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Review Article

Gene mutations and molecularly targeted therapies in acute myeloid leukemia

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Abstract: Acute myelogenous leukemia (AML) can progress quickly and without treatment can become fatal in a short period of time. However, over the last 30 years fine-tuning of therapeutics have increased the rates of remission and cure. Cytogenetics and mutational gene profiling, combined with the option of allogeneic hematopoietic stem cell transplantation offered in selected patients have further optimized AML treatment on a risk stratification basis in younger adults. However there is still an unmet medical need for effective therapies in AML since disease relapses in almost half of adult patients becoming refractory to salvage therapy. Improvements in the understanding of molecular biology of cancer and identification of recurrent mutations in AML provide opportunities to develop targeted therapies and improve the clinical outcome. In the spectrum of identified gene mutations, primarily targetable lesions are gain of function mutations of tyrosine kinases *FLT3*, *JAK2* and *cKIT* for which specific, dual and multi-targeted small molecule inhibitors have been developed. A number of targeted compounds such as sorafenib, quizartinib, lestaurtinib, midostaurin, pacritinib, PLX3397 and CCT137690 are in clinical development. For loss-of-function gene mutations, which are mostly biomarkers of favorable prognosis, combined therapeutic approaches can maximize the therapeutic efficacy of conventional therapy. Apart from mutated gene products, proteins aberrantly overexpressed in AML appear to be clinically significant therapeutic targets. Such a molecule for which targeted inhibitors are currently in clinical development is PLK1. We review characteristic gene mutations, discuss their biological functions and clinical significance and present small molecule compounds in clinical development, which are expected to have a role in treating AML subtypes with characteristic molecular alterations.

Keywords: Acute myeloid leukemia, targeted therapy, mutation, FLT3, NPM1, CEBPA, JAK2

Introduction

Acute myelogenous leukemia (AML) can progress quickly and without treatment can become fatal in a short period of time. However, over the last 30 years fine-tuning of therapies and therapeutic schemes have increased the rates of remission and cure [1]. Currently certain karyotype abnormalities and gene mutations are being taken into consideration to guide treatment and in particular the therapeutic use of allogeneic hematopoietic stem cell transplantation in non-elderly patients. However, AML remains incurable for a significant proportion of adult patients [2-4], while no viable therapeutic option exists for patients with relapsed and refractory AML [5]. In this context it is crucial to develop novel targeted therapies that could improve the clinical outcome in subsets of AML [6].

A better understanding of the molecular basis of cancer during the last two decades has contributed to the development of drugs that target protein products of mutated or chimeric genes, which are linked to various cancers [7-9]. Pivotal example of small-molecule kinase inhibitors that bind to driver oncoprotein and block its function on a potentially curative intent are the BCR–ABL kinase inhibitors, in use for chronic myeloid leukemia (CML) [10]. Acute Promyelocytic Leukemia (APL) is another example where effective targeted therapies, such as all-trans retinoic acid (ATRA) and arsenic trioxide are used and can reinstall differentiation of leukemic promyelocytes by targeting the culprit

PML-RARa fusion protein [11, 12]. However, in non-APL AML, despite the identification of a handful recurrent chimeric genes and gene mutations, development of targeted therapies has been notably sluggish. Here we review characteristic gene mutations, discuss their biological functions and clinical significance and present small molecule compounds in clinical development, which are expected to have a role in treating non-APL AML subtypes with characteristic druggable mutations.

Gene mutations of good prognosis

NPM1 mutations in AML (NPMc+ AML)

Nucleophosmin (NPM1) is a ubiquitously expressed phosphoprotein that belongs to the nucleoplasmin family of nuclear chaperones. It is encoded by the *NPM1* gene located at 5q35.1 that produces 3 isoforms through alternative splicing, of which NPM1 (or nucleolar phosphoprotein B23.1) is a 294-amino acid [13, 14]. NPM1 is a pleiotropic nucleolar protein that shuttles across cytoplasm and nucleoplasm and regulates among others centrosome maturation and the ARF/p53 pathway [15-18].

NPM1 protein as a product of mutated or fused *NPM1* has been associated with certain blood cancers. In Anaplastic Large Cell Lymphoma (ALCL) the t(2;5) translocation results to NPM1–ALK fusion oncoprotein, which anchors to cell cytoplasm and is detected with antibodies against the NPM1 N-terminus [19]. In 2005, it was first discovered that about 35% of adult AML had aberrant nucleophosmin expression in leukemic cell cytoplasm, as a result of *NPM1* gene insertions at exon-12 [20]. In about 80% of AML with mutated *NPM1,* the mutation is a duplication of the 4-base sequence TCTG at positions 956-959 of the *NPM1* gene, the so-called mutation A, which results in a slightly longer protein with a different C-terminal amino-acidic sequence [21]. These C-terminal changes are responsible for cytoplasmic localization of the NPM1 leukemic mutants through generation of new nuclear export signal (NES) motifs and loss of the two tryptophan residues 288 and 290 which cause the unfolding of the C-terminal domain and thus loss of binding capacity to the nucleolus [21, 22]. *NPM1* mutations may rarely occur at exon-9 and exon-11 and these mutants also localize in the cytoplasm through the same mechanism that exon-12 *NPM1* mutants dislocate [23, 24].

