Impact of mutational analysis in acute myeloid leukemia

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A B S T R A C T

During the last 10 years, the classification of acute myeloid leukemia (AML) has changed substantially due the successive integration of novel molecular abnormalities, a process that is ongoing constantly and will lead finally to a segregation of this complex disease into individual, genetically defined entities. The most recently identified mutations in several genes involved in epigenetic regulation (IDH1 and 2, TET2, DNMT3A) using whole genome sequencing approaches has extended the spectrum of abnormalities, clearly indicating the complex nature of the disease. The identification of these novel pathways has extended further the portfolio of potential therapeutic targets to improve treatment in AML.

As already introduced in the first paper by Dr. Delwel, the research of the last 40 years has clarified that the disease called acute non-lymphocytic leukemia initially and now known as acute myeloid leukemia (AML) represents a much more heterogeneous group of hematopoietic stem cell disorders of the myeloid lineage. Especially the cytogenetic and molecular characterization performed in the last 20 years has enabled a much better understanding of the mechanisms responsible for leukemia development, which has already led to first strategies for a targeted treatment and will help hopefully to improve outcome of the disease.

During the last 15 years, we have seen a continuous refinement of the molecular phenotype of AML, with the identification of several important molecular lesions. The most prominent representatives are mutations in N- and K-RAS, MLL, FLT3, RUNX1, CEBPA, NPM1, KIT, and WT1. Some of these changes are now recognized as disease defining lesions[1] and have become important prognostic factors that are used prospectively to stratify patients into treatment protocols (NPM1, CEBPA, FLT3). In addition, some of them represent promising candidates for targeted treatment (FLT3).[2] With the introduction of next-generation sequencing (NGS), this linear process of discovery has turned into an exponential phase. This novel, high throughput technology allows an unbiased view on small genomic abnormalities for the first time. Thus, its use for the analysis of leukemias, as pioneered by Timothy Ley's group from the University of Washington,[3] not only increased tremendously the number of identified abnormalities, but also shifted the spectrum of discovered changes. NGS led to the discovery of numerous novel abnormalities, including mutations in IDH1/2,[4] DNMT3a,[5] BCOR/BCORL1[6,7] and several others, which affect novel pathways and had not been associated with leukemia development so far. This review will focus on the current situation in the molecular typing of AML and will provide an outlook on potential future developments.

Over the last 20 years, AML has been classified using cytogenetics. Based on these findings, the large cooperative groups identified three major cytogenetic prognostic risk groups.[8-10] Patients with core binding factor (CBF)-leukemias, that is, with reciprocal translocations involving chromosomes 8 and 21 (t(8;21)(q22;q22) leading to a fusion of the *RUNX1* gene to the *RUNX1T1* gene or with an inversion of chromosome 16 (inv16) (p13.1q22)/or t(16;16)(p13.1;q22), both leading to the fusion of the *CBF* and *MYH11* genes, belong to the favorable risk group. On the opposite end of the scale, patients with loss of chromosomes 5 or 7, or patients showing complex cytogenetic aberrations (*i.e.*, independent alterations of three or more chromosomes), were shown to have a very poor response to standard chemotherapy and an overall inferior outcome. These patients are therefore considered as high-risk patients. In between, there is the large group of patients with intermediate risk, mostly showing a normal karyotype (CN-AML).

The last two releases of the WHO classification for the first time integrated cytogenetics (WHO 2001), as well as molecular abnormalities (WHO 2008) as disease defining alterations. Some of these abnormalities are sufficient to define the diagnosis AML (Table 1). The presence of CBF-alterations and of alterations involving the retinoic-acid receptor alpha gene (*RARA*) defines the diagnosis, irrespective of the blast count in peripheral blood or bone marrow.

