Principles and Practice

Molecular Aspects of Hematologic Malignancies

Diagnostic Tools and Clinical Applications

Bearbeitet von
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Weitere Fachgebiete > Medizin > Vorklinische Medizin: Grundlagenfächer > Molekulare Medizin, Zellbiologie

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Chapter 2
Genetic Mechanisms and Molecular Markers of Neoplastic Transformation in Acute Myeloid Leukemia

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Abstract Acute myeloid leukemia (AML) constitutes a group of diseases heterogeneous with regard to clinical course, response to therapy as well as genetic features that contribute to disease pathogenesis, progression, and outcome. Numerous molecular lesions found in AML patients serve as prognostic and predictive factors; some of these markers have been included in the latest WHO classification. The analysis of cryptic genomic changes and alterations of gene expression is particularly important in cytogenetically normal AML patients. *NPM1* and biallelic *CEBPA* mutations are favorable prognostic factors, while *MLL* and *FLT3* duplications, *WT1*, *IDH1/IDH2*, *KIT*, *TET2*, and *DNMT3A* mutations, as well

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as overexpression of *BAALC*, *ERG*, or *MN1* have an adverse prognostic impact. Aside from aberrations involving structural genes, mutations of microRNAs, copy number alterations, and altered methylation of gene promoter regions also influence the development and progression of AML.

2.1 Introduction

Acute myeloid leukemia (AML) constitutes a broad range of disorders with marked clinical and biological heterogeneity. It is defined as a clonal proliferation of immature hematopoietic progenitors (myeloblasts) with varying degree of myeloid differentiation in the bone marrow, peripheral blood, or extramedullary tissues (Vardiman et al. 2008). AML accounts for 25 % of all cases of leukemia and shows a bimodal distribution across age groups, with a small peak of incidence very early in life, and then a much more prominent peak in older patients (Zuo et al. 2009). The characterization of the individual subtype of AML, crucial for therapeutic decisions, is nowadays based mainly on cytogenetic and molecular alterations of blast cells, or on morphology and immunophenotype in the absence of a specific genetic marker. The identification of genetic lesions, including gene expression changes, provides the basis for risk stratification and allows for effective treatment design.

2.2 Genetic Pathomechanisms of Acute Myeloid Leukemia

Genetic changes, recognized as primary alterations in AML, fall into two categories: chromosome aberrations, revealed by GTG-banding (*G* bands by *T*rypsin using *G*iemsa) in about 60 % of AML and submicroscopic changes, which occur both in cytogenetically normal AML (CN-AML) and in cytogenetically abnormal AML (CA-AML) patients (Grimwade and Hills 2009).

2.2.1 Classical "Two Hits" Model of AML Transformation

It is now commonly recognized that a cell which becomes an AML blast acquires two distinct kinds of mutations (Gilliland 2001).

Class I mutations confer proliferation and survival advantage. They involve genes coding for receptor and nonreceptor protein tyrosine kinases like *FLT3*, *JAK2*, *C-KIT*, *ABL1* (*C-ABL* oncogene 1, nonreceptor tyrosine kinase), as well as

for proteins of GTPase activity such as *N-RAS* and *K-RAS* (see Sect. 2.5), which alterations affect proliferative signaling pathways, causing abnormal growth of leukemic cells.[1]

Class II mutations lead to impaired differentiation of myeloid progenitor cells by affecting genes of transcription factors (TFs). They block the differentiation with minimal effect on proliferation. These mutations occur earlier in leukemogenesis (primary lesions) than class I mutations. Genes encoding TFs become disrupted either by their fusion, as a result of chromosome aberration (*CBF*, *MLL*, *EVI1*, *TEL* also named *ETV6*—ets variant 6, *RARA*—retinoic acid receptor, alfa), or by point mutation (*CEBPA* and *NPM1*) (Deguchi and Gilliland 2002; Kelly and Gilliland 2002).

While occurring separately, the aforementioned mutations cause excessive myeloproliferation (class I) or differentiation arrest (class II) of hematopoietic progenitors, resulting in the development of myeloproliferative syndrome or myelodysplasia, respectively. However, when occurring sequentially in a single cell, these "two hits" lead to a fully penetrating AML (Kelly and Gilliland 2002).

2.2.2 Extension of "Two Hits" Model by Other Mechanisms Leading to Leukemogenesis

The classical model of leukemogenesis proposed by Gilliland implies only lesions in the genes directly involved in the activation of proliferation and block of differentiation (Gilliland 2001). However, it has been recently shown that other gene classes might also be mutated in AML (Metzeler et al. 2011). These include genes which encode regulators of:

- Genomic stability and DNA repair, e.g.: tumor protein p53 (*TP53*),
- Transcription, e.g.: *TET2*, *IDH1/IDH2*, *DNMT3A*—DNA(cytosine-5)-methyl-transferase 3 alpha
- Translation (microRNAs),
- Protein turnover, e.g.: *C-CBL*—Cas-Br-M(murine) ectopic retroviral transforming sequence.

