review

The challenge of risk stratification in acute myeloid leukemia with normal karyotype

Syed Z. Zaidi,[a] Tarek Owaidah,[b] Fahad Al Sharif,[c] Said Y. Ahmed,[c] Naeem Chaudhri,[c] Mahmoud Aljurf[c]

From the [a]Adult Hematology & SCT Department, Prince Sultan Hematology Oncology Center, King Fahad Medical City, Riyadh, Saudi Arabia, [b]Department of Pathology & Laboratory Medicine and [c]Section of Adult Hematology/HSCT, King Faisal Cancer Center, King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia

Correspondence and reprints: Mahmoud Aljurf, MD, MPH · Section of Adult Hematology & HSCT King Faisal Cancer Center (MBC 64), King Faisal Specialist Hospital & Research Centre · PO Box 3354, Riyadh 11211, Saudi Arabia · T: +966-1-442-3937 F: +966-1-442-3941 · maljurf@kfshrc.edu.sa · Accepted for publication June 2008

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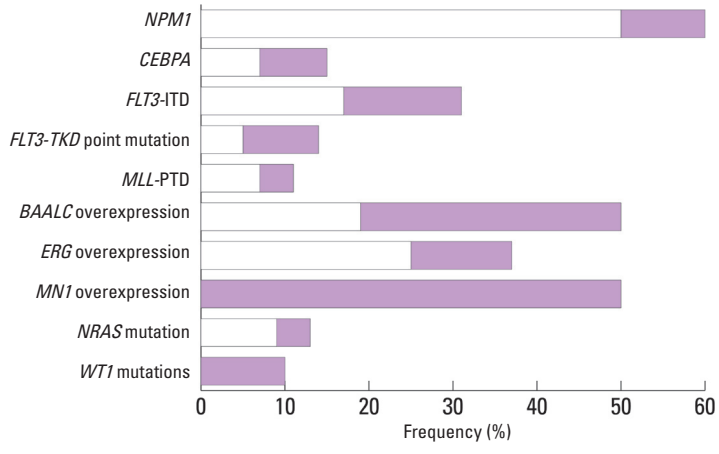
Cytogenetic aberrations have long been recognized as the most important prognostic variable in acute myeloid leukemia (AML) and are now a major stratification tool for post-remission therapy. Cytogenetics-based stratification improves survival. Patients with AML and normal cytogenetics, the largest single subgroup, have had a very heterogeneous outcome with standard chemotherapy in multiple clinical trials. Hence it is difficult to recommend a "one size fits all" kind of treatment for this heterogeneous population of AML patients. New emerging data from preclinical, retrospective, and large, randomized controlled studies indicate that in addition to cytogenetic abnormalities, many other molecular aberrations are operative in the response to treatment as well as in the risk of relapse. Such molecular markers are being tested for developing targeted therapies and may help in improved stratification of patients in the selection of post-remission therapy. Emerging evidence reveals that at the submicroscopic level, AML with normal cytogenetics may carry poor prognostic genetic lesions or "molecular signatures" as is the case with *FLT3* mutations and overexpression of *BAALC, ERG* or *MN1*, or may have aberrations that predict better risk as is the case with isolated *NPM1* or *CEBPA* mutations. Later studies have tried to explore the interaction of various prognostically important genes in this group of AML patients. The utility of the evolving data for bedside management of such patients is expected to improve with the wider application of modern tools, using the proposed clinical outcome models, and probably by development of a risk-scoring system based on the relative risk associated with each molecular aberration. The goals include identifying those patients most likely to benefit from upfront allogeneic HSCT and sparing good-prognosis patients from unnecessary transplant-related morbidity. The following is an outline of the most common molecular changes, their impact on the outcome of AML patients with normal cytogenetics and challenges in their wide scale application in risk stratification.

Acute myeloid leukemia (AML), the most common form of acute leukemia in adults, is a heterogeneous group of diseases that currently are curable in about 30 percent of cases.[1] Advances to improve the long-static treatment outcome in AML are urgently needed. Traditionally, AML is grouped into three prognostically relevant categories[2] based on cytogenetic analyses: First, the balanced translocations, which are associated with a favorable prognosis, including t(15;17), t(8;21) and t(16;16) or inv(16)(p13;q32). Second, a complex aberrant karyotype that confers a poor clinical outcome. And third, an intermediate prognosis group, including normal karyotype and other karyotypic abnormalities that do not fall into a good- or poor-risk karyotype, e.g., t(9;11)(p22;q23). Patients with a normal karyotype comprise the single largest cytogenetic subset in AML as no chromosomal abnormality is visible by conventional karyotyping in 40% to 50% of adult cases and in 25% of pediatric cases. Normal karyotype is associated with 5-year survival rates between 24% and 42%, and biologic features of this large cytogenetic subgroup are still poorly understood.[2,3] Hence most of these cases are presently classified in the World Health Organization (WHO) scheme[4] under the term "acute myeloid leukemia not otherwise characterized". Based on recent findings, discussed below in detail, this clas-

sification is under revision and is expected to incorporate more molecular genetics (personal communication. Clara D Bloomfield MD).

Recent data have shown that this cytogenetic subgroup is genetically heterogeneous.[1,3] A number of submicroscopic genetic lesions with prognostic impact have been identified in patients with AML and a normal karyotype. Such genetic lesions provide some hints towards understanding the biology of this subset of AML patients and hence may provide new approaches for sub-classification of AML with a normal karyotype. More than 100 genes have been reported to be altered in their physiological function by rearrangements, mutations, underexpression or overexpression, and some of these can serve as markers for prognosis, detection of minimal residual disease (MRD) and for the development of targeted therapies for AML patients. These mutations in AML may be divided into two broad categories: mutations that activate signal transduction cascades and mutations in genes that result in dysregulated gene transcription. This article is not intended to provide a comprehensive review of all potentially altered genes, but will focus on the most frequently and most recently recognized dysregulated genes in AML with normal cytogenetics (Figure 1). These include nucleophosmin 1 (*NPM1*), FMS-like tyrosine 3 (*FLT3*), CCAAT/enhancer-binding protein α (*CEBPA*), partial tandem duplication (PTD) of the mixed lineage/myeloid lymphoid leukemia (*MLL*) gene, brain and acute leukemia cytoplasmic (*BAALC*) gene, ETS-related genes (*ERG*), neuroblastoma RAS viral oncogene homolog gene (*NRAS*), meningioma 1 (*MN1*) gene and Wilm's tumor 1 (*WT1*) gene (Table 1); and the role of microarray gene expression profiling in constructing an optimal clinical-outcome prediction model that may prove more accurate in predicting survival among AML patients with a normal karyotype.



NPM1: nucleophosmin 1; *FLT3*-ITD: FMS-like tyrosine kinase 3–internal tandem duplication; *FLT3*-TKD: FMS-like tyrosine kinase 3-tyrosine kinase domain; *CEBPA*: CCAAT/enhancer binding protein alpha; *MLL*-PTD: partial tandem duplication of the mixed lineage leukemia/myeloid lymphoid leukemia; *BAALC*: brain and acute leukemia cytoplasmic gene; *ERG*: ETS-related genes; *MN1*: meningioma 1; *NRAS*: meuroblastoma rat sarcoma gene, *WT1*: Wilms tumor 1 antigen.

Figure 1. Reported frequencies (%) of some genetic alterations in AML patients with normal karyotypes. Smaller values reported in selected references are shown as unshaded portions.

We also describe how these molecular findings can be utilized in stratifying patients for risk-adapted and targeted treatments, including a description of clinical outcome models proposed in recent studies that may help hematologists in identifying patients most likely to benefit from upfront allogeneic hematopoietic stem cell transplantation (HSCT) and spare good-prognosis patients from unnecessary transplant-related morbidity and or mortality. Finally we propose that, with the accumulation of information about the relative impact of each aberration on clinical outcome, there is a need to develop a scoring system incorporating all (or selected) molecular markers for prospective decision making.

Mutations in the *nucleophosmin* 1 (*NPM1*) gene

The *nucleophosmin* (*NPM1*) gene encodes for a multifunctional nucleocytoplasmic shuttling protein that is localized mainly in the nucleolus. *NPM1* mutations generate NPM mutants that localize aberrantly in the leukemic-cell cytoplasm.[5,6] Both tumor-suppressor and oncogenic functions have been attributed to this multifunctional phosphoprotein. NPM promotes the biogenesis of the ribosome by acting as a chaperone that carries preribosomal particles from the nucleolus to the cytoplasm and by favoring the processing and maturation of ribosomal RNA.[7] This may prevent protein aggregation in the nucleolus.[6,8] NPM has been implicated in modulation of the function of tumor-suppressor transcription factors, such as interferon regulatory factor 1 (IRF-1) and the regulation of the alternate-reading-frame protein (ARF)-p53 tumor-suppressor pathway[9-12] and is also a target of CDK2-cyclin E complexes in centrosome duplication.[13] As NPM controls the $p19^{ARF}$-p53 tumor suppressor pathway at multiple levels,[9] this may suggest a tumor-suppressor role for NPM itself. However, NPM, when overproduced, can transform immortalized cells and is highly expressed in various tumors and in actively proliferating cells, again suggesting its role in ribosome biogenesis.