Although the overall classification of the cytogenetic groups did not generally change over the last years, several important refine-

ments have been made. The Rotterdam group[11] could show for the first time that a so-called "monosomal karyotype (MK)", defined by two or more distinct autosomal chromosomal monosomies, represents an independent prognostic factor associated with very poor outcome. This result has been confirmed by other groups.[12,13] In an extended dataset of more than 5000 patients treated in different MRC-protocols, Grimwade *et al.* identified additional favorable (inv16+trisomy 22) and unfavorable subtypes (*e.g.*, abn 3q).[14] In another large dataset of more than 2200 patients, Seifert *et al.* documented the dismal outcome associated with loss of chromosome 17p.[15]

However, the most impressive and important achievements in the prognostic assessment have been made in the large group of patients carrying a CN-AML. Between 40 and 50% of adult AML patients belong to this group. A variety of molecular abnormalities have been identified especially in these patients (Table 2). The most prominent abnormalities of this group are mutations in the NPM1, FLT3, and CEBPA genes. Dr. Delwel has covered the CEBPA mutations extensively in his review. The results on NPM1 and FLT3 will be discussed here in more detail.

FLT3 mutations: one gene, many aspects

Even when located in the same gene, the estimation of the prognostic impact of different mutations is a complex task. This is probably best illustrated by one of the most frequently mutated genes in AML: fms-like tyrosine kinase 3 (FLT3). The FLT3 receptor tyrosine kinase is a protein expressed on early hematopoietic progenitor cells. Mutations of FLT3 have been described in two critical regulatory domains of the protein: the juxtamembrane (JM) region and the second tyrosine kinase domain.[16] Internal tandem duplication (ITD) mutations of the JM-domain encoded by exons 14 and 15 of *FLT3* have been described in 15-25% of patients with AML.[17] The prevalence is significantly higher in certain groups of patients defined by cytogenetics, as CN-AML, t(15;17) and t(6;9), reaching up to 80% in the rare subgroup of patients with t(6;9).[19,20] Also, patients carrying mutations in several other genes, that is, *NPM1*[18] and *DNMT3A*,[5] more often display *FLT3-ITD* mutations. There is now broad consistency in the published results, showing that FLT3-ITD mutations in general are associated with inferior outcome,[21] although there might be several important modulators of the prognostic impact (see below).

The other predominant type of mutations are missense single base pair exchanges in the second tyrosine kinase domain, especially in codon D835, but also in several other codons in close vicinity.[22,23] Mutations have been described in 6-10% of all AML patients. They are also more prevalent in patients with normal karyotype and appear to be especially common in patients with NPM1-mutations and patients with inv(16).[23-25]

Both types of mutations lead to constitutive activation of the FLT3 protein. However, several groups have shown profound differences in the mode of activation and the involved downstream signaling pathways.[26,27] Major differences relate to the activation of the STAT5 pathway, which might be relevant for the biological differences observed.[27]

Table 1. 2008 release of the WHO-classification of AML (modified).[1]

Group	Description	
1	AML with recurrent cytogenetic abnormalities: - t(8;21)(q22;q22), RUNX1-RUNX1T1 - inv(16)(p13.1;q22), t(16;16)(p13.1;q22) CBFB-MYH11 - acute promyelocytic leukemia with t(15;17)(q22;q12); PML-RARA and variants	defining an AML independent of blast count
	- AML with t(9;11)(p22;q23), MLLT3-MLL or other 11q23 (MLL) abnormalities - AML with t(6;9)(p23;q34), DEK-NUP214 - AML with inv(3)(q21;q26.2) oder t(3;3)(q21;q26.2), RPN1-EVI1 - AML (megakaryoblastic) with t(1;22)(p13;q13), RBM15-MKL1 - provisional entities: AML with mutated NPM1, AML with mutated CEBPA	defining an AML only if ≥20% blasts in bone marrow or blood
2	AML with myelodysplasia related changes	
3	Therapy-related myeloid neoplasm (t-AML)	
4	Myeloid sarcoma	
5	Myeloid proliferations associated with Down syndrome	
6	Blastic plasmacytoid dendritic cell neoplasm	
7	AML, not otherwise specified (AML-NOS)	

