Molecular Genetic Pathways as Therapeutic Targets in Acute Myeloid Leukemia

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The heterogeneity of acute myeloid leukemia (AML) results from a complex network of cytogenetic aberrations and molecular mutations. These genetic markers are the basis for the categorization of cases within distinct subgroups and are highly relevant for the prediction of prognosis and for therapeutic decisions in AML. Clinical variances within distinct genetically defined subgroups could in part be linked to the interaction of diverse mutation classes, and the subdivision of normal karyotype AML on the basis of recurrent molecular mutations gains increasing relevance for therapeutic decisions. In parallel to these important insights in the complexity of the genetic networks in AML, a variety of diverse new compounds is being investigated in preclinical and clinical studies. These approaches aim to develop targeted treatment concepts that are based on interference with molecular genetic or epigenetic mechanisms. This review provides an overview on the most relevant genetic markers, which serve as basis for targeted therapy approaches now or might represent options for such approaches in the future, and summarizes recent results of targeted therapy studies.

Introduction

Due to the heterogeneity of acute myeloid leukemia (AML), with its variability of genetic markers a more detailed classification as the basis for therapeutic risk stratification gained importance in recent years. Subclassification and prognostic predictions were first possible based on cytogenetic abnormalities which are identified in approximately 55% of all adult patients with AML. These chromosomal abnormalities are heterogeneous and include numerical aberrations such as gains or losses of whole chromosomes as well as structural abnormalities or balanced translocations. Most recurrent chromosomal alterations could clearly be associated to clinical profiles,[1] such as the reciprocal t(8;21)/*AML1-ETO* (*RUNX1*/*RUNX1T1*), inv(16)/*CBFB-MYH11*, or t(15;17)/*PML-RARA* rearrangements with a more favorable prognostic impact or complex aberrations with ≥ 3 chromosomal abnormalities, which are associated with inferior outcome.[2]

However, in recent years several recurrent molecular markers were identified that allowed further subclassification and prognostic predictions in the vast majority of the 45% of AML patients who are observed with a normal karyotype.[3] These recurrent molecular markers span a wide spectrum of biological functions and range from activating mutations such as length mutations/internal tandem duplications of the *FLT3* gene (*FLT3*-LM/ITD) with the insertion of hundreds of nucleotides[4] to point mutations within the *RAS* protooncogenes.[5] Further examples are alterations of genes encoding transcription factors such as *CEBPA*[6,7] or mutations interfering with tumor suppressor pathways such as *Nucleophosmin (NPM1)* mutations[8] consisting of four basepair insertions in most cases. However, the clinical variety even within distinct molecular subgroups makes it important to consider and integrate the coincidence of different alterations, e.g., a favorable *NPM1* mutation detected together with an unfavorable *FLT3*-ITD.[9-11]

The identification of this variety of molecular markers in AML with normal karyotype led to new concepts that interpret leukemogenesis as the result of cooperating mutations. These theories of interaction,[12] which were based on experiments in the murine model and on large correlation studies, divided the diverse mutation subtypes into two main categories: Mutations of the first category (class I mutations) interfere with transcription and lead to a stop in differentiation—either by direct alteration of transcription factors due to gene fusions, such as in the core-binding-factor (CBF) leukemias or in *PML-RARA*–positive AML, or by indirect interference with transcription processes, e.g., *MLL* rearrangements. The second category (class II mutations) is represented by activating mutations that lead to increased cell proliferation, e.g., by stimulation of protein tyrosine kinases such as the *FLT3*-ITD.[13] This cooperation of diverse mutation classes seems to occur non-randomly, as distinct class I mutations cooperate preferentially with particular class II mutations.[14] In addition, the recent detection of the *NPM1* mutations revealed a third category of mutations affecting genes implicated in cell-cycle regulation or apoptosis.[15]

Thus, an improved molecular characterization of AML not only allows a more detailed subclassification and more exact prognostic predictions in many patients, but also provides the basis for future therapeutic approaches: whereas targeted therapy in AML was previously mainly restricted to the application of all-*trans* retinoic acid (ATRA) in pa-

tients with acute promyelocytic leukemia (APL) with the t(15;17)/*PML-RARA*, deeper insights in the variety of molecular markers, signalling pathways, and cooperating leukemogenic processes opened new perspectives for molecular targets that hopefully will lead to more individualized treatment concepts.

Possible Candidate Target Genes

Given the enormous genetic heterogeneity of AML the development of targeted therapeutic approaches remains challenging. The definition of genetic pathways that could be detected in patients with diverse kinds of mutations might pave the way to therapeutic approaches being suitable for diverse AML subtypes. However, this first requires the standardized categorization of an increasing number of molecular mutations to achieve deeper insights into common principles of leukemogenesis and interaction in the heterogeneous disorder of AML. Such categorization can, for example, be performed according to the two-hit model, which implies that mutations leading to interference with transcription cooperate with mutations resulting in increased cell proliferation.[14]

Mutations interfering with transcription

The first category of genetic alterations that either directly affect the function of transcription factors or show indirect interference with transcription[12] includes a variety of mutational subtypes ranging from reciprocal translocations as in the CBF leukemias over partial tandem duplications as in the *MLL*-PTD to various point mutations, deletions, or insertions as seen for the *RUNX1*-gene mutations.