Cytoplasmic mutant NPM1 contributes to AML development by inactivating p19Arf through delocalization of the tumor suppressor protein. This results in reduced p19Arf activities, both p53-dependent (Mdm2 and p21cip1 induction) and p53-independent (sumoylation of NPM). p19Arf stability is compromised when coupled with NPM1 mutant, which may lead to weaker control of the p53-dependent cell-cycle arrest [25, 26]. Mutated NPM1 bounds to NF-kappaB and dislocates it in the cytoplasm, leading to its inactivation. This inactivation of NF-kappaB is thought to be responsible for the high response rates of AML with NPM1mutant to chemotherapy [27, 28] NPM1c+ (cytoplasmic positive) AML is closely associated with normal karyotype and represent a provisional entity in the WHO 2008 classification.

NPM1 targeted therapy: There are two key points that prompt consideration of nucleophosmin as a therapeutic target: a) *NPM1* mutation is one of the most common recurring genetic lesions in AML with a prevalence of 27%-35% in adult AML and 45%-64% in adult AML with a normal karyotype and b) normal karyotype AML and the genotype 'mutant NPM1 without FLT3-ITD' carry a most favorable prognosis when treated with intensive chemotherapy [29-32]. This data indicate that *NPM1* mutation behaves as a founder genetic lesion in a fraction of AML patients, which makes it an attractive target for therapeutic intervention, primary aiming to increase chemotherapy efficacy [33]. Interestingly it has been shown that the favorable outcome of chemotherapy in *NPM1* mutated non *FLT3-ITD* AML can be improved by incorporating all-trans retinoic acid (ATRA) [34]. Moreover specific inhibitors of NPM1 oligomerization such as NSC348884 may further sensitize leukemic cells of this genotype to apoptosis when exposed to the ATRA plus cytarabine combination [35].

CEBPA mutations in AML

CCAAT/enhancer binding protein alpha (*CEBP-alpha, CEBPA*) is an intronless gene located at chromosome 19q13.1 that encodes for a basic region leucine zipper (bZIP) transcription factor, which can bind as a homodimer to certain pro-

moters and enhancers but can also form heterodimers with the related proteins CEBP-beta and CEBP-gamma [36]. CEBPA functions as key regulator of granulocytic differentiation [37].

CEBPA mutations contribute to leukemogenesis by promoting proliferation and blocking differentiation of myeloid lineage [38, 39]. The two most frequent mutations are: a) N-terminal frame-shift mutations that truncate the p42 form while preserving the p30 form which inhibits the remaining wild-type CEBPA p42 protein in a dominant-negative manner and b) C-terminal in-frame insertions or deletions that disrupt the basic zipper region, thus affecting DNA binding [40]. Most cases carry both types of *CEBPA* mutations: a N-terminal frame-shift mutation and a C-terminal in-frame mutation, with the two mutations typically being located on different alleles [41, 42].

CEBPA-mutated AML usually displays classical features of AML with or without cell maturation but some cases may show monocytic or monoblastic features. Myeloid-associated antigens HLA-DR and CD34 are usually expressed, as is CD7 in a significant proportion of patients. About 70% of cases have normal karyotype and approximately 25% carry concomitant *FLT3-ITD* mutations [43].

Prognosis of cytogenetically normal (CN)-AML patients with *CEBPA* mutations in the absence of an *FLT3-ITD* or *NPM1* mutation, is favorable, similar to AML with inv(16)(p13.1q22) or t(8; 21)(q22; q22) [43-45]. However only patients with double *CEBPA* mutations have favorable clinical course, whereas single *CEBPA* mutations not only do they not differ from *CEBPA* wild-type patients but also they have a tendency toward high-risk *FLT3-ITD* mutations [46]. However, coexistence of *NPM1* mutations with monoallelic *CEBPA* mutations was shown to be associated with prolonged survival in CN-AML patients [47]. Hereditary predisposition is a noteworthy point related to *CEBPA*. Germ-cell mutations appear to occur in 7% of patients with CN AML and myeloid precursor cells from healthy individuals carrying single germ-line *CEBPA* mutation may evolve to overt AML by acquiring a second sporadic *CEBPA* mutation [46, 48]. Adult AML with *CEBPA* mutation is also a provisional entity in the current WHO classification.

CEBPA targeted therapy: Restoring function of particular dysregulated transcription factors appears to be a reasonable target for novel therapeutic strategies in AML [49]. However, no therapies to restore CEBPA function in dysregulated CEBPA CN-AML cases have been currently developed.

Gene mutations of poor prognosis

FLT3 mutations in AML

FLT3 (Fms-like tyrosine kinase 3, CD135) is a member of class III tyrosine kinase (RTKIII) receptor family, which also includes c-FMS, c-KIT, and PDGFR. The *FLT3* gene encodes a 993–amino acid protein in humans, which is composed of an immunoglobulin-like extracellular ligand-binding domain, a transmembrane domain, a Juxtamembrane dimerization domain and a cytoplasmic domain with a split tyrosine kinase motif. It is expressed in immature hematopoietic cells, placenta, gonads, brain, and in lymphohematopoietic organs such as the liver, spleen and the thymus [50].

