with *FLT3-ITD*, *CEBPA*, or *WT1* appears to be less common [99, 102].

## 7. *KIT* (CD117) mutations

The *KIT* gene is located on chromosome 4q12 and encodes transmembrane glycoprotein that belongs to a family of the type III RTK. The structure of RTK consists of five immunoglobulin-like domains in the extracellular portion of the receptor, a transmembrane and juxtamembrane domain, and an intracellular kinase domain [104]. The *KIT* protein is found in the cell membrane and binds with ligand. This binding activates the *KIT* protein, which then activates other proteins inside the cell by adding a phosphate group at specific positions. This phosphorylation leads to the activation of a series of proteins in multiple signaling pathways. The signaling pathways stimulated by the *KIT* control cell growth, proliferation, survival, and migration of cells [104, 105]. The majority of stem cells in the bone marrow express CD117. *KIT* expression and intensity on normal blast cells in the bone marrow decrease during maturation as a strong negative regulation during hematopoiesis. *KIT* is expressed on the surface of leukemic blasts in 80% of AML patients.

Ligand-independent activation of *KIT* can be caused by gain-of-function mutations that have been reported in core binding factor (CBF) AML [106, 107]. In cytogenetically favorable CBF-AML, which is associated with t(8;21)(q22;q22) and inv(16)/t(16;16)(q13;q22), *KIT* mutation is found most frequently within exon 17, which encodes the *KIT* activation loop in the kinase domain, and in exon 8, which encodes a region in the extracellular portion of the *KIT* receptor

[104, 108, 109]. Mutations of *KIT* occur in 20%–25% of t(8;21) and in approximately 30% of inv(16) cases [106].

The clinical significance of *KIT* mutations in CBF-AML has been intensively studied. The clinical significance of c-*KIT* mutations in CBF-AML is potentially related to mutation type, patient age, and type of chromosomal translocation. Paschka et al. reported that *KIT* mutations confer higher relapse risk and adverse OS in AML with inv(16) and t(8;21) AML [106, 110]. Contrary to most published studies, in a single CBF AML group no association between c-*KIT* mutations and prognosis of AML was found [107]. Various further studies confirmed that C-*KIT* mutations linked to adverse outcome in patients with t(8;21) but not in inv(16)/t(16;16) AML [108, 111–113]. National Comprehensive Cancer Network (NCCN) guidelines have defined t(8;21) and inv(16) AML with *KIT* mutations as intermediate risk guidelines, whereas ELN has provided no further recommendation for those with a *KIT* mutation [9, 114].

## 8. *TET2* mutations

The TET (ten–eleven translocation) protein family includes three members (TET1, TET2, and TET3) and is involved in the epigenetic regulation, in particular, responsible for demethylation. *TET2* gene located on chromosome 4q24 and catalytic activity converts 5-methylcytosine to 5-hydroxymethylcytosine in an  $\alpha$ -ketoglutarate-dependent reaction [115]. TET proteins further oxidize 5-hydroxymethylcytosine to formylcytosine and carboxylcytosine, which are replaced by unmodified cytosines through the DNA repair machinery [115]. The data published suggests a role for the TETs in the regulation of gene expression through modification of chromatin at promoter regions [116]. The *TET* family members have two highly conserved regions, an N-terminal cysteine-rich domain followed by a 2-oxoglutarate -Fe(II) oxygenase characteristic double-stranded b-helix [117]. Somatic loss-of-function mutations in *TET2* gene occur in a significant proportion of patients with myeloid malignancies. In AML, *TET2* mutations affect 7%–10% of the adult and 1.5%–4% of pediatric patients [118–121].

*TET2* mutations show loss-of-function phenotype [118, 122] and are anticipated to result in hypermethylation [123]. It was shown that *TET2* mutation samples display low levels of 5-hydroxymethylcytosine compared with normal controls, supporting a functional relevance of *TET2* mutation in leukemogenesis. *TET2* mutant AML displays increased promoter methylation [123]. In addition, it was shown that *TET2* mutants do not suppress the function of the wild-type protein and hence do not show dominant negative traits [118].