Mutations of these genes indirectly contribute to AML transformation by the alteration of either structure or expression of final effector genes, which results in proliferation advantage and differentiation arrest. Thus, the updated version of "two hits" model should comprise any structural or quantitative gene aberration, which deregulates growth and differentiation of hematopoietic cells (Ko et al. 2010; Makishima et al. 2009; Marcucci et al. 2010; Yan et al. 2011).

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[1] Unless included in the text, the names of the genes are presented in the descriptions in Tables 2.1 and 2.2. This refers to the whole chapter.

Table 2.1 Cryptic genetic alterations in AML and their prognostic impact

Gene/mutation	Chromosome localization	AML type/comment	Prognosis	Reference
FLT3-ITD	13q12	CN-AML	Poor	Whitman et al. (2010), Smith et al. (2011)
FLT3-TKD	13q12	CN-AML	Unclear	Whitman et al. (2010), Smith et al. (2011)
NPM1	5q35	CN-AML, distinct WHO entity (provisional)	NPM1(+)/FLT3-ITD(−)—favorable, NPM1(+)/FLT3-ITD(+)—intermediate, NPM1(−)/FLT3-ITD(−)—intermediate, NPM1(−)/FLT3-ITD(+)—poor	Bacher et al. (2010a), Becker et al. (2010), Thiede et al. (2006)
CEBPA	19q13.1	CN-AML, occurs as mono (sm) or biallelic (dm) mutation, distinct WHO entity (provisional)	dm—favorable, sm—intermediate	Dufour et al. (2010), Taskesen et al. (2011)
MLL-PTD	11q23.3	Mostly CN-AML, may associate with trisomy 11	Poor	Bacher et al. (2010a), Foran (2010), Mrózek et al. (2007)
IDH1	2q33.3	CN-AML	Poor in patients with NPM1(−)	Walker and Marcucci (2011)
IDH2	15q26.1	CN-AML	Poor in older patients	Walker and Marcucci (2011)
TET2	4q24	CN-AML	Poor, worsens prognosis in $CEBPA^{dm}$(+) AML, worsens prognosis in NPM1(+) AML	Metzeler et al. (2011)
WT1	11p13	CN-AML	Poor, especially poor in FLT3-ITD(+) patients	Betz and Hess (2010), Foran (2010), Gulley et al. (2010), Walker and Marcucci (2011)
N-RAS	1p13	CA-AML, associates with CBF leukemias	Neutral, may sensitize to cytarabine	Gulley et al. (2010), Smith et al. (2011)
C-KIT	4q12	CBF-AML	Poor	Betz and Hess (2010), Paschka et al. (2010)
BAALC	8q22.3	CN-AML	Overexpression—poor, especially poor in FLT3-ITD(+) patients, especially poor in CEBPA(−) patients	Bienz et al. (2005)

(continued)

Table 2.1 (continued)

Gene/mutation	Chromosome localization	AML type/comment	Prognosis	Reference
ERG	21q22.3	May correlate with Down syndrome-associated leukemia	Overexpression—poor	Marcucci et al. (2005), (2007), (2009)
MN1	22q12.1	CN-AML	Overexpression—poor	Langer et al. (2009)
EVI1	3q26.2	–	Overexpression—poor	Walker and Marcucci (2011)
MIR-181a	9q33.3	Overexpression correlates with CEBPA mutation	Overexpression—favorable	Schwind et al. (2010a), Marcucci et al. (2011) Walker and Marcucci (2011)
MIR-191	3p21.31	–	Overexpression—poor	Garzon et al. (2008)
MIR-199a	19p13.2	–	Overexpression—poor	Garzon et al. (2008)

CN-AML cytogenetically normal AML; CA-AML cytogenetically abnormal AML; *FLT3* fms-related tyrosine kinase 3; *NPM1* nucleophosmin; *CEBPA* CCAAT/enhancer binding protein (C/EBP), alpha; *MLL* myeloid/lymphoid or mixed-lineage leukemia factor (trithorax homolog, Drosophila); *IDH1* isocitrate dehydrogenase 1 (NADP +), soluble; *IDH2* isocitrate dehydrogenase 2 (NADP +), mitochondrial; *TET2* tet methylcytosine dioxygenase 2; *WT1* Wilms tumor 1; *NRAS* neuroblastoma RAS viral (v-ras) oncogene homolog; *KIT* v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; *BAALC* brain and acute leukemia, cytoplasmic; *ERG* v-ets erythroblastosis virus E26 oncogene homolog (avian); *MN1* meningioma (disrupted in balanced translocation) 1; *EVI1* (*MECOM*) MDS1 and EVI1 complex locus