FLT3 receptor exists in a monomeric unphosphorylated status and turns activated when bound by its FLT3 ligand (FL), which promotes its unfolding and homodimerization. Homodimerization of FLT3 switches on its tyrosine kinase activity and recruits a number of intracellular proteins [SHC proteins, GRB2, GRB2-associated binder 2 (GAB2), SHIP, CBL, CBLB (CBLB-related protein)] to its intracellular domain. Each protein becomes activated and a phosphorylation cascade starts resulting in activation of secondary mediators (MAP kinase, STAT, and AKT/PI3 kinase signal transduction pathways), which are transported to the nucleus by HSP90, where they regulate transcription of several genes, which participate in differentiation, proliferation, and apoptosis [51, 52].

FLT3 expression in the normal bone marrow is restricted to early progenitors, including CD34+ cells with high levels of expression of CD117 (c-KIT), and committed myeloid and lymphoid progenitors with variable expression in the more mature monocytic lineage [53]. It is also expressed at high levels in many hematologic malignancies including most of AML subtypes, B-precursor cell acute lymphoblastic leukemia (ALL), some T-cell ALLs, and CML in blast crisis [54, 55].

Mutations of the *FLT3* are of major clinical relevance in AML because they commonly guide treatment decisions as independent indicators of poor prognosis [2, 3].

FLT3 internal tandem duplications (FLT3-ITD)

The most common mutation of *FLT3* in AML is internal tandem duplications (FLT3-ITD). FLT3-ITD results from a duplication of a fragment within the juxtamembrane domain coding region (encoded by exons 14 and 15) of FLT3. It is one of the most common mutations in hematologic malignancies, occurring in CML (5–10%), MDS (5–10%), and AML (15–35%) patients [56]. Nakao et al. first described FLT3-ITD in a high proportion of patients with AML [56]. FLT3-ITD is rare in infant AML, but increases to 5% to 10% in age 5 to 10 years, 20% in young adults, and >35% in AML patients older than 55 years [57]. FLT3-ITD mutations vary in size and region of ITD involvement that ranges from 3 to more than 400 base pairs [58].

Segmental duplication of the Juxtamembrane (JM) domain of FLT3 promotes auto-dimerization and autophosphorylation of the receptor, which turns it constitutively phosphorylated and activating AKT [59, 60]. Some of the effects of FLT3-ITDs are unique to the mutated receptor: cellular proliferation of FLT3-ITD transduced cells is mediated by RAS and STAT5 pathways, while ligand-induced FLT3-WT activation does not lead to STAT5 activation and no STAT5 DNA binding [61].

FLT3 tyrosine kinase domain mutations (FLT3/TKD)

Missense mutations have also been described in the activation loop domain of the tyrosine kinase of *FLT3* (FLT3 activation loop mutation, FLT3/ALM, or FLT3 Tyrosine Kinase Domain mutation, FLT3/TKD) [62]. *FLT3/TKD* are the second most common type of FLT3 mutations found in 5-10% of AML and they can rarely co-exist with FLT3-ITD. The majority of the *FLT3/TKD* occur in codon 835 with a change of an aspartic acid to tyrosine (D835Y or Asp835Tyr), however, other point mutations, deletions, and insertions within codon D835 (Asp835) and its surrounding codons have been described [60, 62-64] *FLT3/TKD* promotes ligand-independent proliferation through autophosphorylation and constitutive receptor activation, similar to that of *FLT3-ITD* but there are significant biological differences between the two types of *FLT3* mutations. They promote activation of different downstream effectors, and trigger different biological responses [65, 66].

Prognostic significance of FLT3-ITD

Many large studies have shown that presence of *FLT3-ITD* is an independent prognostic factor for poor outcome in AML [63, 67]. Kottaridis et al [68] examined the prevalence and prognostic significance of *FLT3-ITD* in a cohort of more than 850 adult AML patients. They found *FLT3-ITD* in 27% of patients and confirmed previous studies showing that *FLT3-ITD*s were associated with leukocytosis and normal cytogenetics. In their study, AML patients with *FLT3-ITD* had a lower remission rate, higher relapse rate (RR), and worse survival. Multivariate analyses showed that *FLT3-ITD* was the most significant prognostic factor with respect to RR and disease free survival (DFS) [68]. In other studies, survival for patients with *FLT3-ITD* was 20% to 30% compared to 50% for those without *FLT3-ITD* and allelic variation (mutant to wild-type ratio) in patients with *FLT3-ITD* seemed to influence outcome. Various thresholds of *FLT3/ITD* allelic ratio established an allelic ratio threshold that demarcated patients with *FLT3-ITD* at high risk of relapse [57]. Similar work in other studies has shown differences in clinical outcome for those with differing allelic ratios [69].