There has been a long debate as to whether FLT3-TKD mutations are also associated with inferior outcome. Although reports have claimed that TKD mutations represent a negative prognostic factor,[28] additional data indicate that TKD mutations may not be relevant for prognosis.[19] Some reports even show that these mutations are associated with an improved outcome in certain subgroups.[23,25] In support of this notion, a recent meta-analysis summarizing results comes to the conclusion that - in contrast to FLT3-ITD mutations - point mutations in the FLT3-TKD domain do not represent an independent prognostic factor.[29]

In addition to the differences between these two principal mutation groups, even within the group of the ITD-mutations, important modulators of the prognostic impact have been reported. Copy number neutral loss of the wild-type allele of *FLT3*, leading to uniparental disomy (UPD) of chromosome 13q, has been associated with a very high risk of treatment failure[19,30,31] and has been confirmed recently in the context of other genetic lesions.[32,33] Experimental data using a knock-in strategy indicate that wildtype FLT3 partially blocks and interferes with the aberrant signaling of the ITD-mutant receptor allele,[34] which might explain this effect. In addition to that, several groups have shown that the site of insertion within the *FLT3* gene further affects the prognostic impact:[35,36] patients with mutations extending into the first tyrosine kinase domain have a considerably higher risk of treatment failure.[36] There is a documented interaction of the insertion site and the length of the inserted sequence,[36] which might explain controversial data that claim that longer stretches of internal tandem duplications are associated with a worse prognosis. Taken together, these data illustrate the complexity of the interpretation of mutational data even within one gene. For many other recently described genes, we are just at the start of understanding the biology and the prognostic and predictive implications. FLT3 mutations are a clear indication that we should not jump prematurely to conclusions and use novel markers for clinical decision-making before sufficient evidence is collected.

NPM1: common and specifically mutated in AML

Among all identified AML gene mutations, *NPM1* is unique in several aspects. So far, it is the most common single gene abnormality in AML. It appears to be specific

Table 2. Recurrent molecular abnormalities in AML.

	Gene	Overall prevalence	Common in	Overall impact on prognosis
1	*NPM1*	30-35%	CN-AML (50%)	↑
2	*FLT3*-ITD;TKD	25%;7%	CN-AML (40%), t(15;17) (40%, t(6;9) (80%)	↓(ITD) →(TKD)
3	*CEBPA*	6%	CN-AML (10-15%)	↑
4	*WT1*	10-15%	CN-AML	↓→
5	*RUNX1*	10-15%	M0, sAML	↓
6	*MLL-PTD*	5-10%	CN-AML (10-15%)	↓
7	*RAS (NRAS/KRAS)*	15-20%	-7, NPM1. inv16	→
8	*PTPN11*	3%	-7, NPM1, inv16	?
9	*KIT*	2-3%	CBF-leukemias (10-10%)	↓
10	*JAK2*	1%	CBF-leukemias (5%)	↓
11	*CBL*	1-3%	CBF-leukemias	?
12	*TET2*	8-12%	sAML	↓
13	*IDH1* (R132) *IDH2* (R140;R172) *PHF6*	7-16% 8-15% 2-4%	CN-AML, NPM1 CN-AML, NPM1 males	↓↑→ ↓↑→ ↓
14	*ASXL1*	3-19%	sAML	↓
15	*DNMT3A*	15-25%	CN-AML (25-35%)	↓
16	*EZH2*	1-3%	sAML	?
17	*TP53*	2-5%	complex karyotype; -17	↓

for AML, with only very few reports existing on mutations found in non-AML patients, mostly patients with CMML or MDS. NPM1 is a nucleolar protein, which is constantly shuttling between nucleus and cytoplasm. The protein has several important functions, including binding of nucleic acids, regulation of centrosome duplication, and control of ribosomal function.[37] NPM1 binds to several proteins, including p53, as well as several other proteins known to interact with and to regulate p53, as, for example, Rb, $p14^{ARF}$ and HDM2. NPM1 is thought to be a major regulator of TP53 function in response to cellular stress factors, including hypoxia, UV-irradiation, or cytotoxic drugs. Brunangelo Falini first described the abnormal localization of the NPM1-protein in AML blasts of about one-third of the patients and identified a 4 bp-frameshift mutation occurring in exon 12 of the *NPM1* gene as the reason.[18] The mutation leads to an elongated protein and converts a nucleolar localization signal into a nuclear export signal. Mutant NPM1 can be detected in about 50% of all patients with CN-AML. NPM1 mutations are associated with a particular mRNA (upregulation of HOX-genes) and microRNA expression signature, and are associated frequently with alterations in other genes (*FLT3, IDH1/2, DNMT3A*). These data prompted the WHO to integrate NPM1 mutations together with CEBPA mutations as provisional entities in the 2008 release in the group of disease defining lesions[1] (Table 1).