The complex physiological functions of NPM include regulation of DNA integrity and inhibition of cell proliferation by interaction with p19ARF.[9] Animal models have established that NPM is essential for embryonic development and the maintenance of genomic

Table 1. Prognostic impact of dysregulated gene(s) described in AML patients with normal karyotype.

Genetic alterations	Gene location	Reported frequency in AML patients with normal karyotype	Impact on clinical outcome
NPM1 mutations	5q35	49.6%,[133] 60%,[3§] 53%[131]	Favorable especially in absence of FLT3-ITD; better CR, EFS, RFS, DFS, and OS
CEBPA mutations	19q13.1	7-15%[99,100,131,133]	Favorable; higher CR duration and OS
FLT3-ITD	13q12	17-31%[44,45,131]	Unfavorable especially if coupled with no expression of a *FLT3* wild-type allele or a high FLT3 mutant to FLT3 wild-type allele ratio; shorter CR duration, DFS and OS
FLT3-TKD point mutations	13q12	5-14%[20,45,131,133]	None but may be favorable if associated with *NPM1* mutation
MLL-PTD	11q23	7-11%[44,76,79,82,131,133]	Unfavorable; shorter CR duration
BAALC overexpression	8q22.3	19%,[83] 50%[44,46,86]	Unfavorable; lower CR rates and shorter DFS and OS
ERG overexpression	21q22.3	25%,[44**] 37%[96]	Unfavorable; lower CR rates, higher relapse and shorter OS
MN1 overexpression	22 q11	50%[107,138]	Unfavorable; shorter relapse-free survival, ATRA resistance in elderly
NRAS mutation	1p13	9.1-13%[110,131]	None but may be favorable OS when other gene aberrations are taken into account
WT1 mutation	11p13	10%[124]	Unfavorable; associated with induction failure

§Cytoplasmic NPM staining[3] ; **Based on quartile expressing the highest ERG level in a group of 84 patients; OS: overall survival; DFS: disease free survival; CR: complete remission; ATRA: all-trans retonoic acid; *NPM1*: nucleophosmin 1; *FLT3*-ITD: FMS-like tyrosine kinase 3–internal tandem duplication; *FLT3*-TKD: FMS-like tyrosine kinase 3-tyrosine kinase domain; *CEBPA*: CCAAT/enhancer binding protein alpha; *MLL*-PTD: partial tandem duplication of the mixed lineage leukemia/myeloid lymphoid leukemia; *BAALC*: brain and acute leukemia cytoplasmic gene; *ERG*: ETS-related genes; *MN1*: meningioma 1; *NRAS*: meuroblastoma rat sarcoma gene, *WT1*: Wilms tumor 1 antigen.

stability.[14] Moreover, NPM haplo-insufficiency accelerates oncogenesis in in vitro and in vivo model systems. Mice expressing a hypomorphic NPM allele develop a hematological syndrome similar to human myelodysplastic syndrome (MDS), which points to a central role for NPM in the pathogenesis of AML.

Chromosomal translocations involving the *NPM1* gene frequently occur in myeloid and lymphoid cancers and result in fusion proteins containing only the NPM N-terminal region.[15,16] Such fusions include NPM-anaplastic lymphoma kinase (NPM-ALK),[17] NPM-retinoic acid receptor α (NPM-RARα),[18] and NPM-myeloid leukemia factor 1 (NPM-MLF1).[19] Rearrangements with the *MLF1* gene occur in AML, chronic myelogenous leukemia, and myelodysplasia.[17-19] NPM appears to contribute to oncogenesis by activating the oncogenic potential of the fused protein partner (MLF1, ALK or RARα). As NPM is thought to have a tumor-suppressor function, abnormalities leading to its movement from the nucleus to the cytoplasm may be critical for malignant transformation. Such changes in the subcellular distribution of NPM and NPM-containing fusion protein can be detected by immunohistochemical methods.[16,18]

Cytoplasmic NPM accumulation is caused by the concerted action of two alterations at a mutant C-terminus, that is, changes of tryptophan(s) 288 and 290 (or only 290) and creation of an additional nuclear export signal (NES) motif.[15] According to recent studies *NPM1* exon-12 gene mutations represent the most common submicrosopic alterations in patients with a normal karyotype (occuring in 50% to 60% of adult acute myeloid leukemia patients) and have a profound diagnostic and prognostic impact.[3,14,15,20] Recently, Falini et al identified a large subgroup of patients with AML who had cytoplasmic NPM in leukemic blasts, a mutated *NPM* gene, a normal karyotype, and a relatively good response to induction chemotherapy.[3] They have shown that an abnormal cytoplasmic localization of the NPM-protein (NPMc+) can be found in about 35% of all patients with primary AML, but not in secondary AML and other hematopoietic or extra-hematopoietic neoplasms other than AML.[3] In this study, NPMc+ did not occur with specific recurrent chromosomal abnormalities. Cytoplasmic NPM staining was associated with responsiveness to induction chemotherapy.[3] The frequency pattern of NPMc+ ranged from 13.6% in M0 tumors (minimally differentiated AML) to 87.5% in M5b specimens (acute monocytic leukemia). Most NPMc+ AML tumors of the M5b and M6 subtypes (acute erythroid leukemia) showed cytoplasmic NPM

in erythroid precursors, particularly proerythroblasts.[3] However, NPM abnormalities may not be the only molecular event in myeloid leukemic blasts; there was a high frequency of *FLT3* internal tandem duplications and an absence of CD34 and CD133 in AML specimens with a normal karyotype and cytoplasmic dislocation of NPM, but not in those in which the protein was restricted to the nucleus ($P<.001$).[3]

Many other European leukemia groups have provided an extensive analysis of *NPM1* and *FLT3* mutations, as well as other mutations with prognostic significance in AML.[20-25] Earlier, in a cancer and leukemia group B (CALGB) study, Whitman et al showed that absence of the wild-type allele predicts poor prognosis in adult de novo AML with normal cytogenetics and the internal tandem duplication of *FLT3* (*FLT3*-ITD).[25] According to the study by Schnittger et al, comprising of 401 patients with a normal karyotype *FLT3*-ITD mutations were found in 40% of patients with *NPM1* mutations and represented the most frequent additional genetic alteration in patients with a mutated *NPM* gene.[20] Interestingly, the favorable impact of *NPM1* mutations on achieving higher complete remission (CR) rate and event-free survival (EFS) was clearly seen in the group of normal-karyotype AML without *FLT3* length mutations (*FLT3*-LM). This favorable effect was lost in the presence of a *FLT3*-ITD since survival of the *NPM1*+/*FLT3*-LM+ double positive was similar to *NPM1*-/*FLT3*-LM+ cases.[20] Hence this study demonstrated that *NPM1*+/*FLT3*-LM- mutations are an independent predictor for a favorable outcome in AML with normal karyotype.[20]

With limitations of significant differences in median time of follow-up, median age, male-female ratio, treatment regimens, and possibly outcome; the essential conclusions related to *NPM1* mutations were concordant in all European reports.[20-23] Of most importance, isolated *NPM1* mutations were found to predict for improved OS,[20-22] relapse-free survival,[20,21] and event-free survival (EFS)[20,22] in AML with normal karyotype by Kaplan-Meier analysis only if *FLT3*-ITD mutations were not coincidentally present (i.e. in the *NPM1*+*FLT3*-subgroup). There were no survival differences between *NPM1*+*FLT3*+, *NPM1*-*FLT3*+, and *NPM1*-*FLT3*-subgroups. Other concordant conclusions were that *NPM1* mutations are not related to age, occur more often in females, are associated with increased WBC count, are more frequently associated with the myelomonocytic phenotype and low CD34 expression, and occur less frequently with coincident partial tandem duplication of *MLL*.[20,21,23] Schnittger et al and Verhaak et al also reported an independent survival advantage for *NPM1* mutation alone by multivariate analysis and a decreased coincidence of *NPM1* and *CEBPA* mutations.[20,22] Very recent data suggests that *NPM1* mutations, which are frequently associated with *FLT3* mutations, are more stable than *FLT3* mutations during the course of disease in patients with AML. Hence these highly prevalent mutations of *NPM1* might provide a suitable marker for monitoring residual disease of AML.[26]