FLT3 targeted therapies: FLT3 tyrosine kinase is thought to be the most reasonable targetable protein in AML [70, 71]. Several potent FLT3 kinase inhibitors are currently in development for AML that harbors *FLT3-ITD* mutations and first results of FLT3 inhibitors in clinical development have already produced encouraging and clinical relevant activity (**Table 1**) [71-73].

Sorafenib is one of the most extensively investigated first generation FLT3 inhibitors. It has shown to specifically reduce the percentage of leukemia blasts in the peripheral blood (7.5% from 81%) and the bone marrow (34% from 75.5%) of AML patients with *FLT3-ITD* but not in patients without this mutation [74]. It has also shown activity in *FLT3-ITD*-positive AML relapsing patients after allogeneic stem cell transplantation [75]. However, development of resistance to TKIs is a well known therapeutic problem [76, 77] and in the case of FLT3-ITD+

Table 1. Commonly mutated or aberrantly expressed genes in non-APL AML for which targeted drugs are being developed

Mutated Gene	Gene pruduct function	Compound	Targets	trial phases	combinations	Trials Registered Clinicaltrials.gov (Nov 2012)	Refs
FLT3	This gene encodes a tyrosine-protein kinase that acts as cell-surface receptor for the cytokine (fms-related tyrosine kinase 3 ligand) FLT3LG and regulates differentiation, proliferation and survival of hematopoietic progenitor cells and of dendritic cells. Activation of wild-type FLT3 causes only marginal activation of proliferation, and differentiation of hematopoietic cells in bone marrow. Mutations that cause constitutive kinase activity promote cell proliferation and resistance to apoptosis via the activation of multiple signaling pathways	Lestaurtinib	FLT3, JAK2	1,2,3	chemotherapy	4 active 4 completed	[89, 211]
		PLX3397	FMS, KIT & FLT3	1	none	1 active	[96]
		Quizartinib (AC220)	FLT3, KIT, PDGFRA, PDGFRB, RET	1,2	chemotherapy	6 active 1 completed	[86]
		Sorafenib	FLT3, cKIT	1, 2		11 active 6 completed	[212-215]
		Midostaurin	Multiple kinases	1,2,3	ATRA, Bortezomib, azacytidine, decitabine, chemotherapy	9 active 4 completed	[90]
NPM1	A nucleolar phosphoprotein that shuttles across cytoplasm and nucleus and regulates centrosome maturation and the ARF/p53 pathway. NPM1 leukemic mutants are characterized by insertions that cause a reading frame-shift and result in a longer protein with a different C-terminal, which is responsible for lossing the binding capacity to the nucleolus. This may contribute to leukemogenesis by inactivating p19Arf through delocalization of the tumor suppressor protein and dislocating NF-kappaB to the cytoplasm.	ATRA	Induces p53 and p21	1		2 active	[34]
PLK1	*PLK1* is the most well characterized member of four serine/threonine protein kinases, which and strongly promotes the progression of cells through mitosis. PLK1 performs several important functions throughout M phase of the cell cycle, including the regulation of centrosome maturation and spindle assembly, the removal of cohesins from chromosome arms, the inactivation of anaphase-promoting complex/ cyclosome (APC/C) inhibitors, and the regulation of mitotic exit and cytokinesis. It plays a key role in centrosome functions and the assembly of bipolar spindles. It als acts as a negative regulator of p53 family members leading to ubiquitination and subsequent degradation of p53/TP53, inhibition of the p73/TP73 mediated pro-apoptotic functions and phosphorylation/degradation of BORA. During the various stages of mitosis PLK1 localizes to the centrosomes, kinetochores and central spindle. Plk1 is found aberrantly overexpressed in a variety of human cancers and in AML, correlated with cellular proliferation and poor prognosis.	Volasertib	PLK1	1,2,3	chemotherapy	2 active	[216]
		BI 2536	PLK1	1,2	chemotherapy	1 completed	[207]

MLL	The MLL gene (mixed-lineage leukemia) encodes a protein plays an essential role in early development and hematopoiesis by acting as a histone methyltransferase and transcriptional co-activator. Among others it activates aberrant trascription DOT1L, which is considered a driver of leukemogenesis	EPZ-5676	DOT1L	1	1 active	[175]
JAK2	The Janus-kinase-2 gene (JAK2) encodes a non-receptor tyrosine kinase involved in relaying signals for hemopoietic cell growth, development and differentiation crossactivated by type I/II cytokine receptors	Ruxolitinib Pacritinib Lestaurtinib (CEP-701)	JAK2 JAK2, FLT3 JAK2, FLT3	1,2 1 1,2	1 active 1 completed 1 active 3 completed	[217] [82] [218]

AML, it appears that stromal niche cells offer sanctuary to early leukemic stem/progenitor cells protecting them from eradication by first-generation inhibitors [78]. Several investigators focus their attempts in developing strategies to prevent or reverse 'acquired' resistance to TKIs. Recent in vitro studies have shown that the anti-leukemic activity of TKIs can be increased when combined with the proapoptotic small molecule Nutlin-3, which inhibits the MDM2/p53 interaction [79, 80]. Moreover fluvastatin, a drug in use for the treatment of hypercholesterolemia, has shown potency to reverse resistance and increase activity of sorafenib [81, 82].