Table 2.2 Guidelines for cytogenetic and molecular risk group stratification in AML according to the European LeukemiaNet (after Döhner et al. 2010, modified)

Genetic risk group	Subsets	AML patients (%)
Favorable	t(8;21)(q22;q22); *RUNX1–RUNX1T1(AML1-ETO)* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD (normal karyotype) Mutated *CEBPA* (normal karyotype)[a]	27
Intermediate-I[b]	Mutated *NPM1* and *FLT3*-ITD (normal karyotype) Wild-type *NPM1* and *FLT3*-ITD (normal karyotype)[c] Wild-type *NPM1* and *FLT3* (normal karyotype)	31
Intermediate-II	t(9;11)(p22;q23); *MLLT3-MLL* Cytogenetic abnormalities not classified as favorable or adverse	19
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* t(6;9)(p23;q34); *DEK-NUP214(CAN)* t(v;11)(v;q23)[d]; *MLL* rearranged –5 or del(5q); –7; 17p abnormalities[e]; complex karyotype[f]	23

a double (biallelic) mutated *CEBPA*

b includes all CN-AMLs, except for those included in the favorable subgroup. The aberrations are associated with intermediate or poor prognosis. However, they should be reported separately because of the potential different response to treatment

c NPM1(–)*FLT3*-ITD(+) AML is associated with poor prognosis, but it has been included in intermediate I group because of potential response to targeted treatment

d v, chromosome breakpoint other than 9p22

e with the rearrangement or deletion of *TP53* locus

f at least three chromosome aberrations, in the absence of one of the WHO designated recurrent balanced aberrations, i.e. t(15;17), t(8;21), inv/t(16)(q22), t(9;11), other 11q23 translocations, t(6;9), inv/t(3)(q26)

RUNX1(AML1) runt-related transcription factor 1; *RUNX1T1 (ETO)* runt-related transcription factor 1, translocated to, 1 (cyclin D-related); *MYH11* myosin, heavy chain 11, smooth muscle; *NPM1* nucleophosmin; *FLT3* fms-related tyrosine kinase 3; *CEBPA* CCAAT/enhancer binding protein (C/EBP), alpha; *MLLT3* myeloid/lymphoid, or mixed-lineage leukemia (trithorax homolog, Drosophila) translocated to, 3; *MLL* myeloid/lymphoid or mixed-lineage leukemia factor (trithorax homolog, Drosophila); *RPN1* ribophorin I; *EVI1 (MECOM)* MDS1 and EVI1 complex locus; *DEK-* DEK oncogene; *NUP214 (CAN)* nucleoporin 214 kDa

2.3 Gross Genetic Alterations in AML

AML-associated chromosome aberrations often affect the components of the core binding factor (CBF) complex, i.e., RUNX1 (CBFA2, AML1) and core-binding factor, beta subunit (CBFB) proteins. This protein complex physiologically activates a number of genes critical for normal myeloid development. The resulting fusion genes include: *RUNX1–RUNX1T1* (*AML1-ETO*) due to t(8;21)(q22;q22), *CBFB-MYH11* due to inv(16)(p13.1q22) or t(16;16)(p13.1;q22), and *RUNX1–EVI1* due to t(3;21)(q26;q22). The first three aberrations are the cytogenetic markers of so-called CBF-AML, the last one is associated with therapy-related AML (Maki et al. 2008; Martens and Stunnenberg 2010). All these chimeric proteins act as dominant negative forms of the CBF complex, leading to differentiation blockade.

RUNX1–RUNX1T1 fusion protein, found in 10 % of AML cases, constitutively represses genes usually activated by RUNX1 transcription factor, including *C-FMS* (*CSF1R*—colony stimulating factor 1 receptor), *P14ARF* (*CDKN2A*—cyclin-dependent kinase inhibitor 2A) and *CEBPA*, thus inhibiting normal myeloid differentiation (Martens and Stunnenberg 2010).

CBFB-MYH11, the result of chromosome 16 inversion/translocation, occurs in 5 % of AML cases and encodes protein that fuses first 165 amino acids of CBFB to the *C*-terminal coiled-coil region of a smooth muscle myosin heavy chain (MYH11). In normal cells, CBFB interacts with the RUNX1, increasing its affinity to DNA, and thus stimulating its activity. CBFB-MYH11 protein interferes with normal RUNX1 activity, which results in the repression of transcription of target genes by the recruitment of corepressors, such as histone deacetylases (HDACs) (Martens and Stunnenberg 2010).

Despite the lesions caused by t(8;21) or inv/t(16), CBF-AMLs are associated with relatively good prognosis in comparison to CN-AML.