The abundant clinical data published in the context of the NPM1 mutations indicate that *NPM1*-mutations are associated with improved outcome, if they are not accompanied by an FLT3-ITD mutation.[38-41] Patients with this favorable constellation show a superior long-term disease free and overall survival. Therefore, this genotype was integrated into the good risk group in the prognostic system recently published by the European leukemia network.[42] In a large dataset, Schlenk and coworkers showed that patients with the NPM1-mut/FLT3-ITD neg genotype do not benefit from an allogeneic transplantation performed in first CR .[43] However, more recent evaluations challenge these data,[44] potentially indicating that even in NPM1-mutant/FLT3-ITD neg patients, an allogeneic transplantation should be considered as consolidation treatment in patients with a fully matched donor and favorable transplantation associated risk scores.

Besides these two very common and established abnormalities, several novel mutations have been identified in the last three years.

Mutations in epigenetic modifier genes: IDH1/2, TET2, DNMT3A, and others

The application of NGS and other genome wide analysis methods in AML has revealed several new recurrent molecular alterations, namely mutations in *IDH1 and 2, DNMT3A, TET2*, and several other genes. Interestingly, the majority of these newly identified changes affect proteins involved in epigenetic regulation, especially DNA-methylation. Methylation of DNA at specific sites is an important mechanism involved in gene regulation. Altered methylation, with hypermethylation and transcriptional silencing of tumor suppressor genes (TSG) and gross genomic hypomethylation, has long been recognized as a common phenomenon in malignant transformation in general and also in leukemia. The mechanisms of altered methylation in tumors have long been unclear, but the emerging data shed new light on these processes.

The first of these novel mutations identified in hematopoietic malignancies is TET2, first described in MDS and CMPD.[45,46] The ten-eleven-translocated gene 2 (TET2) on chromosome 4q24 codes for a protein, which converts 5-methylcytosine into 5-hydroxylmethylcytosine (5-hmC).[47] Although still not understood completely, this process appears to be involved directly in the control of DNA-methylation and can lead to active demethylation of DNA.[48] Loss of TET2 function by mutations reduces the concentration of 5hmC. However, the effect on DNA-methylation appears to be site specific.[47] In hematopoietic malignancies, the TET2 gene is inactivated by mutations and genomic deletions, as well as by uniparental disomy of chromosome 4q. In AML, mutations of *TET2* have been described in 8-12% of patients with *de novo* AML, the prevalence being considerably higher in patients with sAML (up 25%). Due to the much more widespread distribution of *TET2*-mutations along the entire gene, the data on the prognostic implications of mutant *TET2* in AML are still limited. Although several studies showed the mutation to be associated with inferior outcome,[49-51] other groups failed to document an independent effect of the mutation on the response to chemotherapy.[52,53]

The first recurrent mutations identified by NGS were point mutations in the isocitrate dehydrogenase gene 1 (*IDH1*). IDH-proteins are components of the tricarboxylic acid (TCA)-cycle. In the initial report, mutations in IDH1 (R132) were found in about 8% of patients with AML, and in 16% of the CN-AML group.[4] Numerous studies confirmed IDH1 to be recurrently mutated in AML and associated with CN-AML and certain molecular abnormalities (NPM1); the overall reported prevalence is between 5.5 and 14%.[54-56] In addition, mutations in the IDH2 isoform of the protein (R140 and R172) were found subsequently at a similar frequency, with R140 being consistently more prevalent and specific for myeloid neoplasms.[57] In hematopoietic malignancies, IDH-mutations are not confined to AML, but can be found in CMPD[58,59] and MDS.[60]