Most frequent examples are found for CBF leukemias—inv(16)/t(16;16)/*CBFB-MYH11* and t(8;21)/*RUNX1*/*RUNX1T1* with frequencies of 6% to 10% of all AML cases, respectively. These rearrangements result in chimeric proteins with the consequence of suppressed transcription.[16] In APL, the *PML-RARA* fusion disrupts the normal interaction of retinoic acid and *RARA*, so *RARA* cannot be converted into a transcription activator.[17] The prognosis of these three AML subtypes is more favorable,[18] at least when they are not seen in combination with prognostically unfavorable markers, such as *FLT3*-ITD in APL[19] or *KIT*-D816 mutations in *RUNX1*/*RUNX1T1*-positive AML.[20]

Also, interchromosomal rearrangements of the *MLL* gene with a variety of partner genes ("*MLL*/11q23 rearrangements")[21] belong to this category, as they interfere with the normal function of *MLL* in the regulation of transcription and for *HOX*-gene expression.[22] In contrast, partial tandem duplications of the *MLL* gene (*MLL*-PTD) are intragenic *MLL* abnormalities, which occur mainly in normal karyotype AML (5%-10%). They are interpreted as gain-of-function mutations.[23] Both interchromosomal and intragenic *MLL* abnormalities are prognostically adverse.[24-26]

Interference with transcription can further be mediated by somatic mutations of genes that code for transcription factors. Both the *CEBPA* and the *RUNX1* gene mutations (= *AML1*, located on 21q22) can preferentially be detected in normal karyotype AML and have a frequency of ~9% and ~6% in all AML patients, respectively. The *CEBPA* -gene encodes a leucine-zipper transcription factor (CAAT/enhancer-binding protein α) and is essential for regulation and differentiation of granulopoiesis. There are two subtypes of *CEBPA* mutations: one affects DNA binding and dimerization with other CEBP proteins, the other results in premature termination of the CEBPA protein[6] with the consequence of complete loss of *CEBPA* function by dominant negative inhibition of wild-type *CEBPA* DNA binding. When occurring as isolated mutations in AML with normal karyotype, *CEBPA* mutations predict a favorable prognosis.[7,27] In addition, the *RUNX1* gene can be affected by a variety of point mutations, insertions, or deletions (*RUNX1* mutations), which have to be separated from the reciprocal rearrangements within the "classical" CBF leukemias (**Table 1**).

Also, dysregulation of the *HOX* genes, which modify transcription of target genes, contributes to leukemogenesis in AML. A recent study showed aberrant expression of *CDX2* ("caudal-type homeobox gene") in most AML patients. Due to the regulatory function of the *CDX2* gene for *HOX* gene expression during developmental hematopoiesis, aberrant *CDX2* expression might be associated with altered *HOX* gene expression.[28] In patients with normal karyotype AML, *CDX2* expression was determined to be 14-fold higher when compared with the reciprocal t(8;21)/*RUNX1*/*RUNX1T1* and t(15;17)/*PML-RARA* rearrangements.[29]

Activating mutations

The second category of mutation mechanisms in AML is represented by activating mutations. Here, mutations within the *FLT*3 gene, which code for the class-III-receptor kinase FLT3, have to be mentioned first as they are ranked within

Table 1. Examples of cooperating mutations modified according to the two-hit model of Gilliland et al.[13]

Class I mutations (interfering with transcription)	Cooperating class II mutations (mediating proliferation)
PML-RARA/t(15;17)	*FLT3*-ITD *FLT3*-TKD
RUNX1/*RUNX1T1*/t(8;21) or *RUNX1* rearrangements with other partner genes	*KIT*-TKD mutation *JAK2*V617F *FLT3*-ITD
CBFB-MYH11/inv(16)/t(16;16)	*KIT*-TKD mutation *NRAS* mutation
CEBPA mutations	*FLT3*-ITD
RUNX1 mutations (= *AML1*, located on 21q22)	*FLT3-ITD*

the most frequent recurrent known genetic markers in AML. They function by autophosphorylation of the FLT3 receptor tyrosine kinase, which results in cell proliferation, inhibition of apoptosis, and activation, e.g., of the *STAT* signalling pathway.[30] The prognostically adverse *FLT3*-ITD[4,31,32] are seen in 25% to 35% of all patients and occur as variable insertions of 3-200 base pairs. They are localized in the region that codes for the juxtamembranous region of the FLT3 receptor,[33] whereas the less frequent *FLT*3-tyrosine kinase domain mutations (*FLT3*-TKD) are localized in the region coding for the activation loop. In contrast to the *FLT3*-ITD, where the unfavorable prognostic impact was clearly assessed by numerous large studies,[4,31,32] the prognostic impact of the *FLT3*-TKD is still considered controversial[34] but seems to depend on the genetic background of cooperating mutations.[35] Mutations of the *KIT*-TKD represent another class III receptor tyrosine kinase mutation in AML. They are mostly caused by a missense amino acid substitution in exon 17 of the activation loop.[36] Although they occur rarely in AML (less than 2% of all AML), they are more frequently found in the CBF leukemias.[20,37] In the t(8;21)*/RUNX1/RUNX1T1* they confer a negative prognostic impact.[38]