Another aberration contributing to differentiation arrest is t(15;17)(q22;q21), which results in *PML–RARA* fusion. It is specific for acute promyelocytic leukemia (APL), which covers approximately 10 % of all AML cases. In normal cells, promyelocytic leukemia (PML) localizes in nuclear bodies where it functions as a transcription factor and tumor suppressor. *RARA* encodes retinoic acid (RA) receptor alpha. Chimeric PML–RARA protein fails to respond to RA concentration changes, acquiring constitutive transcriptional repressor activity. Additionally, *PML–RARA* rearrangement results in the disruption of nuclear bodies, thus inducing a maturation block. As a result, the cells are blocked at the promyelocytic stage of differentiation and self-renewal. Interestingly, the spectrum of PML–RARA targeted genes is wider as compared to wild-type RARA targets, thus potential implications of this fusion may be much more complex.

Both all-*trans*-retinoic acid (ATRA) and arsenic trioxide (ATO), used in APL treatment, directly target PML–RARA-mediated transcriptional repression, and protein stability, inducing rapid differentiation of the promyelocytes and complete clinical

remission in most APL patients (Licht and Sternberg 2005). Some variant transloca-
tions of chromosome 17, involving *RARA locus*, preserve ATRA sensitivity:

- t(15;17)(q35;q21)/*NPM1–RARA*,
- t(11;17)(q13;q21)/*NUMA1–RARA NUMA1*—nuclear mitotic apparatus protein 1,
- t(4;17)(q12;q21)/*FIP1L1–RARA FIP1L1*—FIP1 like 1 (*S. cerevisiae*).

Other aberrations yield an ATRA—resistant form of disease (Gulley et al.
2010; Licht and Sternberg 2005):

- interstitial duplication of chromosome 17 resulting in *STAT5B–RARA* fusion
  STAT5B—signal transducer and activator of transcription 5B
- t(11;17)(q23;q21)/*PLZF–RARA*
  PLZF also named *ZBTB16*—zinc finger and BTB domain containing 16

Various abnormalities of chromosome 11, involving mixed-lineage leukemia
(MLL) gene *locus* at 11q23.3, i.e.

- t(11;19)(q23;p13)/*MLL-MLLT1*
  MLLT1—myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog,
  Drosophila), translocated to, 1
- t(9;11)(p22;q23)/*MLL-MLLT3*
  are found in about 4 % of adult AML cases and in 15–20 % of pediatric AMLs
  (Balgobind et al. 2011).

MLL may fuse to one of over 50 partner genes, which results in chimeric
proteins of oncogenic potential. The *N*-terminal MLL region of such a chimera is
responsible for DNA-binding, and the fusion protein partner serves as strong
transactivator of target genes (Martens and Stunnenberg 2010). The direct genomic
targets of MLL fusion proteins remain largely unknown; however, gene expression
profiling showed that AMLs with 11q23/*MLL* rearrangements are characterized by
the deregulation of homeobox (HOX) genes (Bacher et al. 2010a). *MLL* aberra-
tions are particularly common in patients with a history of treatment with topo-
isomerase II inhibitors (e.g. etoposide), and are generally related to adverse
outcome, with the exception of t(9;11)/*MLL-MLLT3*, which is associated with
intermediate prognosis (Bacher et al. 2010b; Betz and Hess 2010).

Another *MLL* alteration found in AML is its amplification (reviewed by Haus
et al. 2009). It may present in variable forms, including hsr (homogenously
stained region), dmin (double minute chromosomes), derivative 11q, and others.
MLL is the second most frequently amplified gene in AML and myelodysplastic
syndrome (MDS), with the incidence of less than 1 % of CA-AML, after *C-MYC*
[*C-MYC*–v-myc myelocytomatosis viral oncogene homolog (avian)]. The number
of *MLL* copies ranges from 5 to 50 per cell (Mohamed 2010). Both *MLL*
amplification and its rearrangements result in the inhibition of myeloid
differentiation. Additionally, over 90 % of AML/MDS cases with *MLL* amplifi-
cation show an inactivation of *TP53* by deletion or mutation (Mohamed 2010).
Some abnormalities involving *MLL* gene are cryptic, not detectable by classical
cytogenetics.

C-MYC (8q24) amplification, presenting in the form of hsr or dmin, is observed in about 1 % of AML, but is rare as compared with solid tumors (Haus et al. 2009; Mohamed 2010). C-MYC is one of the pivotal TFs regulating hematopoiesis, which expression decreases during differentiation. However, *C-MYC* amplification does not necessarily lead to its higher expression. In pediatric AML, high expression of *C-MYC* associates with 11q23 rearrangements, while in adults it is related to *RUNX1–RUNX1T1*, *PML–RARA*, and *PLZF–RARA* fusions (Delgado and Leon 2010; Schotte et al. 2011).