IDH-mutations lead to neoformation of 2-hydroxyglutarate (2-HG),[61] which accumulates and can be measured in affected individuals.[57] More recently, Figueroa and coworkers[62] could show that the 2-HG produced by mutant IDH-proteins is able to block the function of the TET2-protein, leading to a decrease in 5-hmC content in the DNA. Interestingly, 2-HG production not only interferes with TET2 function and leads to aberrant DNA-methylation,[62] but also impairs histone demethylation and interferes with normal differentiation of cells.[62,63]

The prognostic impact of IDH-mutations is still controversial. Although several groups have shown that IDH-mutations are associated with inferior outcome, especially in the context of specific lesions,[55,56,64] others have seen no prognostic impact of the mutation[54,65] or even showed a beneficial effect in certain subgroups.[64,66] A potential explanation might be a differential effect of individual mutations, as suggested by some authors.[67]

In contrast to TET2 and IDH proteins, the prognostic effect of DNMT3A appears to be much more consistent between different studies. DNMT3A is one of three enzymatically active DNA-methyltransferases in humans.

DNMT3A and B are involved predominantly in de novo methylation, whereas DNMT1 acts primarily as maintenance methyltransferase. Several groups performing NGS analysis of AML patients recently independently described DNMT3A mutations.[5,68,69] The predominant mutations affect conserved functional regions of the protein, the majority being located in the catalytic domain with R882 accounting for about 50% of all mutations. DNMT3A mutations appear to be a common lesion in adult AML patients. The overall prevalence in unselected series is between 18 and 23%.[5,66,70,71] Interestingly, two publications from Asia report a considerably lower mutation frequency (7-14%),[72,73] potentially indicating an impact of the ethnic background. The highest rate of mutations was found in CN-AML (29-36%),[74,75] which makes DNMT3A mutations one of the most common alterations in CN-AML in adults. In general, mutations were found to be associated with increased age, FAB M4/M5 morphology, and increased WBC counts. In addition, all studies reported an independent prognostic effect of the presence of the mutation. Patients with mutant DNMT3A have a significantly shorter disease free and overall survival.[5,66,70-73,75] although some studies could document this only in certain mutational subgroups.[74]

In addition to these four commonly mutated genes, a number of additional mutations in genes are involved in epigenetic regulation, that is, *ASXL*,[76,77] *UTX*,[78] and *PHF6*.[79] The precise roles and prevalences of these alterations in AML have not been established fully, although preliminary data suggest that these mutations might also be associated with inferior outcome. In addition, first data indicate that some of these mutations may be predictive for the response to treatment with demethylating agents.[80,81]

Integrating the prognostic information of several genes

We are at the end of an era, where single mutations are interrogated to obtain prognostic information. As discussed in the previous paragraphs, combinations of mutations are detected that seem likely to dominate the disease phenotype. Using conventional methods, such as PCR, Sanger sequencing, or fragment analysis, it is very time and cost intensive to characterize all possible alterations in a clinical setting. However, with the invention of next generation sequencing, such multigene analyses are possible and will soon be available for routine testing in a clinical setting. Although even whole genome analysis may soon become feasible technically and financially for diagnostic purposes, the bottleneck for their clinical usefulness will be the development of algorithms for data analysis, as well as for assessment of the biological relevance of the alterations. However, targeted analyses of genes using either PCR-amplicon resequencing or even whole exome enrichment may soon be suitable to perform the constantly increasing demand for molecular analysis of patient samples in clinical practice.