Mutations in the *RAS* oncogenes occur in many malignancies. They have a central role in the regulation of cell cycle and differentiation and lead via the RAS proteins to constitutive activation of the *RAS* pathway.[39] In AML the *NRAS* subtypes are predominant when compared with *KRAS* and *HRAS* mutations as they are detected in 11% to 25% of all cases. Prognosis is influenced by *NRAS* mutations in some cytogenetic and molecular subgroups only.[40]

Another activating mutation is represented by the somatic V617F mutation of the *JAK2* gene, which activates the JAK2 tyrosine kinase and the *JAK-STAT* pathway. This mutation primarily shows a close association to various chronic myeloproliferative disorders (CMPD), whereas in AML the incidence is low (only 2% to 8%). Mutated cases are mostly seen in secondary AML after preceding CMPD[41,42] and seem to be associated with the t(8;21).[42,43]

Mutations interfering with cell cycle and apoptosis

The third category—mutations that interfere with cell cycle regulation and apoptosis—contains two main genetic alterations:[15] *NPM1* mutations and *TP53* deletions. Mutations of the *NPM1* gene are the most frequent known genetic alterations in AML (in 35% of all cases) and have a specific association with normal karyotype (55% mutated). There are more than 40 diverse mutational subtypes, which mostly consist of four basepair insertions.[8] The sole consequence of the diverse *NPM1* subtypes is the generation of a nuclear export signal that relocalizes the NPM1 protein from the nucleus to the cytoplasm. This aberrant cytoplasmatic localization inhibits its normal shuttle function between nucleus and cytoplasm, which is essential for its participation in a certain (ARF-P53) tumor suppressor pathway. However, the leukemogenic mechanism of the *NPM1* mutations is not yet fully understood, as the NPM1 protein is also involved in other cellular processes such as the regulation of centrosome function or the processing of pre-RNA molecules.[44]

If detected alone, *NPM1* mutations have a favorable prognostic impact when compared with *NPM1*-unmutated normal karyotype AML.[8-11,45] It was recently shown that the incidence of the *NPM1* mutations is age-dependent as the mutation has not yet been identified in children younger than 3 years old, whereas the frequency is 10% to 19% in children older than 3 years and exceeds 30% in children older than 10 years. Interestingly, the distribution of the different mutation classes is also different in adults and children: adults show most frequently the mutational subtype A, whereas in children type B is more frequent.

This third category also contains deletions of the *TP53* tumor suppressor gene. This gene is localized on 17p, and the mutations are usually represented by deletions or missense point mutations. In *de novo* AML the frequency is greater than 10%, but the incidence is higher in therapy-associated myeloid malignancies and AML with complex karyotypes,[2,46] and also increases greatly with higher age.[47] Of course, overlaps between these diverse mutation categories have to be assumed. Thus, it might be hypothesized that several class II mutations such as the *FLT3*-ITD might be involved in the regulation of apoptosis or other processes that are associated with the third mutational class.

Other Molecular Markers

In recent years attention has also been focused on additional molecular markers, although their role in leukemogenesis is still not completely defined. One of these is the Wilms' tumor gene (*WT1*), which encodes a zinc-finger DNA binding protein. Since it might act as tumor suppressor gene or as oncogene, functional duality is assumed as it could either be involved in transcriptional activation or in suppression of differentiation. *WT1* is highly expressed in various leukemia types, particularly in AML.[48] In addition, 10% of AML patients carry mutations of *WT1* that show a strong correlation to normal karyotype. There seems to be an association with failure to achieve complete remission; therefore, *WT1* mutations might be included in the risk stratification of patients with normal karyotype.[49]

The *BAALC* (brain and acute leukemia, cytoplasmic) gene is primarily expressed in neuroectoderm-derived tissues and in hematopoietic precursors and encodes a protein with no homology to any known proteins or functional domains. It is overexpressed in different hematologic malignancies. High expression of the *BAALC* gene was found to correlate in normal karyotype AML with a negative prognosis, so this genetic marker might as well be a candidate for risk assessment at diagnosis of normal karyotype AML.[50]

Also, overexpression of the *ERG* (ETS-related),[51] *MN1*,[52] and *EVI* (ecotropic virus integration-1)[53] genes seems to be associated with an unfavorable outcome in normal karyotype AML. Thus, it is possible that for future risk stratification, quantitation of the expression of these genes in patients without cytogenetic abnormalities will be relevant, but further studies will have to be performed for definite conclusions.

Coincidence of Molecular Mutations in Normal Karyotype AML

Risk stratification by molecular markers in patients with normal karyotype AML plays an increasing role at diagnosis. The most relevant molecular markers are the *NPM1* mutations in approximately 55%, the *FLT3*-ITD in approximately 40%, *MLL*-PTD (6%), *NRAS* (8%-10%), *CEBPA* (10%), and *FLT3*-TKD mutations (6%).[7,24,25,27,31,35,40,54] These are either found alone or in non-random combinations (**Figure 1**; see Color Figures, page 506). This can be exemplified by the *FLT3*-ITD, which show frequent coincidence with the *NPM1* mutations[9-11] or the *MLL*-PTD.[55] Thus, a rather limited number of molecular markers further subclassifies more than 85% of normal karyotype AML.[3,56] This gives hope that a limited number of molecular targets or pathways might be sufficient to reach the majority of these patients.