Several studies based on mouse model suggested that *TET2* mutation occurs in progenitor cells, which creates a predisposition to the development of myeloid malignancy. These studies confirm the role of *TET2* mutations in the pathogenesis of myeloid malignancies. Therefore, *TET2* mutation may exist as an early event, and in cooperation with secondary mutations, drives the phenotype of the disease [124, 125].

*TET2* mutations were spread over all cytogenetic subgroups. It was reported that *TET2* alterations are associated with *NPM1* and *FLT3*-ITD mutations [119, 121, 126]. Recent data



observed that *TET2* and *IDH1/2* mutations were mutually exclusive in a large, genetically annotated *de novo* AML cohort, suggesting that these lesions may be biologically redundant [123]. The high incidence of *DNMT3A* mutations in both of these groups was reported [118].

This could indicate of a cooperative mechanism through which mutations impairing DNA hydroxymethylation and DNA methylation contribute to leukemogenesis [118].

The prognostic relevance of *TET2* mutation is still not well established and remains controversial. In some studies no prognostic impact of *TET2* mutations on clinical outcome as well as in CN AML subtype was observed [121, 126]. A study of Cancer and Leukemia Group B Study with a large cohort of AML patients reported an adverse prognostic impact in the molecular favorable-risk cytogenetically CN AML group whereas there was no impact of mutation in the intermediate risk I group [120]. In a study by Chou et al., shorter OS was observed in patients with intermediate-risk cytogenetic [119]. An integrated genetic analysis revealed that mutations of *TET2* gene are associated with poor OS in intermediate risk patients, regardless of the presence of the *FLT3-ITD* mutation. Weissmann et al. also showed a negative impact of *TET2* mutations on survival in favorable risk AML patients with normal cytogenetics [127]. Most recently, the negative effect of *TET2* mutation on OS was confirmed in various risk groups in adult AML patients less than 60 years of age [118].

In addition, recently demonstrated low levels of *TET2* expression as a poor prognostic marker for patients without *TET2* or *IDH1* mutations can suggest that both loss of function mutations and low expression of *TET2* are markers of poor prognosis in AML [118]. The development of target therapies could be beneficial for these patients [118].

## 9. *IDH1* and *IDH2* mutations

Isocitrate dehydrogenases (IDH) 1 and 2 are NADP-dependent enzymes of the citrate cycle that convert isocitrate to  $\alpha$ -ketoglutarate. *IDH1* and *IDH2* genes encode cytoplasmic/peroxisomal isocitrate dehydrogenase 1 and mitochondrial isocitrate dehydrogenase 2, respectively. These are homodimeric, NADP<sup>+</sup>-dependent enzymes that catalyze the oxidative decarboxylation of isocitrate to  $\alpha$ -ketoglutarate ( $\alpha$ -KG), generating NADPH from NADP<sup>+</sup>. NADPH is an important source of synthetic reducing power and has key functions in cellular detoxification processes. Both genes function in a crossroads of cellular metabolism, cellular defense against oxidative stress, oxidative respiration, and oxygen-sensing signal transduction [128].

Somatic mutations in *IDH1* and *IDH2* occur frequently (50%–80%) in adult glioma [129]. In *de novo* CN AML, *IDH1/2* mutations occur in 15% and in 20% in sAML [24, 123, 130]. The frequency is higher in CN AML and in elderly patients. In contrast with glioma, in AML, *IDH2* mutations occur more frequently than *IDH1* mutations, with *IDH2*-R140Q as the most common mutation [131, 132].