The prevalence of the *NPM1* gene mutation in non-M3 childhood AML patients with normal karyotype enrolled in an Italian study was 6.5% (in 107 successfully analyzed patients).[27] *NPM1*-mutated patients were older in age.[27] More recent pediatric data confirms that frame shift mutations in exon 12 of the nucleophosmin gene are relatively uncommon (8%) in childhood AML, particularly in younger children.[28] Both studies confirm that NPM mutation does not abrogate the negative prognostic influence of *FLT3*-ITD mutations, but may contribute to risk stratification in children who lack *FLT3*-ITD mutations by identifying a group with superior prognosis.[28]

NPM1 mutations are associated with several pretreatment characteristics of patients with AML and normal karyotype, including a predominance of female sex, wide morphologic spectrum, multilineage involvement, higher white blood cell and bone marrow blast percentages, higher lactate dehydrogenase levels, as well as high CD33-antigen expression but low or absent CD34-antigen expression, high frequency of *FLT3*-ITD, and a distinct gene-expression profile.[15]

In summary, at present *NPM* mutations represent the most common submicrosopic alterations in adult patients with a normal karyotype and have a profound diagnostic and prognostic impact in constructing an optimal clinical-outcome prediction model.[3,14,20] Analysis of mutated NPM has important clinical and pathologic applications. Immunohistochemical detection of cytoplasmic NPM predicts *NPM1* mutations and helps rationalize cytogenetic/molecular studies in AML. Due to their frequency and stability, *NPM1* mutations may become a new tool for monitoring MRD in AML with normal cytogenetics.[15]

Obviously these data will have a major impact on molecular investigative and clinical approaches in AML with normal karyotype. For example, the high-risk procedure of allogeneic stem cell transplantation may not be indicated in first CR for this newly defined, favorable-prognosis subgroup as it did not improve the long-term 60% relapse-free survival rate in *NPM1*+*FLT3*- cases but, as reported by Dohner et al,[21,23] it did so in the other subgroups. On the other hand, it is potentially possible that treatment with recently developed *FLT3*

inhibitors[25,29-33] might be effective in *NPM1*+*FLT3*+ patients by producing pharmacologic conversion to a more chemotherapy-sensitive *NPM1*+*FLT3*- status.[23]

A recent in vitro study suggested that abrogation of the levels or reversing the aberrant cytosolic localization of m*NPM1* may be a promising anti-leukemia treatment strategy, especially for targeting AML that co-express *FLT3*-ITD and mutant *NPM1*.[34]

Mutations of the FMS-like tyrosine 3 (*FLT3*) gene

FLT3 (FMS-like tyrosine kinase) is a transmembrane tyrosine kinase, belonging to class III of receptor tyrosine kinases (RTK), in which ligand binding triggers a pro-proliferative signaling cascade. The *FLT3* gene belongs to the same family as *FMS, KIT* and the two genes encoding PDGFR α and β. A mutation of *FLT3*, is the most common tyrosine kinase mutation in AML.[35] At mRNA level, it is expressed in 80%-100% of blasts from patients with AML,[36] while combined data from multiple studies shows 66% positivity by flowcytometry in AML cases.[36] *FLT3* is expressed at high levels in 50% of such patients with AML.[37] Kuchenbauer et al showed that *FLT3* expression levels were different in certain FAB types with a minimum in FAB M3 and maximum in FAB M5. Their results also correlated with the *FLT3* receptor surface expression (CD135) detected by flow cytometry ($P<.001$), showing the highest CD135 expression in FAB M5.[38]

Approximately 30% of the AML patients have activating mutations in *FLT3*. The most frequent (~25%) is an internal tandem duplication (ITD) within the cytoplasmic juxtamembrane (JM) region. Less frequent (~7%) are point mutations in the activation loop of the tyrosine kinase domain (*FLT3*-TKD mutation), such as the D835Y mutation.[37] Both types of mutations result in ligand-independent kinase activation of *FLT3* and its downstream signaling pathways such as signal transducer and activator of transcription 5 (STAT5), RAS/mitogen-activated protein kinase (RAS/MAPK), phosphoinositide 3-kinase (PI3K), src homologous and collagen gene (SHC), SH2-containing inositol-5-phosphatase (SHIP), and cytoplasmic tyrosine phosphatase with 2 Src-homology 2 (SH2) domains (SHP2), providing the cells with a proliferative and survival advantage.[37,39]

According to the Gilliland model,[37] type I mutations represent genetic alterations which induce a proproliferative and anti-apoptotic signal (e.g. gain of function mutations of protein tyrosine kinase [PTK] and ras), whereas type II mutations interfere with differentiation and often involve myeloid transcription factors (loss of function mutations). This model is supported by experimental data indicating that AML1-ETO and *FLT3*-ITD mutations are both insufficient on their own to cause leukemia in animal models.[40,41] Additional mutations, such as those transcription factor abnormalities seen in good prognosis subtypes of AML, are required to cooperate with such tyrosine kinase mutations to cause full-blown murine leukemia in model systems.[40,41]

Most studies have shown that internal tandem duplication mutations in the *FLT3* confer adverse prognosis.[42,43] This is particularly true for AML patients with a normal karyotype. Activating mutations of *FLT3* tend to be more common in those with a normal karyotype (17-30%).[44,45] However these have also been reported in patients with the t(15;17) typical of acute promyelocytic leukemia (APL), the t(6;9) translocation found in the rare entity of AML with basophilia and also with some other recurring cytogenetic abnormalities.[37,45,46] Clinical trials showed that *FLT3*-LM are strongly associated with leukocytosis, high blast counts, normal cytogenetics and t(15;17).[38,47,66] In contrast, the *FLT3*-LM are rarely found in patients with a complex karyotype and CBF-leukemias (CBF-MYH11 and AML1-ETO). In almost all cytogenetic subgroups, the presence of a *FLT3-LM* represents a negative prognostic risk factor for OS and EFS. It is controversial whether a *FLT3* mutation is an independent negative prognostic factor in APL;[48] moreover, studies have failed to show a consistent correlation between activation loop mutations and prognosis.[49] However, Whitman et al have shown that the loss/deletion of the residual *FLT3*-wild type (WT) allele predicts poor prognosis in adult de novo AML with normal cytogenetics and the internal tandem duplication of *FLT3*.[25] These findings are also supported by data from Spiekerman et al showing that deletions of the *FLT3*-WT allele are more frequently found in patients at relapse as compared to patients at initial diagnosis suggesting that these genetic alterations are associated with disease progression.

Remarkable strategies to inhibit *FLT3* signaling in development include antibodies[50] and chaperone protein inhibition[51] to decrease the half-life of the molecule, but the greatest interest has been in the small molecule tyrosine kinase inhibitors including SU11248,[30,52] SU5416,[31] CEP701,[53] PKC412[54] and MLN518. These tyrosine kinase inhibitors specifically kill leukemic cell lines transformed with either of the activated constructs of *FLT3*.[29] Moreover, these agents have been shown to prolong the survival of mice with a fatal activated *FLT3*-mediated murine myeloproliferative disorder.[55] A number of structurally different *FLT3* tyrosine kinase inhibitors (TKIs) have reached clinical trials in

Table 2. Summary of FLT3 inhibitors already tested in clinical trials.

FLT3 inhibitor	Class	*FLT3* IC50**	Other receptors	Phase of clinical trial
CEP-701 (lestaurtinib)	Indolocarbazole	2 nM	TRKA	Phase III
PKC412 (midostaurin)	Indolocarbazole	10 nM	KIT	Phase III (Intergroup trial is planned)
MLN518 (tandutinib)	Quinazoline	30 nM	KIT, PDGFR	Phase II
SU5416 (sexamanib)	3-substituted indolinone	100 nM	KIT, VEGFR	Phase II
SU11248 (sunitinib)	3-substituted indolinone	50 nM	KIT, PDGFR, VEGFR	Phase I
CHIR-258	Benzimidalzole - quinoline	10 nM	KIT,FMS, FGFR, VEGFR	Phase I
ABT-869	NR	4 nM	KIT, KDR, PDGFR	Phase I
KW-2449	NR	6 nM	FLT3, KIT, aurora kinase	Phase I