Recently, Schlenk et al demonstrated that patients with normal karyotype AML under the age of 60 years with isolated *NPM1* mutations and without *FLT3*-ITD did not benefit from allogeneic stem cell transplantation (SCT) in first remission. This was also true for those with isolated *CEBPA* mutations. However, when the *FLT3*-ITD and the *NPM1* mutation occurred together, survival was significantly improved when allo-SCT was performed. Patients with the prognostically unfavorable *MLL*-PTD also had a significant benefit from SCT.[45] In conclusion, these data suggest that molecular screening may allow "targeted allo-SCT" in AML with normal karyotype.

Coincidence of Target Genes with Other Molecular and Cytogenetic Alterations

The above coincidence of the *NPM1* mutations with other molecular markers in normal karyotype AML illustrates that genetic alterations cannot be seen in isolation—their combination is also important. This is true not only with respect to their role in leukemogenesis, but also with respect to possible targeted therapeutic approaches. Thus, research activities should focus first on the coincidence of diverse molecular alterations and also on the coincidence of molecular and chromosomal aberrations for deeper insights into genetic pathways as possible targets for therapy. Further, the coincidence of diverse genetic alterations reveals some explanations for the varying clinical courses within distinct genetic subgroups: *FLT3*-ITD have a negative prognostic impact in cases with *NPM1* mutation when compared *NPM1*-mutated/FLT3 wild-type AML.[9-11,45,57] *FLT3*-ITD are also observed in more than 30% of all *MLL*-PTD–positive cases[55] and in 40% of all t(15;17)/*PML-RARA* AML. The coincidence of the *FLT3*-ITD and the *PML-RARA* seemed to confer an inferior prognostic impact in patients with APL, which might also have been due to the higher frequency of the FAB M3v subtype in these comutated cases. Further, these double-mutated cases are more frequently observed with the *PML* breakpoint bcr3 when compared to bcr1,[19] which suggests that the *FLT*3 mutations cooperate specifically with certain *PML-RARA* transcript subtypes. This coincidence of a class III receptor tyrosine kinase mutation with a type I mutation according to the model of cooperation should be considered in future therapy concepts for *FLT*3-ITD$^+$ APL,[58] as targeting the *FLT3*-ITD in a subgroup of APL patients might improve outcome in this less favorable subgroup.

A similar example is represented by the coincidence of mutations of the *KIT*-receptor class III mutation with the CBF leukemias, as they are found in more than 40% of cases of the t(8;21)/*RUNX1*/*RUNX1T1* and the inv(16)/*CBFB-MYH11* in contrast to less than 2% in overall AML.[20,37] Also, activating *NRAS* mutations are significantly more often observed in inv(16)/*CBFB-MYH11* with a predominance of codon 61 in the double-mutated cases.[40,59]

However, these are only some of the various coincidences of molecular genetic alterations that were discovered in AML in the last decade. The various correlations of molecular aberrations with distinct cytogenetic aberrations also deserve attention: deletions of the *TP53* tumor suppressor gene are strongly linked with abnormalities of the chromosomes 5q and 7q, and often occur in the context of a complex aberrant karyotype,[2,46,47] which means that a common molecular target is available in a high proportion of prognostically adverse AML cases. The frequent occurrence of the *MLL*-PTD in trisomy 11 cases or of *RUNX1* mutations in trisomy 13[60] are further examples that emphasize the need for a combined molecular and cytogenetic approach when possible targets for novel therapies are investigated (**Table 2**).

Targeted Therapy

Although the vast majority of AML patients can be individually characterized on the basis of distinct chromosomal aberrations and molecular markers, treatment of AML is still based on quite unspecific cytotoxic therapy. For a long time targeted therapy was only available for APL. However, the increasing potential of known genetic markers and of altered signalling pathways provides the background for novel targeted concepts, and various compounds and strategies are currently being investigated in various clinical studies (**Table 3** and **Table 4**).

Targeting mutations interfering with transcription

APL represented the first model of targeted therapy in leukemia.[61] High-dose ATRA is able to overcome the differentiation stop that is mediated by the *PML-RARA* fusion,[17] and in combination with anthracycline-based chemotherapy more than 80% of patients are cured. In contrast, arsenic trioxide induces degradation of the fusion protein encoded by the *PML-RARA* oncogene and induces differentiation of leukemic cells.[62] It was suggested that the combination of ATRA and arsenic trioxide might be able to cure the disease in patients with a low risk profile, and that anthracycline- or cytarabine-based therapies with their toxicity profiles might be restricted to patients with an increased relapse risk.[63] Indeed, a recent study in 78 patients with APL showed that the omission of anthracyclines in case of peripheral leukocytes $< 10 \times 10^9$/L at diagnosis did not worsen prognosis. Thus, it seems that in the t(15;17)/*PML-RARA*$^+$ AML not only a targeted but also risk-adapted therapeutic approach is feasible.

Table 2. Correlation of molecular markers with diverse cytogenetic subgroups in AML.