Main gross genetic alterations in AML and their clinical relevance are presented in Table 10.1.

2.4 Cryptic Molecular Alterations Associated with Cytogenetically Normal AML

Aside from gross chromosomal rearrangements resulting in gene fusions, an increasing number of submicroscopic alterations have been identified which are essential for AML pathogenesis and serve as independent prognostic factors (Bacher et al. 2010b; Smith et al. 2011). Among 40 % of AML patients without cytogenetic abnormalities, more than 85 % have submicroscopic genetic alterations (Bacher et al. 2010b). Cryptic rearrangements may also be found in other cytogenetically defined subgroups of AML and may influence the outcome (Table 2.2).

2.4.1 Intragenic Aberrations

2.4.1.1 FLT3

The *FLT3* gene (fms-like tyrosine kinase receptor 3), located on chromosome 13q12, encodes the class III transmembrane tyrosine kinase receptor (Zuo et al. 2009). *FLT3* is normally expressed by hematopoietic stem cells, early myeloid and lymphoid precursors and both immature and mature monocytic cells. After activation by its ligand (FL), FLT3 supports the proliferation and survival of hematopoietic progenitors (Betz and Hess 2010).

Mutations in the *FLT3* gene are documented in approximately 30 % of all AML cases, across all French-American-British (FAB) subtypes, with an increased incidence in CN-AML, *MLL* partial tandem duplication (*MLL*-PTD)-positive AML, APL, and *DEK-CAN*(+) AML (Libura et al. 2003). They fall into two categories: internal tandem duplications of the 3–400 bp gene fragment (*FLT3*-ITD), that involve mainly the juxtamembrane (JM) domain (35–45 % of CN-AML; incidence in total AML population: 25 % for adults and 15 % for children) and less frequent point mutations (15 % of all *FLT3* mutations; 5 % of adult and 3 %

of pediatric AML), involving the kinase domain (*FLT3*-TKD) (Meshinchi et al. 2008). Both *FLT3*-ITD and *FLT3*-TKD result in ligand independent, constitutive activation of FLT3 (Gulley et al. 2010; Betz and Hess 2010).

FLT3-ITD has an adverse effect on relapse risk (RR) and overall survival (OS) of AML patients, even in prognostically favorable cytogenetic subtypes (e.g., APL with *PML–RARA* fusion gene), while little effect on remission induction is described. Prognostic significance of *FLT3*-TKD mutation remains unclear (Whitman et al. 2010; Smith et al. 2011).

2.4.1.2 *NPM1*

The nucleophosmin 1 gene (*NPM1*) is located on chromosome 5q35. Its mutations occur in 55 % of adult CN-AML cases and 30 % of the whole adult AML group. Their incidence is lower in pediatric AML (7.5 %) (Bacher et al. 2010b). NPM1 is a multifunctional protein shuttling between nuclear compartments and cytoplasm. It is localized predominantly within nucleolus, taking part in ribosome assembly and the regulation of ARF (CDKN2A—cyclin-dependent kinase inhibitor 2A) and p53 tumor suppressor function (Betz and Hess 2010). *NPM1* frameshift mutations retain the protein in the cytoplasm, which inhibits the ARF-p53 pathway (Mardis et al. 2009; Zuo et al. 2009). *NPM1* mutations are associated with an increased white blood cells count and leukemia of a monocytic M4/M5 lineage. Mutations of *NPM1* commonly coexist with *FLT3*-ITD. Only *NPM1*(+)/*FLT3*-ITD(−) configuration is recognized as a favorable prognostic factor, especially in patients aged ≥70 years. AML with *NPM1*(−)/*FLT3*-ITD(+) is associated with the worst outcome, while *NPM1*(+)/*FLT3*-ITD(+) and *NPM1*(−)/*FLT3*-ITD(−) AML—with the intermediate outcome (Bacher et al. 2010b; Becker et al. 2010; Thiede et al. 2006). *NPM1*-mutated AML presents distinct gene and microRNA expression profiles, and is provisionally recognized by the revised WHO classification (4th edition, 2008) as a separate entity (Becker et al. 2010; Vardiman et al. 2009).

2.4.1.3 *CEBPA*

The *CEBPA* gene maps to chromosome band 19q13.1 and encodes a CCAAT/ enhancer binding protein (C/EBP) alpha, a transcription factor important in the regulation of myelopoiesis (Zuo et al. 2009). CEBPA regulates the transcription of genes encoding for interleukin 6 (IL-6), granulocyte colony-stimulating factor (G-CSF) receptor, myeloperoxidase, and others (Dufour et al. 2010). *CEBPA* mutations occur in approximately 5–15 % of CN-AML (Foran 2010). A frameshift and nonsense mutations in the *N*-terminal region result in a truncated, dominant negative protein. In-frame mutations involve a leucine zipper motif at the

C-terminus of CEBPA, which impairs dimerization and DNA-binding ability (Zuo et al. 2009).