Upregulation of JAK2 in FLT3-TKI-resistant AML cells appears to be a potential mechanism of resistance to selective FLT3 inhibition [83, 84]. Second-generation potent multi-targeted FLT3/JAK2 inhibitors are thought to address this important therapeutic issue. A number of such compounds, such as quizartinib, lestaurtinib and midostaurin are currently in early phases of clinical development.

Quizartinib (AC220) is such a second-generation FLT3 inhibitor, which exhibits low nanomolar potency, good bioavailability and exceptional kinase selectivity [85]. Early clinical results of quizartinib were promising. They showed meaningful reductions in marrow blasts in a substantial proportion of patients with both refractory and relapsed FLT3-ITD+ AML [86]. Lestaurtinib (CEP701) a dual FLT3 and JAK2 inhibitor has shown activity as monotherapy in AML, but although it produced high remission rates, it failed to increase survival in combination with cytarabine and idarubicin in young patients with relapsed or refractory AML [87-89]. Midostaurin (PKC412), a semi-synthetic multitargeted tyrosine kinase inhibitor, has demonstrated activity as monotherapy in patients with FLT3-mutant and wild-type AML and high complete response and survival rates when given in combination with standard chemotherapy in newly diagnosed young adults with AML [90, 91]. Pacritinib (SB1518) is another novel potent JAK2/FLT3 inhibitor, which has demonstrated promising activity and clinical benefits in refractory AML patents treated in a phase I trial [82]. Pacritinib in combination with pracinostat (SB939), an oral HDAC inhibitor showed synergy in reduction of tumor growth and JAK2 and FLT3 signaling [92]. Another oral multikinase inhibitor that has showed antileukemic activity in preclinical models is TG02 that inhibits CDKs 1, 2, 7 and 9 along with JAK2 and FLT3 [93].

In addition more specific and potent anti-FLT3 compounds such as PLX3397 and FLT3-Aurora kinase inhibitor CCT137690, are in early phases of clinical development [94-96] and others such as DCC2036, CCT241736 have produced in vitro very promising data for the treatment of FLT3-ITD+ AML [97].

KIT mutations in core binding factor leukemia (CBF) AML

Core-binding factor (CBF) AML patients when compared to other cytogenetic groups have a favorable prognosis, particularly when treated with high-dose cyrabine consolidation regimens and do not require stem cell transplantation. However, relapses do occur and approximately 50% of patients with these cytogenetic abnormalities are alive at 5 years [98]. Mutations that have been found in this group of

patients and have been correlated with adverse outcome are related to c*KIT* and *JAK2* genes [99-101].

The *c-KIT* gene (stem cell factor) encodes for a tyrosine kinase with a structure similar to platelet growth factor and is expressed in hematopoietic progenitor cells and AML blasts [102]. Upon binding of the ligand stem cell factor to c-kit, phosphorylation of several cytoplasmic proteins occurs and pertinent downstream pathways get activated. Those include the JAK/STAT pathway, the PI-3 kinase pathway and the MAP kinase pathway [103]. Mutations in c-KIT receptor result in constitutive phosphorylation and activation of the receptor in absence of the ligand. Mutations in the *KIT* and *FLT3* genes are associated with unfavourable prognosis in AML patients with t(8; 21). In particular, patients with *c-KIT* mutated have been reported to have a higher incidence of relapse (80 versus 13.5%) and a lower 6-year progression free survival (PFS) compared to unmutated [104]. Patients with t(8; 21), but not those with inv(16) have a shorter relapse free survival when harbouring a mutated c-kit [105]. KIT mutation was recently found to be related to PFS in patients with inv(16) or t(16; 16) AML [106]. The prognostic impact of c-KIT mutations in patients with inv(16) remains controversial since some studies failed to establish a link [105], while others found that exon 8 mutations increased the relapse rate but did not affect overall survival (OS) [107].

KIT targeted therapies: Multi-kinase inhibitors imatinib and sunitinib beside their indications for the treatment of CML and renal cancer respectively, have also been licensed for the treatment of gastrointestinal stromal tumors, because they effectively inhibit mutated c-KIT, which is the characteristic molecular abnormality in these tumors [108, 109]. However, not all c-KIT mutations respond to the same agent. Exon 8 and the exon 17 N822 *c-KIT* mutations but not the D816 are sensitive to imatinib *in vitro*, therefore assessment of the exact *c-KIT* mutational status is important and may have direct therapeutic consequences. Initial clinical studies with imatinib in a small number of patients with refractory AML did not show beneficial results [110], however, when tested in c-KIT positive AML patients results were more promising [111]. Several studies have investigated the activity of imatinib alone or in combination with chemotherapy in *c-KIT* positive AML patients and results are awaited.