Molecular marker	Correlation to cytogenetic subgroup	Frequency of molecular alteration, %
KIT mutation	t(8;21)/*RUNX1*/*RUNX1T1*	~25
	inv(16)/t(16;16)/*CBFB-MYH11*	~25
FLT3-ITD	t(15;17)/*PML-RARA*	~40
	t(6;9)/DEK-CAN	~90
NRAS mutation	inv(16)/t(16;16)/*CBFB-MYH11*	~35
	inv(3)/t(3;3)	~25
RUNX1-mutation	Trisomy 13	~80
TP53 mutation/ deletion	Complex karyotype, deletion 5q/-5, deletion 7q/-7	10-15

Targeting Receptor Tyrosine Kinase Signalling

Receptor tyrosine kinase (RTK) signalling pathways play a central role in the pathogenesis of AML. Thus, various approaches focus on targeting RTKs, e.g., the FMS-like tyrosine kinase (FLT3), KIT, and signal transduction via the phosphoinositide 3-kinase (*POI3K*) and mitogen-activated protein kinase (*MAPK*) pathways.[64]

Targeting FLT3-kinase signalling

First, the high incidence of the *FLT*3-mutations in AML (35%-40%) drew attention on the development of specific FLT3-tyrosine kinase inhibitors. A wide range of compounds targeting the FLT3-RTK was investigated,[65,66] which all have potent activity for *FLT3*-ITD but vary in their selectivity and specificity with respect to inhibition of other kinases. The indoline tyrosine kinase inhibitors SU5416 and sunitinib (SU11248),[67-69] the small molecular FLT3-inhibitor PKC412,[70,71] and sorafenib (Bay 43-9006)[72] are considered rather unspecific inhibitors of FLT3 kinase signalling. In comparison, CEP-701 or tandutinib (MLN518)[73] seem to be more selective for the FLT3 kinase.

Table 3. Concepts for targeted therapy for diverse subentities of acute myeloid leukemia (AML).

Target / AML Subentity	Compound	Mechanism
PML-RARA in acute promyelocytic leukemia with the t(15;17)(q22;q21)	All-*trans* retinoic acid (ATRA)	- Suppression of the differentiation stop mediated by the *PML-RARA* fusion - Induction of differentiation
	Arsenic trioxide (AS_2O_3)	- Degradation of the fusion protein encoded by the *PML-RARA* oncogene - Induction of differentiation
FLT3 mutated AML	Unspecific FLT3-inhibitors: - indoline tyrosine kinase inhibitors (SU5416; sunitinib - SU11248) - small molecular compounds: PKC412 Specific FLT3-inhibition: - tandutinib (MLN518), CEP701	- Inhibition of downstream signaling *via MAPK* and *STAT* pathways - Reduction of cellular proliferation - Increase of apoptosis - Induction of cell cycle arrest
CD117- positive or *KIT*-mutated AML *KIT* mutations in CBF leukemias (*RUNX1*/*RUNX1T1*; *CBFB-MYH11*)	Imatinib Dasatinib	- Prevention of autophosphorylation of the c-KIT receptor tyrosine kinase - Activation of downstream signaling *via* MAPK and Akt pathways
Constitutive activation of *MAPK* (Mitogen-activated protein kinase) signaling, e.g., in *RAS* mutated AML	Farnesyltransferase inhibitors: - tipifarnib (R1157777) - lonafarnib	- Inhibition of farnesyl transferases - Inhibition of Ras binding to the cell membrane - Induction of apoptosis - Inhibition of anchorage-independent growth
DNA hypermethylation	Demethylating agents: - 5-azacytidine - decitabine (5-aza-2'-deoxycytidine)	- Reduction of hypermethylation - Reexpression of tumor suppressor genes
Deacetylase activity	Histone deacetylase (HDAC) inhibitors: - valproic acid	- Induction of histone hyperacetylation - Induction of apoptosis and differentiation - Downregulation of *c-MYC* expression
NPM1 mutated / *FLT3*wt AML without adverse chromosomal abnormalities	ATRA (in addition to cytotoxic therapy)	- remains to be determined

Table 4. Results of some recently published targeted clinical trials in acute myeloid leukemia (AML).