**IC50 is the concentration required for 50% inhibition of *FLT3* autophosphorylation; NR: not reported

elderly AML patients and/or relapsed refractory AML patients, some or all of whom had *FLT3* mutations (Table 2).[32,39,40,52-54] In most studies, the drugs have been well tolerated as monotherapy as outpatient oral medications. Usually there is more frequent response to *FLT3* TKI in the mutant *FLT3* patients. The responses in all patients, however, are usually short lived and restricted to a clearing of peripheral blasts for only few weeks to months. For example, clinical activity manifested as transient reductions in bone marrow and peripheral-blood blasts or longer periods of transfusion independence, was seen in 3 (60%) of 5 patients with mutated *FLT3* in a phase 2 trial of the *FLT3* inhibitor lestaurtinib (CEP701) used as first-line treatment for older patients with AML who were not considered fit for intensive chemotherapy.[56] Phase 1 clinical trial results with tandutinib (MLN518), another novel *FLT3* antagonist, in patients with AML or high-risk MDS have also been reported.[57] Eight of 40 patients had *FLT3*-ITD mutations. Two of the 5 evaluable patients, treated at 525 mg and 700 mg twice daily, showed evidence of anti-leukemic activity, with decreases in both peripheral and bone marrow blasts. Authors recommended tandutinib at the maximum tolerated dose (525 mg twice daily) should be evaluated more extensively in patients with AML with *FLT3*-ITD mutations to better define its anti-leukemic activity.[57] However, major bone marrow responses are uncommon with these *FLT3* inhibitors. This could be a result of genomic instability leading to multiple pathways of escape from dependence on *FLT3* signaling. Each of these molecules inhibits *FLT3* in the nanomolar range, but they vary in terms of chemical structure, preclinical side effect profile and most conspicuously, the spectrum of kinase which they inhibit. For example, PKC412 not only inhibits the *FLT3* tyrosine kinase but it also inhibits c-kit, and the serine-threonine protein kinase C.[58] Using an in vitro mutagenesis screen, Cool et al identified four different positions in the ATP-binding site of *FLT3* that, when mutated, confer resistance to PKC412 (also called midostaurin), further documenting that resistance to PKC412 can develop due to mutations in the kinase domain of *FLT3*.[59] Most recently, Barry et al have reported that PKC412 is a potent inhibitor of a spectrum of *FLT3* activation loop mutations, and that AML patients with such mutations are potential candidates for clinical trials involving midostaurin.[60] Midostaurin has shown activity in a phase 1 trial in combination with daunorubicin and cytarabine in newly diagnosed AML, in particular those patients with *FLT3*-ITD mutations.[61]

Levis et al have recently developed a useful surrogate assay for monitoring the efficacy of *FLT3* inhibition in patients treated with oral *FLT3* inhibitors. The plasma inhibitory activity (PIA) for *FLT3* correlates with clinical activity in patients treated with CEP-701 and PKC412.[62] The application of this assay has revealed that the metabolite CGP52421 may contribute a significant portion of the anti-leukemia activity observed in patients receiving oral PKC412. Additionally, the results suggested that non-selectivity may constitute an important component of the cytotoxic effect of *FLT3* inhibitors in *FLT3*-mutant AML.[62]

With target identification followed by the demonstration that activating mutations of *FLT3* are patho-

physiologically relevant in AML, the development of *FLT3* inhibitors as therapeutic agents to AML is an important progress. Although complexity of the disease and patient population precluded their standard use as single agents in AML, it appears that the most widespread use of one or more of the *FLT3* inhibitors in development in AML will come in conjunction with chemotherapy. Certainly randomized Phase III trials of chemotherapy with or without the *FLT3* inhibitors are required but the major controversy is whether or not such trials should be conducted in selected groups of AML patients with an activating mutation of *FLT3* where the greatest activity would be expected; or whether all AML patients should be included in such an evaluation because of the preliminary results showing activity in non-*FLT3* mutant patients as well as the possibility that the off-target effects of these drugs might also be beneficial.[58] Thus, it appears prudent argument to explore the interactive effect of *FLT3*-ITD mutations with other genetic aberrations in AML patients with normal cytogenetics. For example, Schnittger et al have raised the possibility that the cytoplasmic displacement of *NPM1* protein by a multiplicity of frame shift mutations that introduce a common nuclear export signal may increase susceptibility to chemotherapy-induced apoptosis.[20] This suggests that dynamic molecular analyses at both transcriptional and posttranscriptional levels early after exposure to chemotherapeutic agents with or without modulators, such as *FLT3* inhibitors, might show differences between $NPM1^{+}FLT3^{-}$ and $NPM1^{+}FLT3^{+}$ AML cells with normal cytogenetics that could implicate important target molecules.[23]

Current trials are combining *FLT3* inhibitors with conventional chemotherapy, but combinations of *FLT3* TKI with other molecularly targeted agents affecting the other pathways could be the alternative goal. It is hoped that such trials will help in reversing the poor prognosis for AML patients with *FLT3*-ITD mutations by preventing relapse and reducing the toxicities associated with nonselective cytotoxic agents.

FLT3 may also be targeted specifically through anti-*FLT3* antibodies.[50,63] Fully human phage display antibodies were generated and selected for binding to human *FLT3* and the ability to block FL binding to *FLT3* in an ELISA assay. They have the ability to block signaling through wild-type *FLT3* and also through some mutated forms of *FLT3*. They have been shown to induce antibody dependent cellular-mediated cytotoxicity (ADCC) and mediate killing of some, but not all, cell lines and primary samples of both AML and acute lymphoblastic leukemia (ALL) when used in vivo in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice engrafted with these cells.[64,65] Anti-*FLT3* antibody trials are also being considered in AML patients especially those expressing mutant *FLT3*. Besides the potential for interfering with *FLT3* signaling, anti-*FLT3* antibodies can also induce ADCC as an additional mechanism for inducing cytotoxicity.[63]

As mentioned earlier, in addition to *FLT3*-ITD, two types of point mutations in *FLT3* localized within the activation loop of the tyrosine kinase domain (TKD) have been reported in 5% to 14% of AML patients with normal karyotype.[20,45,66,67] In contrast to *FLT3*-ITD, the *FLT3*-TKD has not been reported to have an adverse prognosis among this subgroup of AML patients.[45] Rather interestingly, a small group of such patients bearing *FLT3*-TKD who concurrently had a mutation in the *NPM1* gene had significantly longer EFS than patients without these mutations.[20] However, further studies are necessary to determine the prognostic role of *FLT3*-TKD, especially in relation to other molecular prognostic factors in AML patients with normal karyotype.

Regarding the effect of transplantation on the outcome of *FLT3*-mutated patients, a consensus did not emerge from two retrospective studies[68,69] In the MRC study conducted by Gale et al[68] (35 patients with a mutation and 135 with no mutation), *FLT-3* mutated status was also an adverse prognostic factor in the recipients of transplants. In this intent-to-treat analysis, (donor vs no-donor) transplantation reduced the relapse risk and improved the DFS, but the OS was not significantly improved. On this basis this study found no evidence to recommend transplantation based on *FLT-3* status alone. On the other hand study by Bornhauser et al (Ulm group),[69] the negative impact of *FLT-3* mutation status was seen in patients who received chemotherapy or autologous transplantation, but not in the recipients of allogeneic HSCT. A major difference in the analysis was that the assessments were based on treatments given, rather than on an intent to treat that attempts to reduce selection of better-risk patients entering the transplantation option.[70]

Partial tandem duplication (PTD) of the mixed lineage leukemia (*MLL*) gene

The mixed lineage/myeloid lymphoid leukemia (*MLL*) gene is located in chromosome band 11q23 covering a genomic region of approximately 100 kb of DNA and represents the human homologue of the *Drosophila* trithorax gene. The *MLL* gene is frequently involved in translocations that occur in AML and in ALL.[71] Rearrangement of the *MLL* gene detected by Southern

blot analysis was the first molecular marker described in patients with AML and normal cytogenetics.[44]

The majority of the translocation breakpoints cluster in an 8.3-kb region (breakpoint cluster region) that is represented by exons 5 to 11.[71-73] *MLL* gene translocations, deletions, and duplications most often result in gene fusions between the 5'-end of the *MLL* gene and the 3'-end of a partner gene. The submicroscopic rearrangements of the *MLL* gene are the partial tandem duplications of *MLL* (*MLL*-PTD). At the level of transcription *MLL*-PTD result in a unique in-frame fusion of exons 11 or 12 upstream of exon 5. Functional data support the hypothesis that *MLL*-PTD induce a loss of *MLL* WT function via monoallelic repression, thereby contributing to the leukemic phenotype by the remaining mutant allele.

However, the pathogenetic relevance of this gene rearrangement in AML is still not clear as Marcucci et al[74] and Schnittger et al,[75] using nested reverse transcriptase polymerase chain reaction (RT-PCR), demonstrated detectable PTD of the *MLL* gene with different frequencies in peripheral blood (PB) and BM of healthy donors.