Small molecules such as SU5416 and SU6668 have activity against c-KIT [109] although neither is selective. Both were developed as angiogenesis inhibitors and also inhibit FLT3, KDR and FGFR [109]. In addition SU5416 inhibits VEGFR2, while SU6668 inhibits PDGFR. SU6668 has shown antiangiogenic properties and inhibition of c-KIT in preclinical models, whereas SU5416 reached later stages of drug development; however it showed modest activity in patients with relapsed/refractory AML or MDS [112]. Further investigating these molecules has been halted. APcK110 is a novel KIT inhibitor with potent proapoptotic and antiproliferative activity in AML cell lines and primary samples whereas in an AML xenograft mouse model it was shown to extend survival [113].

JAK2 mutations in CBF AML

The Janus-kinase-2 gene (*JAK2*) encodes a non-receptor tyrosine kinase involved in relaying signals for hemopoietic cell growth, development and differentiation [114]. JAK proteins consist a family of four non-receptor tyrosine kinases (JAK1, JAK2, JAK3 and Tyk2) that are closely associated with type I/II cytokine receptors. When activated via association to cell surface receptors they phosphorylate and translocate STATs to the nucleus to activate gene transcription [115, 116]. Among the family members JAK2 associates with the IFN-1, IL-6, 12/23 cytokine and EPO receptors [116].

JAK2 is commonly mutated in myeloid neoplasias. The *JAK2*V617F gain of function mutation in the cytoplasmic tyrosine kinase domain is a common finding in myeloproliferative neoplasms [117]. The same mutation has been found in a small number of AML patients, more commonly in t(8; 21) AML [100, 101, 118]. AML t(8; 21) patients harbouring *JAK2*V617F in addition to *KIT* and *FLT3* mutations have poorer disease-free survival compared to wild type *JAK2* [119-121]. Moreover activating *JAK2* gene fusions with the *TEL*(ETV6) (TEL-JAK2) and *PCM1* genes have been found in leukemia patients [122-124]. Beside the detected mutations, a recent immunohistochemical study found JAK2 to be invariably activated (phosphorylated) in AML, while high of p-JAK2 levels were found to be a predictor of poor response

to chemotherapy (45% in patients with high p-JAK2 vs. 78% in patients with low p-JAK2, $p < 0.003$) and a factor of poor prognosis ($p=0.023$) which justifies its consideration as a therapeutic target in AML [125].

JAK2 targeted therapy in AML: JAK inhibitors constitute a new class of drugs with activity in a wide range of diseases, primarily in myeloproliferative neoplasias and autoimmune disorders [126]. Ruxolitinib, the first JAK inhibitor that recently received marketing authorization by FDA and EMA for the treatment of myelofibrosis, is now investigated in patients with relapsed or refractory acute leukemia (ClinicalTrials.gov Identifier: NCT01251965) [127].

Following ruxolitinib approval, several highly potent next generation JAK2/FLT3 inhibitors, such as pacritinib and lestaurtinib, entered clinical evaluation for patients with advanced myeloid malignancies (NCT00719836, NCT00469859) [126]. First available data suggest that blockade of JAK2 in conjunction with FLT3 can enhance clinical benefit for AML patients harboring a *FLT3-ITD* mutation and provide a strong basis for a clinical evaluation of these targeted small molecule therapeutics in AML patients particularly to those who are resistant to FLT3 directed TKI therapy [82].

Gene mutations of unclear prognostic value

RAS mutations in AML

RAS proto-oncogene belongs to the GTPase family and has 3 isoforms: N-Ras, K-Ras, and H-Ras. Mutant *RAS* isoforms are found in various types of tumors and leukemia [128]. Point mutations are mostly found at codons 12, 13, and 61 of RAS proto-oncogene. De novo AML patients harbour activating mutations in the *RAS* proto-oncogenes (*N-RAS* and *K-RAS*) in about 25% of cases [129]. *HRAS* mutations are extremely rare in myeloid leukemia [130]. *RAS* mutations seem to contribute to leukemogenesis (class I mutations). Several reports have suggested that AML patients harboring *RAS* mutations have worse, similar or more favourable clinical outcomes than those with wild-type *RAS* genes [129, 131, 132]. The presence of *RAS* mutations seems to sensitize AML cells to high-dose cytarabine therapy in vivo and these patients when treated with chemotherapy alone probably benefit from high-dose cytarabine postremission treatment [133]. *NRAS* mutations are frequently detected in patients with inv(16)/t(16; 16) [133, 134].

RAS targeted therapies: The product of mutated *RAS* gene is an abnormal Ras protein that is constitutively active. Activated Ras anchores on the cell membrane and stimulates a critical network of signal transduction pathways involved in cellular proliferation, survival and differentiation. Wild type Ras proteins require post-translational modifications by farnesyltransferase (FTase) to get attached to binding sites in the cell membrane to become biologically active. Farnesyl transferase inhibitors (FTIs) are the best-studied class of Ras inhibitors in hematologic malignancies. However Ras can escape FTI suppression and become activated through geranylgeranylation [98]. Tipifarnib), is the main FTI tested in patients with AML. However, inceased toxicity and suboptimal activity in elderly patients did not justify further investigation of this drug [135-137]. The same drug was also proven inactive in young AML patients [138]. Negative was also a phase 2 trial of lonafarnib, which is another FTI in patients with MDS or secondary AML [139].