The typical *IDH1* mutation affects the evolutionary conserved arginine residue 132 (*IDH1* R132) and the analogous amino acids 172 (*IDH2* R172) and 140 (*IDH2* R140) of the *IDH2* gene [133]. A great variety of *IDH1/2* mutants were reported (*IDH1*-R132, *IDH2*-R140, *IDH2*-R172



and their variants). All of them possess varying enzymatic properties. The most common *IDH1/2* mutants in AML, *IDH2* R140Q, and another mutant *IDH1*-R132H are weak *D*-2HG producers, as compared with other variants. Different mutant, *IDH1* R132C, produces relatively high levels of *D*-2HG [129]. It has been hypothesized that in oncogenesis, the intracellular *D*-2HG concentration that gives the largest growth advantage varies depending on the tumor's cell type of origin. This could explain why each type of cancer has a specific *IDH1/2* mutation. In addition, an *IDH1/2* mutation and the subsequent high *D*-2HG levels may affect which specific type of cancer is being formed [129, 134]. Acquired somatic mutations of *IDH1* and *IDH2* genes contribute to abnormal metabolic processes. Despite these effects of 2-HG on DNA and histone methylation, there is a growing consensus that, while obviously important, *IDH1/2* mutations are insufficient to drive neoplasia [134]. *IDH1* and *IDH2* missense mutations were infrequent in patients with preleukemic disorders, particularly in MDS, Paroxysmal nocturnal hemoglobinuria (PNH), and Aplastic anemia (AA) patients, suggesting that these genetic alterations may not be essential in the development and transformation of preleukemic disorders to AML [135].

In AML, *IDH1* mutations correlate with worse patient survival, whereas *IDH2*-R140Q mutations are associated with a moderately prolonged survival [136, 137]. Prognostic effect is known to depend on certain biological factors as well as a combination of cytogenetics and other mutations such as those in *FLT3* and *NPM1* [24, 130-132].

*IDH* mutations in AML are predominantly associated with CN AML and *NPM1* mutations.

*IDH1* and *IDH2* mutations are mutually exclusive of each other, or with *TET2* mutations, which suggests functional redundancy [123].

## 10. *DNMT3A* mutations

Somatic mutations in the *DNMT3A* have been reported approximately in 22% of *de novo* AML and 36% of CN AML [138]. Mutations in *DNMT3A* were first described by Ley et al. using whole genome sequencing [139]. *DNMT3A* belongs to the mammalian methyltransferase gene family which is responsible for tissue-specific gene expression [140]. DNA methyltransferases are the key enzymes for genome methylation, which plays an important role in epigenetically regulated gene expression and repression. *DNMT3A* together with other methyltransferase conducts *de novo* methylation of cytosine residues in CpG islands by the enzymatic addition of methyl residues from S-adenosyl-L-methionine to the 5-carbon atom of the cytosine ring. CpG islands are often located proximate to gene promoters thereby regulating their activation. Actively transcribed genes display unmethylated CpG islands that supports the euchromatin structure whereas methylated CpG islands are associated with untranscribed genes stabilizing the heterochromatin structure [141, 142]. Cancer genomes are most commonly characterized by global DNA hypomethylation. However, cancer cells also typically exhibit distinct regions of DNA hypermethylation, which are particularly well characterized in the CpG islands of promoter regions of tumor-suppressor genes. *DNMT3A* mutations are typically heterogenous. More common, mutations affect residual R882 within the methyltransferase domain [139, 143].

The biology of *DNMT3A* is not fully understood. Holz-Schietinger et al. reported that mutations in *DNMT3A* could retard its function by multiple mechanisms as changes in the catalytic properties, its processivity, and the disruption of interaction with binding partners [144]. Furthermore, Russler-Germain et al. found that mutations in the position R882 inhibit the formation of active tetramers of *DNMT3A* [145]. The impaired function of mutated *DNMT3A* leads to a hypomethylated genome of myeloid cells possibly promoting leukemogenesis and influencing disease outcome [146]. *DNMT3A* mutations are typically heterozygous. Continued expression of both mutated and wild-type *DNMT3A* in heterozygous cells, observed *in vitro*, suggests a dominant negative or possibly a neomorphic gain of function role for *DNMT3A* mutations in AML [147].