In a recent comprehensive analysis of mutations in a cohort of almost 400 patients with AML treated in the E1900 protocol of the Eastern Cooperative Group (ECOG), Patel and coworkers[66] reported for the first time a simultaneous mutational analysis of 18 genes, covering most abnormalities currently discussed as relevant for AML prognosis. Their results show that somatic mutations in any of the 18 genes can be found in more than 97% of all patients. In line with previously published data, *FLT3, NPM1*, and *DNMT3A* are the most frequently mutated genes. An analysis of the impact of all mutations showed that the alterations in *FLT3-ITD, MLL-PTD, ASXL1*, and *PHF6* are independently predicting an inferior outcome. In contrast, the good prognosis of NPM1 mutant patients was restricted to patients with NPM1 mutations in the presence of an IDH2 mutation. This study represents an important step towards a novel molecular classification of AML. However, based on 400 patients, the group size in certain subgroups necessarily became small, so that several important aspects could not be covered completely. For example, mutations in *IDH1* and 2 have previously been intensely investigated. Several groups have shown substantial differential biological and clinical effects of specific mutations in these two genes. For example, alterations in the codons R140 and R172 of *IDH2*, respectively, confer markedly different biological effects.[67] Taken together, interpretation of the data presented by Patel has to be confirmed in larger patient series, before they can reliably be the basis of a novel, molecular classification of AML.

As outlined above, the complex interaction of individual markers shows that the prognostic information that can be deducted from single aberrations is context dependent. However, given the complexity of next generation sequencing data, assessment of the clinical meaning of the results of complex genetic testing on a given patient urgently requires additional tools and algorithms. Such algorithms should result in risk scores that will have to be re-evaluated constantly in the proper clinical context. Several groups tried to establish such scoring systems,[82,83] and have shown that in principle, integrated information of multiple abnormalities is significantly superior in its prognostic validity over the reporting of individual lesions. However, to be definitive, such analyses will have to be performed again, once all potential genetic alterations are discovered and sufficiently characterized. In addition, one should keep in mind that these scoring systems are by definition treatment dependent. Mutations, which do not have a major prognostic role in standard chemotherapy, might become highly relevant in the context of targeted treatment. A first example of such an association was demonstrated recently in the context of treatment with the potent FLT3-specific inhibitor quizartinib/AC220. As stated above, mutations in the second tyrosine kinase domain of FLT3 have been shown to have no prognostic value in standard chemotherapy (see above). However, in contrast to FLT3-ITD mutations, they confer resistance to quizartinib and have been described to occur as secondary mutations in the course of quizartinib treatment.[84] In addition, CEBPA mutations, associated with better outcome in patients undergoing standard treatment, might confer resistance against TKI-induced differentiation.[85] Taken together, these data indicate that the prognostic impact of lesions can change substantially in the context of novel treatment approaches.

About 10 years ago, Speck and Gilliland[86] proposed a model to illustrate the general mode of action of mutations in AML, with class I mutations primarily affecting proliferation and class II mutations interfering with nor-

mal differentiation due to the loss of critical myeloid transcription factors. In general, this scheme has shown to be valid to improve our understanding of leukemia development in AML. However, based on the recent results, the model needs refinement. The abundance of genetic lesions interfering with epigenetic regulation suggest that these might represent a third important mechanistic pathway (class III; Figure 1). In addition, the fact that between 8-12 mutations are consistently found in the AML genomes[3-5] suggests that the picture that will finally have to be drawn is much more complex than the idea that affecting two or three functional pathways of the normal myeloid hierarchy is sufficient to result in such a highly malignant disease as AML.

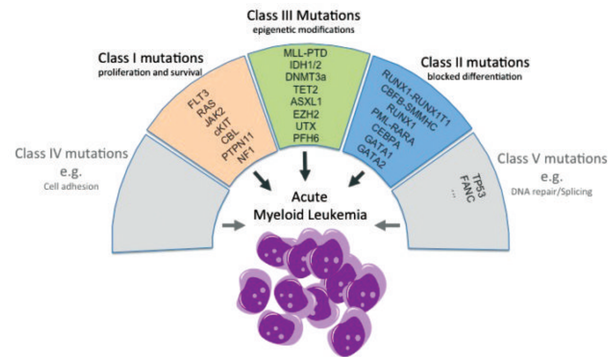

Figure 1. Molecular pathogenesis of AML.

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