Gene mutations in epigenetic modifiers

IDH mutations in AML

Isocitrate dehydrogynase (IDH) isoenzymes catalyse an essential step in the Krebs cycle that catalyzes conversion of isocitrate to α-ketoglutarate [140]. In mammalian cells three classes of IDH exist: nicotinamide adenine dinucleotide (NAD)-dependent IDH, mitochondrial nicotinamide adenine dinucleotide (NADP)-dependent IDH, and cytosolic NADP-dependent IDH [141]. IDH1 gene is located at chromosome band 2q33.3 and its product is NADP-dependent and localized in cytoplasm and peroxisomes while IDH2 gene is located at chromosome band 15q26.1 and encodes the mitochondrial NADP-dependent IDH2 enzyme [142, 143].

Recurring mutations in *IDH1* and *IDH2* have been described in more than 70% of World Health Organization grade 2 and 3 astrocytomas, oligodendrogliomas, and glioblastomas [144-146] and in approximately 30% of patients with normal karyotype AML [147-150]. Mutations in the *IDH1* occur at R132 while at

IDH2 at R172. The mechanisms underlying causal association of mutated IDH with cancer pinpoint to deleterious metabolic alterations although intervention with epigenetic homeostasis through remodeling of the methylome has also been suggested [151-154]. Dang et al showed that *IDH1* mutation leads to the overproduction of 2-hydroxyglutarate, a putative oncometabolite that has been associated with a high risk of brain tumors in patients with inborn errors [147, 155] and Zhao et al found that mutant IDH1 contributes to tumor growth by activating hypoxia-inducible factor-1α [156].

Somatic mutation at IDH1 R132 was originally described by Mardis et al., who sequenced the entire genome of a CN AML. They subsequently screened 187 AML cases and showed a heterozygous *IDH1R132* mutation in 15 cases (8.0%) [147]. In AML, mutant IDH enzyme activity converts α-ketoglutarate to 2-hydroxyglutarate (2-HG), which leads in accumulation of the cancer-associated metabolite 2-hHG [153, 157, 158]. Several studies have studied the IDH1/2 mutational status in patients with AML and a statistically significant co-occurrence with NPM1 mutations has been reported [159, 160]. In the two largest studies correlations *IDH1/2* mutations with outcome in AML by the UK MRC, except for the IDH1/2 mutation enrichment in the NPM mutant group, it was reported that patients with the IDH R140Q mutation had an improved OS and decreased response rates. In contrast, IDHR172 mutations did not correlate to outcome or response to therapy, whereas presence of the *IDHR132* mutation had an impact on worsened outcome in patients with the FLT3 wild type genotype [160, 161]. It becomes obvious that since the number of co-occurring mutations increases, further investigation is needed to better define the prognostic impact of the *IDH1/2* mutations in patients with AML.

IDH targeted therapy: It is thought that small-molecule inhibitors with a potential to block the synthesis of 2-HG could be developed given that IDH mutations lead to a gain-of-function mutation but to date no such therapies have been discovered [162]. However it has been observed that *IDH*-mutant AMLs have a unique methylation profile characterized by global promoter hypermethylation, which renders these cases reasonable candidates for demethylation therapies [163].

MLL mutations in AML

The *MLL* gene (mixed-lineage leukemia) encodes a protein that plays an essential role in early development and hematopoiesis by acting as a histone methyltransferase and transcriptional co-activator. One of its domains, the SET domain, mediates methylation of 'Lys-4' of histone H3 (H3K4me) complex and acetylation of 'Lys-16' of histone H4 (H4K16ac). H3K4me mediates epigenetic transcriptional activation of specific target genes, including many of the HOX genes [164, 165].

Aberrant expression of MLL is usually associated with leukemogenesis [166, 167]. Mutations and chromosomal translocations involving the MLL gene identify a unique group of acute leukemias, and often predict a poor prognosis. Partial tandem duplication of the MLL gene (MLL-PTD) was the first mutation observed in de novo AML with a normal karyotype or trisomy [168]. These duplications consist of an in-frame duplication of MLL exons. MLL-PTDs are named according to the fused exons e.g. e9/e3. Some, PTD seem to be generated by mispairing of Alu elements, which are repetitive regions with high homology [169].

The incidence of MLL-PTD is around 6% in unselected AML cases but it is higher in cases with normal karyotype (up to 8%) and even higher in cases with trisomy 11 (up to 25%), while favorable karyotypes (e.g. t(8; 21), t(15; 17), inv(16)/t(16; 16)) are MLL-PTD negative [170, 171]. MLL-PTD may also be associated with *FLT3-ITD* and *FLT3* point mutations [171, 172]. Patients with MLL-PTD expression seem to have shortened remission duration and shorter disease-free survival (DFS) [168].

MLL targeted therapy: MLL specific therapies are optimally targeting mislocated enzymatic activity of DOT1L, which is considered a driver of leukemogenesis in aberrantly expressing MLL leukemias. DOT1L is a histone methyltransferase recruited by rearranged/mutated MLL that methylates lysine-79 of histone H3 and drives expression of the leukemia-causing genes HOXA9 and MEIS1 [93, 173, 174] The first small molecule inhibitor of DOT1L that

entered human clinical development just recently is EPZ-5676 [175].