Treatment approach	Reference	Patients	Treatment	Results	Interpretation /conclusion
FLT3 inhibitor PKC412 (phase 2)	Stone et al, 2005[71]	20 *FLT3* mutated refractory or relapsed AML pts	3 × 75 mg daily p.o. until toxicity or disease progression occurs	Clinical benefit: 70% PR: 33%	Toxicity acceptable (2 pts fatal pulmonary events); promising for *FLT3* mutant AML.
ATRA plus ATO in APL (+ GO)	Estey et al, 2006[63]	25 pts with low-risk APL (leukocytes =10 × 10^9/L); (leukocytes >10 × 10^9/L)	Low-risk APL: • induction: ATRA (45 mg/m^2 daily from d 1) • post-remission: ATRA + ATO without chemotherapy up to 28 wks; MRD at 3 mos positive: + gemtuzumab ozogamicin (GO) High-risk APL: as low risk and always + 9 mg/m^2 GO d 1	Complete remission: low-risk APL: 96% Relapses: 8% Post-induction GO only in 3 high-risk pts necessary	ATRA plus ATO represents an alternative to chemotherapy in low- may improve outcome in high-risk APL.
FLT3-antagonist tandutinib (MLN518) (phase 1)	DeAngelo et al, 2006[73]	40 pts with refractory or relapsed AML/high-risk MDS; of these 8 *FLT3*-mutated	2 × 50 – 2 × 700 mg daily p.o. (dose escalation) maximum 12 mo	Antileukemic effects in 2/5 evaluable pts with FLT3-ITD; no effect in FLT3-wildtype	Tandutinib should be evaluated more extensively in pts with *FLT3*-mutant AML.
Imatinib and low-dose cytarabine in older patients with c-KIT–positive AML/high-risk MDS (phase 2)	Heidel et al, 2007[81]	34 AML, 6 MDS; 38 pts evaluable; median age 73 years	Imatinib 600 mg daily p.o. Cytarabine 10 mg s.c. daily days 1–21 every 28 d Maximum 12 mo	Stable disease: 21% Hematological response: 8% 2 year OS: 20%	Imatinib + low-dose cytarabine seems comparable to myelosuppressive therapy, but was not superior to low-dose Ara-C monotherapy; pts have to be selected better
Decitabine alone or in combination with valproic acid in AML (phase 1)	Blum et al, 2007[95]	25 AML: at diagnosis (12) or relapse (13); 21 pts - evaluable; median age 70 y	Decitabine 20 mg/m^2 iv. d 1–10 alone or combination with dose escalating valproic acid d 5-21: 20-25 mg/d	Hematological response: 52% CR: 19%	Low-dose decitabline seems safe and is encouraging in AML. Additional valproic acid led to in encephalopathy relatively low doses.
Farnesyltransferase inhibitor tipifarnib in refractory or relapsed AML (phase 2)	Harousseau et al, 2007[89]	252 pts: refractory (117), at relapse (135), median age 62 y	Tipifarnib 2 × 600 mg d1-21 p.o.; every 4 weeks Treatment possible until progression or unacceptable toxicity Dose reduction to 2 × 200 mg, or escalation to 2 × 900 mg possible	OR: 12% CR: 4% Median CR duration: 17.3 mo Median survival of CR pts: 369 d	Tipifarnib was associated with prolonged survival in pts with refractory or relapsed AML. Higher response rate, however, requires combination with other active agents.
Farnesyltransferase inhibitor tipifarnib in untreated, poor-risk elderly AML (phase 2)	Lancet et al, 2007[90]	158 elderly pts with poor-risk AML; median age 74 y	Tipifarnib 2 × 600 mg d 1–21 p.o.; rest period up to 42 d before second course maximum 4 cycles for responding pts	OR: 23% CR: 14% Median CR duration: 7.3 mo Median survival of CR pts: 18 mo	Tipifarnib is active and well tolerated in older pts with poor-risk AML. Responders might have a survival benefit.

Abbreviations: ATRA, all-*trans* retinoic acid; ATO, arsenic trioxide; GO, gemtuzumab ozogamicin; APL, acute promyelocytic leukemia; MDS, myelodysplastic syndrome

Although these FLT3 inhibitors are very heterogeneous with respect to chemical structure, pharmacokinetics, toxicity profile, and efficacy, all compounds share some characteristics: They reduce the proliferation rate and induce an increase of apoptosis due to upregulation of proapoptotic proteins.[72] The higher *in vitro* efficacy against *FLT3* mutants when compared to *FLT3* wildtype suggests a selective effect toward *FLT3* mutant cells.[66]

However, when applied as monotherapy in clinical studies, response to FLT3 inhibitors was limited and transient mostly due to the development of resistance.[66,68,69,71,73] So far there is also no clear evidence that relates response to these agents, when observed, to inhibition of farnesyl transferase enzyme activity. As the mechanism through which responses are observed is not yet understood, more information may help to select patients for this treatment option. Furthermore, approaches are developed to enhance the effects of FLT3-kinase inhibitors by combining them with compounds showing interference with phosphatidylinositol 3-kinase signaling.[74] When FLT3 inhibitors are combined with other anticancer drugs,[75,76] it should be kept in mind that FLT3 inhibitors show different effects on the cell cycle when *FLT3* mutations are present: in cell line experiments, the small molecular compound PKC412 induced massive apoptosis when *FLT3* mutations were present, whereas in AML cell lines with *FLT3* wildtype the G_2 phase was blocked.[75]

Heidel et al performed screening for resistance mechanisms in 6 patients with AML who developed relapse during PKC412 treatment. Persisting tyrosine kinase phosphorylation of *FLT3* was detected despite sufficient serum levels of PKC412. An algorithm of additional analyses to reveal the underlying resistance mechanism allowed the identification of a single amino acid substitution—an N676K—within the N-lobe of *FLT3* as the sole cause of resistance to PKC412 in 1 of 6 patients.[77]

Recently, a study using a high-throughput next-generation sequencing platform revealed a variety of previously unknown *FLT3* mutations within the juxtamembranous domain, the catalytic domain, and, rarely, in the activation loop. Although these novel *FLT3* mutations are quite rare, this study suggests that novel sequencing strategies could identify some additional patients who might benefit from FLT3 inhibition.[76]