Two-thirds of *CEBPA*(+) AML cases harbor mutations affecting both *C*- and *N*-terminal region simultaneously, each occurring at a different allele (dm, double allele mutation). The remaining *CEBPA*(+) AML cases exhibit mutation of only one allele (sm, single allele mutation). *CEBPA* double mutant cases present a unique gene expression signature, are rarely associated with *FLT3*-ITD and confer a favorable prognosis, as compared with $CEBPA^{sm}$(+), and *CEBPA*(−) AML. This suggests that $CEBPA^{dm}$(+) AML constitutes a distinct disease entity and it has been provisionally recognized as such in the latest WHO classification (Dufour et al. 2010; Taskesen et al. 2011).

2.4.1.4 MLL

Partial tandem duplications of *MLL* were the first molecular change recognized to be of prognostic value in CN-AML. They are found in 5–10 % of cases and have a negative impact on disease outcome, especially in older patients (Whitman et al. 2007). In CA-AML, *MLL*-PTD is frequently associated with chromosome 11 trisomy (Bacher et al. 2010b; Foran 2010; Mrózek et al. 2007).

2.4.1.5 *IDH1/2*

IDH1 and *IDH2* genes, located at 2q33.3 and 15q26.1, encode two isoforms of isocitrate dehydrogenase (NADP+), the enzyme associated with the tricarboxylic acid (TCA) cycle. Genome sequencing of de novo CN-AML showed the prevalence of *IDH1* and *IDH2* mutations of 16 and 14–15 %, respectively (Marcucci et al. 2010; Mardis et al. 2009). In pediatric AML mutations of *IDH* genes are rare (3 %) (Paschka et al. 2010; Smith et al. 2011; Walker and Marcucci 2011).

The precise role of *IDH* point mutations in AML pathogenesis is not clear, but they have been shown to alter DNA and the histone methylation profile, as well as to have an adverse prognostic impact in some groups of patients (see Table 2.1) (Dang et al. 2010; Figueroa et al. 2010a; Walker and Marcucci 2011).

2.4.1.6 *TET2*

Mutations in tet oncogene family member 2 (*TET2*) were recently described in several myeloproliferative neoplasms. *TET2 locus* at 4q24 is involved in some AML-associated aberrations, such as t(3;4)(q26;q24). Additionally, many different *TET2* point mutations that spread over the whole gene were discovered in 10–26 % of MDS or secondary AML and 12 % of de novo AML (Nibourel et al. 2010). Recent studies revealed that *TET2* encodes methylcytosine dioxygenase, an

enzyme involved in the epigenetic regulation of myelopoiesis. TET2 influences the promoter methylation profile, changing the expression of target genes. *TET2* missense or nonsense mutations, resulting in a truncated or sequence-altered protein, lead to a promoter hypermethylation of target genes, and their silencing (Mohr et al. 2011). Similarly, *IDH1/IDH2* point mutations result in hypermethylation phenotype which suggests that both *IDH1/2* and *TET2* mutations represent a third type of genetic alteration important for AML leukemogenesis, different from class I and class II mutations (Figueroa et al. 2010a; Walker and Marcucci 2011).

TET2 mutations were found to have an adverse impact on the prognosis of CN-AML patients who have *CEBPA*[dm] and/or mutated *NPM1* without *FLT3*-ITD, classified by European LeukemiaNet (ELN) to the favorable-risk group (Metzeler et al. 2011).

2.4.1.7 Other Intragenic Aberrations Associated with CN-AML

Many other genes were found to be mutated in AML, i.e. *WT1*, *DNMT3A*, *ASXL1* [additional sex combs like 1 (Drosophila)], *CBL*, *HOXA9* (homeobox A9), *PTPN11* (protein tyrosine phosphatase, nonreceptor type 11), and *RUNX1* (*AML1*). Mutations of *WT1* are observed in 10 % of CN-AML and associate with the worse OS and primary chemotherapy resistance. Recently, mutations in *DNMT3A* gene encoding DNA methyltransferase were found in about 30 % of patients with intermediate-prognosis AML. They confer a poor prognosis that may be partially attributed to simultaneous *FLT3* and *IDH* mutations (Ley et al. 2010). Point mutations in *RUNX1* gene, found in 7–15 % of de novo AML, mainly of intermediate prognosis, are inversely correlated with NPM1 mutations and worsens the prognosis, especially in relation to event-free survival (EFS) (Gaidzik et al. 2011). The precise role of other aforementioned gene mutations in AML pathogenesis and their prognostic significance has to be further clarified.