Strout et al[76] have shown that molecular rearrangement in patients with normal cytogenetics is a tandem duplication of an internal portion of the *MLL* gene that spans exons 2 to 6 or exons 2 to 8 in most of the cases. This PTD was detected in approximately 10% of AML with normal cytogenetics and in 90% of AML exhibiting trisomy 11 (+11) as a sole chromosome abnormality.[72,77] By dosage analysis, it was shown that in the cytogenetically normal cases and in the cases with +11, only one chromosome 11 contained the mutated allele.[78]

Caligiuri et al[79] analyzed 98 patients with de novo AML and normal karyotype for PTD of the *MLL* gene by Southern blot analysis and single-round RT-PCR. In this study, 11 (11%) of 98 patients showed a PTD. PTD-positive patients who achieved CR had a statistically significant shorter CR duration (7.1 months) compared with the PTD-negative patients (23.2 months). In another study,[80] the incidence of *MLL* PTD was lower than previously reported, but in agreement with the findings of Caligiuri et al.[79] PTD of the *MLL* gene was associated with an unfavorable prognosis.

Lately, Dohner et al in a large prospective series of newly diagnosed young adult patients (16 to 60 years of age) with AML treated within the multicenter treatment trials AML-HD93 and AML-HD98A of the AML Study Group[81,82] have shown that within the subgroup of patients with normal karyotype, *MLL* PTD is associated with short remission duration.[82] *MLL* PTD was identified in none of the 129 patients with t(8;21), inv(16), and t(15;17); in 19 (7.7%) of 247 patients with normal karyotype; and in 10 (8.5%) of 119 patients with all other abnormalities, while 30 cases of t(11q23) were excluded. The median remission durations of the PTD-positive and the PTD-negative groups were 7.75 months and 19 months, respectively ($P<.001$) and multivariate analysis identified the *MLL* PTD status as the single prognostic factor for remission duration.[82]

In summary, *MLL*-PTD are frequently found in adult de novo AML and are associated with a worse prognosis (i.e., shorter duration of remission) when compared with normal karyotype AML without the *MLL* PTD. In addition, several studies have shown that *MLL*-PTD are more frequently found in *FLT3*-ITD positive compared to *FLT3*-ITD negative patients.

BAALC (brain and acute leukemia, cytoplasmic) overexpression is a gene located at chromosome 8q22.3. It encodes a protein of yet-unknown function. Normally, *BAALC* is almost exclusively expressed in neuroectoderm-derived tissues. In addition, CD34-positive progenitor cells from normal and abnormal bone marrow express *BAALC*, identifying it as another gene whose expression is shared by neural and hematopoietic cells. In hematopoiesis, *BAALC* reflects a stage-specific marker as downregulation of *BAALC* occurs with cell differentiation. As all subtypes of CD34+ cells express *BAALC*, it is postulated that *BAALC* represents a novel marker of an early progenitor cell common to the myeloid, lymphoid, and erythroid pathways. It is aberrantly expressed in a subset of acute leukemias.[46,83,84] Tanner et al reported *BAALC* overexpression in 12 of 63 patients with normal karotype (19%). Overexpression of *BAALC* was an adverse prognostic factor.[83] In a CALGB study of 86 de novo AML patients younger than 60 years with a normal cytogenetics and more favorable *FLT3* mutation status, high mRNA expression levels of *BAALC* were shown to be an adverse risk factor.[85] There was no significant difference in response to induction therapy between the two cohorts as 81% of low *BAALC* and 77% of high *BAALC* expressers achieved CR.[85] However, AML patients with high *BAALC* expression tended to relapse more frequently than those with low *BAALC* expression (52% versus 29%). High *BAALC* expression predicted significantly shorter OS (median, 1.7 versus 5.8 years, $P=.02$). Event-free survival also was significantly shorter in patients with high *BAALC* expression compared to those with low *BAALC* expression (median, 0.8 versus 4.9 years, $P=.03$), as was DFS (median, 1.4 versus 7.3 years, $P=.03$).[85]

A recent study by Baldus et al[46] on 307 AML patients (60 years of age with normal cytogenetics enrolled in the Deutsche Studies Initiative Leukämie (DSIL)

has confirmed that as compared to patients with low-*BAALC* expression, patients with high-*BAALC* expression had a higher rate of primary resistant leukemia (16% v 6%; *P*=.006),[46] a higher cumulative incidence of relapse (CIR; *P*=.018) and an inferior OS (3-years, 36% v 54%; *P*=.001). High *BAALC* expression was associated with a higher percentage of blood blasts (*P*=.004) and more immature FAB subtypes M0/M1 (*P*=.001) while monocytic differentiation FAB M5b and gingival hyperplasia were more frequent in low *BAALC* patients (*P*=.001 and .003, respectively). Although high *BAALC* expression was associated with a higher percentage of circulating blasts, and both variables were associated with poor outcome in the univariate analysis, in multivariate analyses only high *BAALC* was independently predictive of resistant disease (P=.019).[46] Additionally this study demonstrated that high *BAALC* is significantly associated with a higher *FLT3* ratio (*FLT3* mutant:wild type ratio). For most AML patients (i.e. those with a low risk *FLT3* mutation status) *BAALC* expression allowed further discrimination of high- and low-risk patients. Importantly, in multivariable analysis high *BAALC* and a high *FLT3* mutant:wild type ratio were independently predictive of inferior OS and higher CIR. As both factors independently influenced the clinical course, different molecular pathways may be involved.[46] This study showed high *BAALC* expression as one of the important independent risk factors associated with resistant disease, a high CIR, and inferior survival. Modulation of induction therapy and intensification of postremission therapy may be critical to improve outcome for these high-risk patients. This study suggested that patients with high *BAALC* expression may benefit from consolidation with allogeneic SCT but not with autologous SCT. This is because in high *BAALC* patients undergoing allogeneic SCT a low 3-year CIR (16%) was observed compared with high-*BAALC* patients receiving autologous SCT (52%; *P*=.007).[46]

Recently CALGB group confirmed that high *BAALC* expression is an independent adverse prognostic factor and is associated with a specific gene-expression profile.[86] They analyzed *BAALC* expression in 172 primary CN-AML patients younger than 60 years of age, treated similarly on CALGB protocols. High *BAALC* expression was associated with *FLT3*-ITD (*P*=.04), wild-type *NPM1* (*P*<.001), mutated *CEBPA* (*P*=.003), *MLL*-PTD (*P*=.009), absent *FLT3*-TKD (*P*=.005), and high ERG expression (*P*=.05). In a multivariate analysis, high *BAALC* expression independently predicted lower complete remission rates (*P*=.04) when adjusting for ERG expression and age, and shorter survival (*P*=.04) when adjusting for *FLT3*-ITD, *NPM1*, *CEBPA*, and white blood cell count. A gene-expression signature of 312 probe sets differentiating high from low *BAALC* expressers was identified. High *BAALC* expression was associated with overexpression of genes involved in drug resistance (MDR1) and stem cell markers (CD133, CD34, KIT).[86]

ETS-related gene (*ERG*) overexpression

ETS-related gene (ERG), which is located at chromosome band 21q22, is frequently overexpressed in AML patients with complex karyotypes and cryptic amplification of chromosome 21.[87] ERG is one of more than 30 members of the ETS gene family, most of which are downstream nuclear targets of signal transduction pathways regulating and promoting cell differentiation, proliferation, apoptosis and tissue invasion.[88-90] Although ERG rearrangements have been found in AML[91] and Ewing sarcoma[92] and its overexpression has been observed in prostate cancer,[93] little is known regarding how ERG contributes to malignant transformation.[94] Baldus et al reported earlier that high ERG expression was not always associated with genomic amplification, thereby leaving ERG overexpression mechanistically unexplained.[87] Nevertheless, the recurrent presence of ERG overexpression in AML with complex karyotypes, a prognostically unfavorable subgroup, suggests that ERG overexpression might not only be a nonrandom event in myeloid leukemogenesis, but also might contribute to an aggressive malignant phenotype.