Targeting the KIT receptor kinase

Targeting of the *KIT*-RTK is another promising approach because the c-KIT stem cell factor CD117 is expressed in more than 70% of all AML[78] and *KIT* mutations occur in more than 40% of the CBF leukemias. Imatinib, which is known primarily because of its inhibitory function on the BCR-ABL tyrosine kinase, also inhibits *KIT* by prevention of autophosphorylation and by inhibition of downstream signaling *via* the *MAPK* and *AKT* pathways. However, the results of preclinical studies of imatinib in *c-KIT*$^+$ AML were controversial—in some studies there was little effect in *KIT*-mutated AML,[79] whereas another study showed an *in vitro* dose-dependent increase in apoptosis in *KIT*-mutated cells.[80] Several case studies demonstrated a potential benefit of imatinib treatment for *KIT* non-mutated but CD117-expressing AML.[78] In a study of 40 elderly patients with AML or high-risk MDS showing *c-KIT* expression on the blasts (as determined by CD117 expression by multiparameter flow cytometry), the combination of low-dose cytarabine and imatinib was associated with 2-year survival of 20%, which was comparable to the results of standard myelosuppressive therapy when compared with historical studies.[81]

Due to the negative prognostic impact of *KIT* mutations in CBF leukemias imatinib represents a potential drug in comutated cases.[36] In addition, PKC412 has been shown to be effective for AML with *KIT* tyrosine kinase domain (TKD) mutations in *CBFB-MYH11* as in the t(8;21).[20] Additionally, a subset of pediatric patients might benefit from imatinib therapy, as juxtamembrane mutations in *KIT* were recently reported in 7% of children with AML,[82] whereas adults show mutations in the kinase domain. In cell lines that had been transfected with this novel *KIT* mutation, a combination of imatinib and rapamycin had a synergistic effect to suppress proliferation.[82] However, the effectivity of imatinib is limited, as the majority of mutated cases in AML show D816 mutations within the tyrosine kinase domain (in exon 17) that are intrinsically resistant to imatinib,[83] although they seem to confer an inferior prognostic impact when compared with other *KIT* mutation subtypes in exons 8 and 11.[38] Cell line experiments suggest that the dual SRC/ABL kinase inhibitor dasatinib might have clinical efficacy against both activation loop and juxtamembranous KIT mutants in hematological malignancies by inhibition of autophosphorylation and downstream signaling.[84]

Targeting farnesyltransferase inhibitors

Also, the mitogen-activated protein kinase (*MAPK*) signaling cascade is constitutively activated in a high proportion of AML cases. Activation of the MAPK signaling cascade can be mediated by the *RAS* mutations—*NRAS*, *KRAS*, or *HRAS*—which are found in approximately 15% in AML, when all subtypes are summarized.[85] Besides being targeted by inhibitory compounds such as sorafenib, which have rather unspecific effects on several pathways of tyrosine kinase signaling, the MAPK pathway can be attacked by interference with RAS protein function. Binding of the RAS proteins to the cell membrane requires structural modifications that are catalyzed by specific enzymes. Inhibition of these enzymes by farnesyltransferase inhibitors (FTI) was demonstrated to result in induction of apoptosis and to decrease proliferation.[86] Several farnesyltransferase inhibitors have already entered clinical trials such as tipifarnib (R1157777) as monotherapy, which showed clinical re-

sponse rates of 29% in 35 patients with refractory or relapsed AML.[87,88] Even complete remissions were seen in single patients.[89] Also, in 158 elderly patients with poor-risk AML, tipifarnib as monotherapy was well tolerated and achieved response rates of > 20%.[90] Although these first results seem encouraging, tipifarnib so far has not been approved for the application in AML.

Combinations of various compounds targeting distinct pathways are currently investigated. Cell line experiments suggested that a combination of tipifarnib and bortezomib has synergistic effects in AML.[91]

Epigenetic Pathways

Targeting hypermethylation

Research is also focused on epigenetic strategies. DNA methylation is relevant for the control of gene expression, whereas aberrant DNA hypermethylation in leukemias results in suppression of gene function.[92] Therefore, concepts arose that DNA demethylation might result in the reactivation of aberrantly silenced genes, e.g., with tumor suppressor function, and thus might indirectly lead to suppression of the leukemic clone.[93] Indeed, hypomethylating compounds such as the pyrimidine analogues 5-azacytidine or decitabine (5-aza-2'-deoxycytidine) showed significant benefit in myelodysplastic syndrome with respect to survival and transformation rates in several Phase III trials.[94] Also, in 158 patients with AML, more than 40% of patients at diagnosis or at relapse showed response to decitabine, which suggests that demethylating agents are a promising approach in myeloid malignancies.[95]

Targeting histone acetylases

Inhibition of the activity of histone deacetylases (HDAC) is another promising approach. HDAC are part of processes for coiling and uncoiling of DNA around histone proteins as a precondition for gene expression. Blockade of these coiling/uncoiling mechanisms can be mediated by HDAC inhibitors, leading to histone hyperacetylation and to an alteration of chromatin structure and gene expression. A variety of HDAC inhibitors are being investigated at this time, e.g., valproic acid,[96-98] vorinostat (suberoylanilide hydroxamic acid),[93] and LBH589.[99] The antileukemic effects of HDAC derive partly from various epigenetic mechanisms such as the induction of DNA damage and reactive oxygen species or acetylation of the heat shock protein (hsp90; a chaperone protein that is required for the proper folding and maintenance of diverse signaling protein kinases).[100,101]