2.4.2 Gene Overexpression

Overexpression of several genes involved in the regulation of normal hematopoiesis was shown to be an important prognostic factor in CN-AML. These include Brain and acute leukemia, cytoplasmic (*BAALC*), ETS-related gene (*ERG*), and *MN1*.

2.4.2.1 BAALC

Brain and acute leukemia, cytoplasmic gene (8q22.3) expression in normal hematopoietic precursors is downregulated during differentiation, which may point at its role in leukemogenesis (Gregory et al. 2009). Its overexpression is associated with the *HOX* gene family and *HOX* gene embedded microRNAs downregulation

(Schwind et al. 2010b). Many studies revealed poor prognostic significance of this marker in CN-AML. The strongest prognostic impact of *BAALC* overexpression seems to be restricted to patients lacking both *CEBPA* mutations, and *FLT3*-ITD (Bienz et al. 2005). It was suggested that dichotomization of CN-AML cases with regard to *BAALC* expression and *FLT3* mutation status presents the best risk stratification approach (Baldus et al. 2006).

2.4.2.2 ERG

ETS-related gene (21q22.3) is a member of the ETS family of TFs. High *ERG* expression correlates with the upregulation of many genes involved in cell proliferation, differentiation, apoptosis, and DNA methylation (Gregory et al. 2009; Schwind et al. 2010b). It was shown that *ERG* overexpression determines lower complete remission (CR) rate, as well as shorter disease-free survival (DFS), and OS (Marcucci et al. 2005). *ERG* expression provides additional prognostic information for patients who were already stratified according to *NPM1* and *FLT3*-ITD status, excluding the high risk *FLT3*-ITD(+)/*NPM1*(−) group (Marcucci et al. 2007; Metzeler et al. 2009).

Overexpression of both, *BAALC* and *ERG* predicts an adverse clinical outcome in de novo CN-AML. It is also associated with distinct gene and miRNA expression signatures (Schwind et al. 2010b).

2.4.2.3 MN1

Transcriptional coactivator MN1 is encoded by the meningioma-1 gene (*MN1*), mapped to 22q12.1. Like *BAALC*, *MN1* expression in normal hematopoietic cells depends on the stage of differentiation (Gregory et al. 2009). Constitutive overexpression of *MN1* in murine bone marrow rapidly induces myeloid leukemia, which is accompanied by an early-onset severe anemia, suggesting that *MN1* alone efficiently blocks megakaryocyte-erythroid differentiation (Gregory et al. 2009). Additionally, *MN1* gene is involved in translocation t(12;22)(p13;q12), resulting in *MN1-TEL*(*ETV6*) fusion.

In multivariate analysis, the overexpression of *MN1* in adult CN-AML was an independent marker of poor prognosis (Langer et al. 2009). It has strong prognostic value for CN-AML patients, especially in cases other than *NPM1*(+)/*FLT3*-ITD(−) (Heuser et al. 2006). *MN1* overexpression is commonly found together with overexpression of *BAALC* and with expression of wild-type *NPM1*. It was found that patients with overexpression of *BAALC* or *MN1* show similar gene expression signatures, which may suggest potential functional interplay between these genes in myeloid leukemogenesis (Langer et al. 2009).

2.5 Intragenic Aberrations Associated with Cytogenetically Abnormal AML

C-KIT (*SCFR*—stem cell factor receptor, *C-KIT* proto-oncogene; 4q12), *N-RAS* (neuroblastoma RAS viral oncogene homolog; 1p13), and *K-RAS* (Kirsten rat sarcoma viral oncogene homolog; 12p12) mutations in AML belong to class I mutations (Betz and Hess 2010). They were found to be associated with CBF leukemias, where they present the "second hit" in the course of transformation.

2.5.1 C-KIT

C-KIT protooncogene encodes type III receptor tyrosine kinase, important for signal transduction in normal hematopoiesis. Gain-of-function *C-KIT* mutations, involving mainly exons 8 and 17 (mut*KIT*8 and mut*KIT*17), were found in 2 % of overall AML, and in over 30 % of CBF-AML (Foran 2010). Both mutations confer higher RR for otherwise favorable CBF-leukemias; however, mut*KIT*17 has stronger adverse impact, which is more evident in inv(16)/*CBFB-MYH11*(+)AML (Betz and Hess 2010; Paschka et al. 2010).

2.5.2 N-RAS and K-RAS

RAS proteins have been shown to influence proliferation, differentiation, transformation, and apoptosis by transmitting mitogenic and growth signals into the cytoplasm and the nucleus. Usually inactive in normal cells, RAS molecules become activated by substitutions. Overall *N-* and *K-RAS* mutations (most commonly seen at codons 12, 13, and 61) are observed in about 15 % of AML and associate with inv(3) or inv(16) (Gulley et al. 2010). They may enhance the response to high dose cytarabine, but their general prognostic impact seems to be neutral (Gulley et al. 2010; Smith et al. 2011).