Marcucci et al from CALGB[44] recently tested the prognostic significance of ETS-related gene (ERG) expression in cytogenetically normal primary AML patients (N=84) who were uniformly treated on the Cancer and Leukemia Group B (CALGB) 9621 protocol. ERG overexpression predicted an increased relapse risk and short survival in AML patients with normal karyotype by both univariate and multivariate analyses and seemed to be associated with a specific molecular signature.[44] With a median follow-up of 5.7 years, patients with the upper 25% of ERG expression values had a worse CIR (*P*<.001) and OS (*P*=.011) than the remaining patients. In a multivariate analysis, high ERG expression (*P*<.001) and the presence of *MLL* PTD (*P*=.027) predicted worse CIR. With regard to OS, an interaction was observed between expression of ERG and *BAALC* (*P*=.013), with ERG overexpression predicting shorter survival only in low *BAALC* expressers (*P*=.002). ERG overexpression was an independent prognostic factor even when the unfavorable group of *FLT3*-ITD patients lacking an *FLT3* wild-type allele was included. High ERG expression was associated with upregulation of 112 expressed-sequenced tags and

named genes, many of which are involved in cell proliferation, differentiation, and apoptosis.[44]

More recently, Marcucci et al have validated ERG overexpression as an adverse predictor in cytogenetically normal AML patients (seen in 37% of such patients).[96] Moreover, by using ERG expression levels, previously proposed molecular-risk classification of cytogenetically normal AML (based on the presence or absence of *FLT3*-ITD and *NPM1* mutations) could be improved as they identified subsets with different outcome among *FLT3*-ITD-negative patients, *NPM1*-positive patients, and *FLT3*-ITD-negative/*NPM1*-positive patients according to ERG expression level.[96]

Mutations of the CCAAT enhancer-binding protein α (*CEBPA*) gene

CEBPA is a single exon gene located in the chromosome region 19q 13.1 that encodes CCAAT enhancer-binding protein α (CEBPα), a member of the basic region leucine zipper (bZIP) class of transcription factors and consist of highly homologous C-terminal DNA-binding (basic region) and dimerization (leucine zipper) motifs and two less-conserved N-terminal transactivation domains. On the basis of the observation that CEBPα-deficient mice lack mature granulocytes,[97] it has been speculated that *CEBPA* mutations might contribute to the differentiation block specific to AML. Conditional expression of CEBPα induces a number of granulocyte specific genes that induce differentiation, while knockout mice exhibit an early block in maturation.

An alternative mechanism for CEBPα inactivation has been described in AML patients with t(8;21), where, the AML 1-ETO fusion protein suppresses CEBPα transcription and hence blocks granulocyte differentiation.[98] Mutations in the CEBPα gene can be detected in 7% to 15% of patients with AML mostly in the FAB-M2 subtype and the majority of patients with mutations had normal cytogenetics.[99,100] The favorable prognostic impact of *CEBPA* mutation has been suggested by several studies. Preudhomme et al[101] examined pretreatment samples from 135 adults and identified different types of mutations in 15 (16%) of 91 patients with intermediate-risk cytogenetics. The presence of a *CEBPA* mutation was associated with significantly better clinical outcome. van Waalwijk from the Netherlands, screened 277 patients for mutations in the bZIP domain. In-frame insertions were identified in 12 patients (4%; eight patients with normal cytogenetics and four patients with other chromosome abnormalities) and were subsequently shown to coincide with N-terminal mutations on the other allele. Among the 187 patients with intermediate-risk karyotypes, patients with the mutations had significantly increased EFS and OS.[102]

More recently, Frohling et al assessed the prognostic significance of *CEBPA* mutation on 236 AML patients, 16-60 years of age with normal cytogenetics.[103] *CEBPA* mutations were detected in 15% of the 236 patients and 9% had mutations predicted to result in loss of *CEBPA* function.[103] Remission duration and OS were significantly longer for the 36 patients with *CEBPA* mutations (P=.01 and P=.05, respectively). By multivariate analysis, wild-type *CEBPA* was an independent adverse prognostic marker affecting remission duration (hazard ratio, 2.85; P=.01) and OS (hazard ratio, 1.87; P=.04). In their analysis of cooperating mutations (both types of activating *FLT3* mutations and *MLL* partial tandem duplications) showed that *FLT3* mutations had no significant prognostic influence in patients with *CEBPA* mutations.[103] The above data consistently support the favorable outcome of *CEBPA* mutations in AML patients with normal cytogenetics.

Meningioma 1 (*MN1*) overexpression

The meningioma 1 (*MN1*) gene, localized on human chromosome 22, was first cloned in 1995 from a patient with meningioma with translocation t(4;22)(p16;q11).[104] It is disrupted in balanced translocation. The translocation t(12;22) involves TEL and *MN1* and is rarely found in AML. It has been shown in a mouse model that the fusion protein TEL-*MN1* can promote growth of primitive hematopoietic progenitor cells (HPCs)[105] and, in cooperation with *HOXA9*, which is overexpressed in patients with AML,[106] may induce AML.[105] Heuser et al from Germany recently quantified *MN1* expression by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in 142 adult patients with AML with normal cytogenetics treated uniformly in trial AML-SHG 01/99.[107] AML samples were dichotomized at the median *MN1* expression. High *MN1* expression was significantly correlated with unmutated *NPM1* (P<.001), poor response to the first course of induction treatment (P=.02), a higher relapse rate (P=.03), and shorter relapse-free (P=.002) and OS (P=.03). In a multivariate analysis, *MN1* expression was an independent prognostic marker (P=.02) in addition to age and Eastern Cooperative Oncology Group (ECOG) performance status. Excluding patients with *NPM1*mutated/*FLT3*-ITD negative, high *MN1* expression was associated with shorter relapse-free survival (P=.057). *MN1* was highly expressed in HPCs compared with differentiated cells and was down-regulated during in vitro differentiation of CD34+ cells, suggesting a functional role in HPCs. Heuser et al concluded that *MN1* overexpression may

serve as a new prognostic marker in AML with normal cytogenetics.[107] However, these findings are yet to be verified by further studies. The negative impact of *MN1* overexpression in elderly AML patients (leading to all-trans retonoic acid resistance) is further discussed below.

RAS mutations

RAS (rat sarcoma) gene mutations were first reported in AML patients 18 years ago,[108,109] but the prognostic impact of *NRAS* mutations in AML is still under debate. RAS oncogenes encode a family of membrane-associated proteins that regulate signal transduction on binding to a variety of membrane receptors. There are three functional RAS genes: N-(from a neuroblastoma cell line), K-(Kirsten), and H-(Harvey) RAS, each containing 4 exons and all homologs carry mutations nearly exclusively in codons 12, 13, and 61, conferring constitutive activation of the RAS protein. *NRAS* mutations seem to be the most prominent RAS mutations in patients with AML and have been reported in 11% to 30% of patients.[110-118] They lead to increased activity of the RAS pathway, resulting in increased proliferation and decreased apoptosis rates.[114]

Some studies indicate an association with poor outcome in general,[119,120] or in only those AML patients who had a favorable karyotype.[112] In contrast, Neubauer et al[111] found *NRAS* mutations associated with a favorable prognosis, whereas in some studies a prognostic impact of *NRAS* mutations could not be defined at all.[114-116] Some studies did not find a significant association of *NRAS* mutations with certain cytogenetic subgroups.[111,113] To clarify the biologic and prognostic impact of *NRAS* mutations, Bacher et al[110] recently analyzed a large cohort of 2502 patients with AML who were well characterized with respect to cytomorphology, cytogenetics, and other molecular mutations. Two hundred fifty-seven (10.3%) of 2502 patients had *NRAS* mutations (*NRAS*mut). Most mutations (112 of 257; 43.6%) were found at codon 12, mostly resulting in changes from glycine to asparagine. These included 109 of 1198 patients with normal karyotype, (9.1%). Overall, they did not find a significant prognostic impact of *NRAS*mut for OS, EFS, and disease-free survival DFS. However, detailed analyses of Kaplan-Meier plots in the *NRAS*mut cohort showed a trend to reach a plateau after 1 year of follow-up in normal karyotype, t(8;21) and inv(16) according to OS analysis.[110]

Taking other molecular markers (*FLT3*-LM, *MLL*-PTD, *NPM1*) into account in this group indicated an even better trend for a more favorable prognosis. In the total cohort without *FLT3* mutations, EFS was slightly improved for patients with *NRAS* mutations (426 ± 73 days) compared with patients negative for *NRAS* mutations (378±21 days) (P=.060).[110]

Mutations in Wilms tumor 1 gene (*WT1*)

The Wilms tumor gene (*WT1*) located on chromosome 11p13 encodes a transcription factor, which is involved in control of growth and differentiation of various cell types including hematopoietic cells.[121] Another study revealed that *WT1* expression levels in normal hematopoietic progenitor cells were at least 10 times less than those in leukemic cells indicating an aberrant overexpression of the *WT1* gene in leukemic cells and implying the involvement of this gene in human leukemogenesis.[122] *WT1* mutations in AML were first reported in 1998.[123] Disruption of *WT1* function by mutation of the gene could either promote proliferation or induce a block in differentiation. In a recent study of 70 patients having AML with normal cytogenetics, *WT1* mutations were detected in 10% of the patients.[124] They found that the mutations consisted of insertions or deletions that mainly clustered in exons 7 and 9. The *WT1* mutations were associated with *FLT3*-ITD and failure of standard induction chemotherapy in this study.[124] However, the impact of *WT1* mutations needs to be evaluated in larger series. Murine and early human studies suggest that *WT1* protein can serve as a target antigen for tumor-specific immunity.[125] The *WT1* is a reliable marker for MRD assessment in acute leukemia patients. The evaluation of *WT1* in peripheral blood samples after induction chemotherapy can distinguish the continuous complete remission patients from those who obtain only an "apparent" complete remission and who could relapse within a few months. *WT1* helps identify patients at high risk of relapse soon after induction chemotherapy allowing post-induction therapy in high-risk patients to be intensified.[126]

Many other genes mutations, such as inactivating mutations of the ubiquitin ligase CBL, have been implicated to have clinical relevance in AML with normal cytogenetics, but the prevalence and clinical relevance, if any, of such gene mutations, remain to be determined.[127-129]

Interactions among prognostically important genes

Most of the above cited studies have focused on the prognostic value of single markers, not taking into account their potential interactions. However, as our knowledge is improving, later studies have tried to explore interaction of various prognostically important genes in AML patients.[20-24,110,130,131] For example, in the

study by Bacher et al[110] the impact of *NRAS* mutations on OS in AML patients with normal karyotype was favorable after 1 year and when other molecular markers (*FLT3*-LM, MLL-PTD, *NPM1*) were taken into account the cohort indicated an even better trend for a more favorable prognosis.