EZH2 mutations

EZH2 is the enzymatic component of the the Polycomb repressive complex (PRC) components and is an Histone 3 Lysine 27 (H3K27) methyltransferase. Overexpression of EZH2 has been reported in both solid tumors and blood cancers [176, 177] and has been shown to be due to, at least in part, the loss of transcriptional repression of specific microRNAs [177]. Missense, nonsense and frameshift mutations have been reported mainly in MDS [178, 179], while recently it was shown that almost half cases of early T-cell precursor acute lymphoblastic leukemia present mutations in histone-modifying genes, including EZH2 [180]. In AML, *EZH2* mutations have been described in a single case of acute myelomonocytic leukemia out of 143 cases screened [181], in a case with childhood AML [182] and recently in a male with CN-AML out of 50 screened [183]. The contradictory findings of overexpression of EZH2 in epithelial cancers and lymphomas and inactivating mutations in myeloid malignancies raises the possibilty that alterations affecting the methylation of H3K27 may be tumor specific. The effects of EZH2 mutations are still unknown and have only recently started to be under investigation. Initial findings though suggest that except for histone modifications, DNA methylation might also be affected, since EZH2 serves as a recrutiment platform for DNA methyltransferases and seems to be a prerequisite for DNA promoter methylation [184].

EZH2 targeted therapy: Development of selective inhibitors of histone methyltransferases, such as EZH2 have only recently begun. An S-adenosylhomocysteine hydrolase inhibitor named 3-Deazaneplanocin A (DZNep) has been shown to induce efficient apoptotic cell death in cancer cells and not in normal cells and to effectively deplete cellular levels of PRC2 components such as EZH2 while inhibiting associated histone H3K27 methylation [185, 186]. Combined DZNep and panobinostat treatment induced more depletion of EZH2 and more apoptosis in AML cells compared to normal CD34(+) bone marrow progenitor cells [187]. This compound has not reached yet the clinical trial setting.

Mutations lacking targeted therapy

It should be noted that not all known recurrently mutated in AML genes have been considered as possible targets for developing novel targeted therapeutics. A number of clinically relevant AML related mutations such of *TET2*, *ASXL1, WT1, p53* and *BCOR*, although of prognostic significance, are currently lacking known drug discovery activities [183, 188-190].

Other novel targeted therapies of interest

PLK1 aberrations in AML

Polo like kinases (PLK) are a family of four serine/threonine protein kinases that are critical regulators of cell cycle progression, mitosis, cytokinesis, DNA damage response and apoptosis [191]. They bind and phosphorylate proteins are that already phosphorylated on a specific motif recognized by the POLO box domains and interplay with Aurora kinases [192, 193].

PLK1 is the most well characterized member of PLK1 family and considered to be a master player of cell-cycle regulation during mitosis strongly promoting the progression of cells through mitosis. Characteristically, PLK1 regulates the mitotic licensing of centriole duplication in human cells and also DNA replication under stressful conditions, and anti-apoptotic activity through phosphorylation of Bcl-x(L) [194-196].

Overexressed PLK1 is thought to behave as oncoprotein [197]. PLK1 is commonly found overexpressed in a majority of samples from patients with acute myeloid leukemia compared with normal progenitors [198].

PLK1 targeted therapy: Early observations that PLK1 depletion could induce apoptosis in cancer cells led to discovery and development of PLK1 inhibitors with potent antitumor activity against solid and blood cancers [199-204]. PLK inhibition is now considered a promising strategy for the treatment of AML preferably combined with conventional antileukemic chemotherapy [205, 206]. First PLK1 inhibitors are currently in early clinical development in AML with promising early results. The first PLK1 inhibitor BI 2536 showed interesting clinical activity in patients with relapsed and treatment refractory AML in an early clinical trial [207]. Its

successor volasertib (BI 6727) demonstrated more favorable toxicity profile and potent anti-leukemic activity as monotherapy and in combination with low dose aracytin in heavily pre-treated AML patients and was taken to a current phase III clinical investigation [208, 209].

Conclusions

AML is a highly agressive heterogenous malignant disease, classified by recurrent genetic abnormalities that define subgroups of distinct biological and clinical features. However, therapeutic approaches have stuck to "one-size fits all" conventional chemotherapy because of lack of targeted therapeutic options. Although in solid cancers a few targeted therapies have advanced to the clinical practice during the last decade, AML has notoriously been left behind despite the fact that this disease was the first human cancer genome to be sequenced and molecularly characterised. Advancements of applied technologies in molecular biology and drug discovery offer hopes that progress will be made towards more rational therapeutic approaches in AML patients. This milestone in AML therapy can only be reached through well-designed clinical trials conducted by expert teams and targeted to well characterized disease subsets. Such studies must follow resource sparing approaches because of the rarity of target patient subgroups and the highly demanding nature of such trials [210].

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Conflict of interest

The authors have no conflict of interest to declare.

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