Since the *DNMT3A* mutations are present in the early preleukemic cells, this alteration seems to be a “founder” mutation, which can be implicated as functional components of AML evolution [148, 149]. *DNMT3A* mutations are highly associated with *NPM1*, *FLT3*, *IDH1*, and *IDH2* [150, 151]. Patients with *DNMT3A* mutations are typically older than average, have a higher white cell count, and are more likely to have monocytic or myelomonocytic leukemia (FAB M4/M5) [8].

Several studies reported a negative prognostic impact of *DNMT3A* mutations [150-154]. Prognostic effect is known to depend on certain biological factors as well as a combination of cytogenetics and other mutations such as those in *FLT3* and *NPM1*.

Some authors have found stability of *DNMT3A* mutations during the course of disease; therefore those aberrations could be a potential marker for minimal residual disease (MRD). Furthermore, the presence of *DNMT3A* mutations seems to be associated with the incidence of *FLT3*-ITD-positive clones at relapse possibly influencing the responsiveness of *FLT3*-positive cases to chemotherapy [155, 156]. In contrast, Hou et al. reported persistence of *DNMT3A* mutations at CR in AML patients, which later achieved relapse and died of disease progression. These data could relate the persistence of *DNMT3A* mutations and high risk of relapse [156]. Recently, Pløen et al. have identified persistence of *DNMT3A* mutations in long-term remission of patients with AML that received cytoreduction or palliative therapy [157]. Using cell-sorting, the authors showed that *DNMT3A* mutations were present in T-cells and B-cells at diagnosis in some patients, and also in T-cells several years after diagnosis. The presence of *DNMT3A* in both B-cells and T-cells could lead to assumption that mutation had occurred in an early pre-leukemic stem cell prior to the acquisition of other genetic events, and could be resistant to chemotherapy [157]. Therefore, further exploration of the role of *DNMT3A* R882H mutations for the progression of AML disease is needed.

Recent discoveries utilizing whole-exome sequencing in a large cohort of persons unselected for cancer or hematologic phenotypes have demonstrated somatic mutations in significant proportion of persons particularly older than 65 years. Moreover, *DNMT3A* gene together with *TET2*, *ASXL1* (additional sex combs like transcriptional regulator 1), and *PPM1D* (Protein phosphatase 1D) had disproportionately high numbers of somatic mutations [5, 158]. The data suggest that mutations in pre-leukemic cells could precede leukemia. Furthermore, *DNMT3A* mutations could drive clonal expansions.

## 11. Conclusion

The whole gene analysis has revealed that leukemic cells carry hundreds of mutated genes. Most of them are “passenger” mutations, which do not provide a selective advantage, and a less number of mutations are “driver” mutations. The latter can cause the tumor. The simultaneous presence of genetic alterations with different functional effects on hematopoietic progenitors led to the concept of leukemogenesis as a multi-step process that ultimately gives rise to malignant transformation. Evidence from many murine models confirmed that a single genetic change is not sufficient for the occurrence of AML. Moreover, two modern studies have demonstrated that somatic mutations that drive clonal expansion of blood cells were a common finding in the elderly and most frequently involved *DNMT3A*, *TET2*, or *ASXL1*. The age-related clonal hematopoiesis is a common premalignant condition that is also associated with increased overall mortality [5, 158]. Overall this knowledge has provided useful elements to stratify AML patients into different subgroups, resulting in better prognosis and therapy.

## Author details

Olga Blau\*

Address all correspondence to: [olga.blau@charite.de](mailto:olga.blau@charite.de)

Charité Universitätsmedizin Berlin, Department of Hematology, Oncology and Tumourimmunology, Berlin, Germany

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