Very recently Schlenk et al registered 872 patients (16 to 60 years of age) with normal karyotype AML130 from four AMLSG treatment trials (AML-2/95, AML-1/99, AML HD93, AML HD98A) and obtained tumor samples from most. Several genes were examined for mutations including: *NPM1*, *FLT3* (*FLT3*-ITD and *FLT3*-TKD), *CEBPA*, *MLL* (PTD), and *NRAS*. Using a logistic regression model, two mutation combinations were identified as predicting response to induction therapy: *NPM1*+/*FLT3*-ITD- and *CEBPA*+. Cox proportional hazard models were then constructed to identify prognostic factors for relapse-free survival (RFS) and OS. Age <48 years, the availability of a matched-related donor, and in particular the *NPM1*+/*FLT3*-ITD- (hazard ratio of 0.34 and 0.43 for RFS and OS survival respectively) and *CEBPA*+ genotypes (hazard ratio 0.42 and 0.36), were all associated with improved outcomes. Interestingly, in those patients with the *NPM1*+/*FLT3*-ITD- genotype combination, the availability of a matched-related donor did not appear to confer a significant survival advantage (HR 0.89 and 0.93 for RFS and OS, respectively). In contrast, for those patients possessing all other combinations of *NPM1* and *FLT3*-ITD, 4-year RFS was significantly higher in those with a matched-related donor (47%) versus those without a donor (23%). This implies that allogeneic HSCT in first CR does offer benefit over standard post-remission therapy in normal karyotype AML, but only in those without the *NPM1*+/*FLT3*-ITD-genotype.[130]

A study by Schlenk et al is pioneering in that it concurrently included multiple genetic markers in its prognostic modeling. The hazard ratios for the *NPM1*+/*FLT3*-ITD- and *CEBPA*+ genotypes are particularly impressive and appear to strongly predict a subset of normal karyotype AML patients destined for good clinical outcomes. More recently Schlenk at al compared the mutational status of the *NPM1*, *FLT3*, *CEBPA*, *MLL*, and *NRAS* genes in leukemia cells with the clinical outcome in 872 adults younger than 60 years of age with cytogenetically normal AML.[131] The overall CR rate was 77%. The genotype of mutant *NPM1* without *FLT3*-ITD, the mutant *CEBPA* genotype, and younger age were each significantly associated with higher CR rate.[131] Of the 663 patients who received postremission therapy, 150 underwent HSCT from an HLA-matched related donor. The risk of relapse or the risk of death during complete remission were lower with the leukemia genotype of mutant *NPM1* without *FLT3*-ITD (hazard ratio, 0.44), the mutant *CEBPA* genotype (hazard ratio, 0.48), while such risks were higher with the *MLL*-PTD genotype (hazard ratio, 1.56).[131] Receipt of a transplant from an HLA-matched related donor lowered the risk of death and relapse (hazard ratio, 0.60) in the subgroup of patients with the prognostically adverse genotype *FLT3*-ITD or the genotype consisting of wild-type *NPM1* and *CEBPA* without *FLT3*-ITD.[131]

As two or more genetic alterations can be present simultaneously in AML patients with normal cytogenetics, part of the validation process of a newly discovered predictor is to prove that it can maintain an independent prognostic value when assessed against other molecular markers. However, one of the limitations of this type of approach, often lies in the small number of patients available for subset analyses, so researchers have attempted to combine data from various trials to get meaningful results.[96]

Recent data from Marcucci et al[96] indicate that *FLT3*-ITD-negative/*NPM1*-positive patients, considered to have a favorable prognosis, can be separated into prognostic subsets based on expression levels of ERG. They suggest that *FLT3*-ITD-negative/*NPM1*-positive/low ERG patients are likely to benefit from high-dose cytarabine and autologous peripheral-blood stem-cell transplantation-based therapies as administered in CALGB 9621 and 19808.[96] In contrast, *FLT3*-ITD-negative/*NPM1*-negative/high ERG patients perhaps should be considered for alternative and/or more aggressive treatments, because their estimated 2-year EFS is less than 40%. Similarly, cytogenetically normal AML patients with *FLT3*-ITD who have a poor prognosis should be considered for clinical trials investigating compounds that directly inhibit *FLT3* tyrosine kinase activity or for allogeneic SCT, although a definitive role for the latter in first CR remains to be established.[96]

A few markers are about to enter clinical practice, in particular in the context of clinical trials. For instance, activating *FLT3* mutations, in particular in AML with normal cytogenetics, have prognostic significance and, importantly, are attractive targets for molecularly targeted therapy. The first randomized studies using TK inhibitors in these patient subsets have started. Furthermore, two genotypes, *NPM1*^{mut}/*FLT3*-ITD^{neg} and *CEBPA*^{mut}, have been identified that are associated with a favorable risk profile, comparable with that of core-binding factor (CBF) AML. Patients whose AML harbor such genotypes may not benefit from allogeneic

SCT in first-line treatment. These data have important implications since the two genotypes represent approximately 45% of patients with cytogenetically normal (CN)-AML and 20% of all patients with AML. As a consequence, *NPM1*, *FLT3* and *CEBPA* mutational screening should become part of the initial work-up of a newly diagnosed AML.

Although such clinical outcome models will ultimately require further refinement and prospective validation, they appear to provide a new tool with which hematologists can identify those most likely to benefit from upfront allogeneic HSCT and spare good-prognosis patients from unnecessary transplant-related morbidity.[130,132] There have been suggestions that a classification of AML incorporating molecular markers will emerge soon.[133,135]

We propose that as the number of such genetic aberrations is growing and we are accumulating information about the relative impact of each aberration on clinical outcome, a scoring system incorporating all such markers may be desirable for prospective decision making. This exciting new information may allow for a more tailored therapeutic approach to such patients in the near future.

Some peculiarities of genetic alterations in elderly patients

AML occurring in patients older than 55 to 60 years of age is characterized by profound biologic differences, including the distribution, but not the spectrum, of cytogenetic abnormalities. In particular, favorable-risk aberrations are relatively uncommon in the elderly, as opposed to normal and complex karyotypes. However in absolute terms the intermediate-risk cytogenetics group including those with normal karyotype constitutes the largest proportion (58%) of even the elderly patients with AML.[136]

At the molecular level, mutations in the *FLT3*, RAS, and TP53 (p53) genes are relatively common in elderly patients with AML, with estimated prevalences of 27%, 16%, and 10%, respectively.[137] However, it is not clear if these *FLT3*-ITD and point mutations in the activation loop of the *FLT3*-TKD (such as the D835Y mutation) are independent predictors of the prognosis of elderly AML patients. In a SWOG study on the outcome of 140 elderly patients treated with daunorubicin and cytarabine on SWOG S9031.[137] The *FLT3* ITD was detected in 34% of these patients. However, in 105 patients with complete cytogenetic and MDR1 data, *FLT3* mutational status did not provide any additional prognostic information for achievement of CR after controlling for age, leukocytosis, blast karyotype, and MDR1 expression. *FLT3* ITD was associated with a lower risk of resistant disease. Similarly the presence of *FLT3* ITD did not significantly affect the CR rate, CR duration, or overall survival of patients more than 60 years old treated with high-dose cytarabine-based regimens at M. D. Anderson Cancer Center.[67]

Another study comparing 381 patients having MDS and 4130 patients with AML (de novo: n=3139; secondary AML following MDS: n=397; therapy-related: n=233; relapsed: n=361) has shown that the frequency of *FLT3*-length mutations, *FLT3*-TKD, *MLL*-PTD, *NRAS*, and KITD816 increase from low- to high-risk MDS, to secondary AML, and to relapsed AML emphasizing the value of these mutations as markers of progressing disease.[95] As AML arising from MDS is a more common event in the elderly, progressive age intuitively may also lead to accumulation of molecular genetic lesions in the elderly AML patients having normal cytogenetics.