It seems attractive to combine HDAC inhibitors, such as sodium phenylbutyrate, with demethylating compounds such as 5-azacitidine. This approach produced an overall response rate of 38% in 29 evaluable patients with MDS and AML in a study by Gore et al.[102] However, the tolerance for the combination of HDAC and demethylating compounds seems to be limited by side effects such as encephalopathy.[95,103] The combination of valproic acid and the anti-CD33 compound gemtuzumab ozogamicin might represent another promising strategy, as suggested by *in vitro* studies on AML cell lines.[104]

Other Approaches

In 2004, Schlenk et al published a study on 242 patients with AML older than 60 years with diverse cytogenetic and molecular subgroups other than APL where the addition of ATRA to induction and consolidation therapy improved CR rates and outcome significantly.[105] Now, retrospective correlation studies in this cohort showed that the subgroup of *NPM1*-mutated but *FLT3*-unmutated patients and without adverse karyotype alterations had the only significant benefit from the addition of ATRA. No other subgroups—those without *NPM1* mutations or those with *FLT3* mutations or *MLL*-PTD—benefitted from additional ATRA when compared with patients treated with standard protocols. The AMLSG (Ulm, Germany) will soon address this topic in clinical studies in younger adults. The relevant mechanism underlying the apparent interaction of isolated *NPM1* mutations and ATRA has not yet been determined.

Application of antisense drugs abolishing the function of distinct genetic sequences has only recently entered clinical studies. The antisense oligonucleotide oblimersen, which was first investigated in chronic lymphocytic leukemia, targets overexpression of the antiapoptotic protein BCL-2. Clinical trials were also performed in AML, either in combination with cytotoxic chemotherapy[106] or in combination with gemtuzumab ozogamicin,[107] but definite conclusions cannot yet be drawn.

Perspectives and Conclusions

The vast majority of AML cases can by now be categorized in clinically and prognostically relevant subgroups by the integration of cytogenetic aberrations and molecular mutations. The variability of clinical courses within certain subgroups is at least in part due to deeper insights into coincidences of these chromosomal and molecular aberrations. Thus, leukemogenesis is conceived as resulting from the interaction of diverse mutational subtypes[13] (**Table 1**) and from complex interactions of signaling pathways. Also, epigenetic mechanisms are part of this novel perception of leukemogenesis.[92,98]

In parallel to the improved understanding of genetic and epigenetic pathways it became obvious that the complex system of leukemogenesis of AML shows many points of convergence: these refer to common signalling pathways, e.g., *STAT,* which is activated in most AML cases,[41] coincidences of diverse molecular markers as of molecular and cytogenetic aberrations, or common epigenetic mechanisms, e.g., hypermethylation.[95] This all indicates that a

limited number of targeted strategies might be able to reach a specific group of patients across the borders of the cytogenetically defined subtypes only.

Understanding of the molecular pathogenesis of AML further benefits from gene expression studies. This can be illustrated by a recent study in normal karyotype AML, where an association between a distinct microRNA signature and the expression of genes known to be involved in innate immunity was shown. It seemed that the downregulation of a microRNA family could be linked to an aggressive leukemia phenotype. The relevant mechanisms were associated with the activation of pathways that are controlled by toll-like receptors and cytokines such as interleukin-1β.[108]

These detailed insights into leukemogenic aberrations and pathways provide the basis for the development of compounds and strategies to target genetic mutations or epigenetic pathways. Examples are the FLT3-tyrosine kinase inhibitors for *FLT3*-mutated cases[68,69] or imatinib for *KIT*-mutated cases[36] or demethylating compounds.[95] These are in part already transferred to clinical application; others are still being tested in preclinical studies.

Thus, future therapy in AML will hopefully be based more on genetic and epigenetic targets that will be individually defined for each patient. However, many scenarios remain to be tested: (1) Should these compounds be applied solely or in combination with cytotoxic chemotherapy? (2) How should they be combined with conventional strategies, e.g., should demethylating compounds be combined with standard induction therapy? (3) Should these novel strategies also be tested in combinations such as hypomethylating agents and farnesyltransferase inhibitors? (4) Is post-remission therapy or maintenance treatment successful?

One shortcoming also has to be mentioned: The discrepancy between the efficacy of potential drugs in preclinical studies and the limited or transient response when these compounds are applied to patients. The existence of as yet unknown cofactors of genetic or epigenetic origin might provide one explanation. Hopefully, novel technologies such as gene expression analyses on a microarray basis[109] might be able to fill these gaps in the near future by the definition of novel molecular targets or by medication-specific sensitivity assays.[110,111] Raponi et al determined a distinct gene expression ratio (*RASGRP1*/*APTX*) that was predictive for the response to the farnesyl transferase–inhibitor tipifarnib with the greatest accuracy using a "leave one out" cross-validation. The utility of this classifier to predict the response to tipifarnib was validated in an independent set of 58 samples from patients with relapsed or refractory AML.[111] This example of a 2-gene expression assay illustrates the potential of gene expression analyses to identify new classifiers for a more accurate definition of treatment strategies.

Thus, the development of new diagnostic techniques and research for new therapeutic targets should be conceived as integral parts, as only their perfect interaction will be able to pave the way to targeted treatment for patients with AML.

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