As described above, *MN1* is a unique oncogene in hematopoiesis that both promotes proliferation/self-renewal and blocks differentiation. It may become useful as a predictive marker in AML treatment, particularly in elderly patients. Heuser et al recently evaluated whether *MN1* expression levels in patients with AML (excluding M3-AML) correlated with resistance to ATRA treatment in elderly patients uniformly treated within treatment protocol AMLHD98-B.[138] Strikingly, patients with low *MN1* expression who received ATRA had a significantly prolonged event-free (P=.008) and overall (P=.04) survival compared with patients with either low *MN1* expression and no ATRA, or high *MN1* expression with or without ATRA. Hence in this study high *MN1* expression in patients with Non-M3AML was associated with ATRA resistance, whereas patients with low *MN1* expression survived significantly longer when ATRA was added to standard chemotherapy.[138]

Overall, these data suggest that the ability of elderly patients to tolerate induction chemotherapy may be a more important factor in addition to age, leukocytosis, blast karyotype, and MDR1 expression.[67,137] Obviously, these facts have great implications in planning therapies for this special population. There is a need to further explore therapeutically relevant molecular genetic lesions and more effective but less toxic targeted therapies for the elderly AML patients with normal cytogenetics. As described above, *NPM1*, *FLT3* and *CEBPA* mutational screening and *MN1* expression assessment may soon become part of the initial work-up of a newly diagnosed AML patient and may help in improving the stratification of elderly AML patients with normal cytogenetics.

Gene expression profiling in the subclassification of AML with a normal karyotype

Genomic approaches have altered the landscape of medical research and have potential to transform clinical practice. Although the established genetic markers like NPM, *FLT3*, *CEBPA*, *BAALC*, *MLL* and NM1 have substantially improved the potential for subclassification of AML with a normal karyotype further insights into the molecular heterogeneity are necessary. To find molecular signatures that identify prognostic subgroups, microarray gene expression profiling has been used as it may help not only in the development of models of outcome prediction, but also in predicting therapy-related toxicity, identifying new pathways of disease pathogenesis and potential targets for therapy.

The DNA microarray (complementary DNA and oligonucleotide) has been a critical tool for genomic-based approaches to generating information about tens of thousands of genes for a given sample. This generation of vast amounts of data has much potential but many challenges. One of the early challenges has been the development of analytical tools to interpret thousands of data points for relatively few samples. Analytical techniques have principally focused on supervised and unsupervised learning. Supervised learning approaches use known class labels (i.e. tumor type and relapse status) to identify genes whose expression correlates with the class label. As such, supervised learning has been used for class prediction and the the assignment of a sample to an already defined class. Unsupervised learning assumes no prior knowledge of sample label and rather groups samples together based solely on gene expression status. Unsupervised learning has been used for new class discovery.[139]

Few recent studies have addressed this topic in relation to cytogenetically normal AML patients. In a study by Valk et al, AML patients with a normal karyotype were grouped into several clusters, three of which accounted for at least 75% of patients.[140] Using cDNA microarrays Bullinger et al profiled 116 diagnostic AML samples and could show that AML patients with a normal karyotype can be divided in two subgroups with different OS.[141] Bullinger et al, with unsupervised and supervised learning algorithms, developed a clinical outcome predictor validated in an independent data set. This expression-based predictor identified good versus poor outcome with statistical significance whether applied to patients with known cytogenetic abnormalities or to those with a normal karyotype. Radmacher et al from CALGB recently validated prognostic association of Bullinger's signature in normal karyotype AML patients, using a different platform (Affymetrix U133) for measuring oligonucleotide microarrays gene expression.[142] This expression signature is the first outcome predictor for patients with intermediate-risk AML. However, clustering can be applied to a set of patients but not for subsequent individual patients as adding a patient can affect formation of clusters and thus change the rules by which each patient is classified. For outcome prediction of individuals, we need a rule unaffected by adding new patients. For example, compound covariate prediction (CCP) is one of many statistical techniques that may be used to assign individual patients to poor or good outcome groups. Radmacher et al used CCP to assign patients to poor or good outcome groups using the Bullinger signature[139,142] and they were able to develop a classifier that predicts outcome (good vs poor) in terms of DFS and OS. Notably this particular signature seems to be highly affected by *FLT3* ITD as a classifier does not discriminate *FLT3* ITD+ patients well, but appears to identify patients in *FLT3* WT group who will have lower survival but prediction accuracy here was moderate.[139,142] Very recently Bullinger et al have discovered the gene-expression patterns that correlated with *FLT3*-ITD mutation and evaluated the utility of their *FLT3* signature (composed of 20 genes) for prognostication in AML patients with normal cytogenetics.[134] Their predictor exhibited modest performance (73% sensitivity; 85% specificity) in classifying *FLT3*-ITD status. The signature outperformed *FLT3*-ITD mutation status in predicting clinical outcome. These findings support the potential clinical utility of a gene expression-based measure of *FLT3* pathway activation in AML.[134]

Bullinger et al earlier profiled gene expression of 138 samples of adult AML patients with normal karyotype using DNA microarray technology.[143] All samples analyzed were derived from AML patients entered within the randomized multicenter treatment trial HD-98A of the German-Austrian AML Study Group (AMLSG). Based on supervised data analyses they were able to identify a 116-gene comprising expression pattern correlated with *NPM1*-mutated and *FLT3* ITD-negative AML cases. The *NPM1*-mutated/*FLT3* ITD-negative pattern was also in part characterized by a prominent *HOX* gene cluster, which clearly separated the *NPM1*-wild type from the *NPM1*-mutated cases. As expected, the *NPM1*-unmutated cases displayed higher *BAALC* and *MN1* expression and the newly defined signature also defined a *NPM1*-mutated group that did not contain many *FLT3* ITD-positive samples. Moreover, they identified several other genes of potential pathogenic relevance which also have been previously shown to be

predictive in normal karyotype AML. These findings support a distinct molecular mechanism associated with the favorable outcome of *NPM1*-mutated/*FLT3* ITD-negative AML cases.[143] Such reported signatures might contribute to improved risk stratification and clinical management of AML patients with normal karyotype. While many challenges remain to be overcome, a combination of gene expression profiling with other microarray-based applications, high-throughput mutational analyses and proteomic approaches will also give important insights into the true pathobiologic nature of this type of leukemia.

DNA microarray technology matured in the mid-1990s, and the past decade has witnessed a tremendous growth in its application as a powerful tools for researchers seeking to describe, classify, and understand human disease. There are great expectations that the technology would advance the AML research. Some challenges remain in translating this technology in clinical practice to confirm if different signature-based classifiers more accurately predict outcome for individual patients and determine how it can be used to stratify patients for risk-adapted treatments.

Conclusions

For adult patients with AML, karyotype is the strongest predictor of treatment outcome. AML with normal karyotype is usually grouped in the "intermediate risk" category. The treatment outcome of AML with normal karyotype is extremely heterogeneous. Accordingly, there has been a recent interest in using molecular markers to risk stratify these patients. Available new technologies such as gene expression profiling have improved our knowledge about potentially altered genes and their prognostic impact. It can be foreseen that these newly recognized molecular markers will soon become part of diagnostic/prognostic workup routine for AML patients with normal cytogenetics. There have been suggestions that soon a classification of AML incorporating molecular markers will emerge.

Eventually, these molecular findings will be used to stratify patients for risk-adapted and targeted treatments. With target identification followed by the demonstration that activating mutations of *FLT3* are pathophysiologically relevant in AML, the development of *FLT3* inhibitors (and other tyrosine kinase inhibitors) as therapeutic agents for AML patients with normal cytogenetics is an important step. It appears that the most widespread use of these tyrosine kinase inhibitors showing activity in AML patients will come in conjunction with chemotherapy.

Although proposed clinical outcome models incorporating the new molecular findings require further refinement and prospective validation, these appear to provide a new tool with which hematologists can identify patients most likely to benefit from upfront allogeneic HSCT and spare good-prognosis patients from unnecessary transplant-related morbidity. As the number of such genetic aberrations is growing and we are accumulating information about the relative impact of each aberration on clinical outcome, we propose that a scoring system incorporating all (or selected) such markers may be desirable for prospective decision making. There is a need to further explore therapeutically relevant molecular genetic lesions and more effective but less toxic targeted therapies for elderly AML patients with normal cytogenetics.

The above review provided a summary of a limited number of recently recognized dysregulated genes and their prognostic impact. More knowledge about these dysregulated genes and identification of additional genes is expected in the next few years and will be extremely instrumental in providing more tailored therapies for the heterogeneous subset of AML patients with normal karyotype.

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