Beside *RAS* and *C-KIT* mutations, the aforementioned *FLT3* alterations also represent an additional type of activating mutations in CBF-AML and are indicators of poor prognosis. In practical terms, as *RAS*(+) CBF-leukemias retain their profile of prognostically favorable AML, identification of *C-KIT* and *FLT3* mutations enables prognostic restratification of CBF-AML patients.

2.6 MicroRNAs in AML

MicroRNAs (miRNAs) are small (18–25 nucleotides), noncoding RNAs that negatively regulate gene expression on a posttranscriptional level. miRNAs hybridize to the 3′-untranslated (3′-UTR) regions of protein-coding, messenger RNAs (mRNAs), and lead to their degradation or inhibition of translation (Seca et al. 2010). It is estimated that over 30 % of structural genes are regulated by miRNAs. They are involved in all crucial physiological processes, like proliferation, differentiation, apoptosis, signal transduction, etc.

As regulators of expression of structural genes miRNA may function indirectly, both as oncogenes (*oncomirs*) or tumor suppressors. Some miRNAs are localized in genomic regions associated with cancer, e.g. breakpoint regions in specific chromosome aberrations, hot-spots, minimal regions of loss of heterozygosity (LOH), and minimal regions of amplification (Marcucci et al. 2011).

Alterations of miRNA expression were described in many types of solid tumors and leukemias. It has been shown that miRNA expression profiles better characterize particular cancer subtypes than gene expression profiles (Seca et al. 2010). miRNA signatures were found to distinguish normal cells from AML blasts and AML from ALL cases. In AML they have also been shown to be associated with several chromosome aberrations, e. g. t(15;17), t(8;21), inv(16), t(11q23)/*MLL*, with *FLT3*-ITD, mutations in *NPM1* and *CEBPA*, as well as *BAALC* and *MN1* overexpression (Marcucci et al. 2011). Many miRNAs target genes inhibiting differentiation and apoptosis, or promoting cell proliferation. While downregulated they contribute to leukemogenesis (Schotte et al. 2011). These include:

- *miR-34b* targeting cyclin-dependent kinase 4 (*CDK4*) and cyclin E2 (*CCNE2*),
- *miR-15a* targeting *BCL2* (B-cell CLL/lymphoma 2; apoptosis regulator),
- *let-7* family targeting oncogenes *RAS* and *MYC*.

Microarray studies have shown that miRNA profiles are also associated with the outcome of AML patients. Overexpression of any of the following: *miR-20a*, *miR-25*, *miR-191*, *miR-199a*, or *miR-199b* was an independent adverse prognostic factor in intermediate and poor-risk cytogenetic groups (Garzon et al. 2008). On the contrary, high *miR-182* expression was found to improve prognosis in cytogenetically normal, poor molecular risk patients with *FLT3*-ITD, and/or wild-type *NPM1* (Schwind et al. 2010a). Sequential miRNA profiling may also be helpful in monitoring the response to therapy (Marcucci et al. 2011).

Although miRNA studies in AML are still at the initial stage, novel technologies enable quick transfer of the results from the bench to the clinic. Importantly, miRNA expression should not only be considered as putative prognostic factor, but also as potential target for novel therapeutic strategies.

2.7 Acquired Copy Number Alterations Identified by SNP Arrays

An important limitation of conventional cytogenetics is the inability to detect genomic alterations below 5 Mb. High resolution genomic single nucleotide arrays (aSNP) enable the measurement of gene copy number and the distinction of individual genotypes to detect a LOH. Although, like other array-based methods, SNP arrays are not useful for the detection of balanced aberrations, or changes present in <25 % cells, they facilitate refining karyotype-based risk stratification by identification of subtle acquired Copy Number Alterations (CNAs) and acquired copy neutral LOH (ACN-LOH) (Parkin et al. 2010). ACN-LOH, which may be caused by acquired somatic uniparental disomy (AS-UPD), arises during the development of the neoplastic clone, when a segment (partial UPD), or a whole chromosome is lost and replaced by its homolog. In any case it may result in LOH, thus influencing the outcome.

In AML, acquired CNAs of regions important for cell survival and differentiation, including both subchromosomal, or entire chromosome gains/losses, may associate with other molecular markers: most UPD(+) patients harbor also *NPM1* or *CEBPA* mutations (Bullinger et al. 2010; Heinrichs et al. 2010; Parkin et al. 2010). The presence of CNAs was shown to worsen the prognosis of AML patients with regard to OS, EFS, and progression-free survival (PFS) (Tiu et al. 2011).

2.8 Epigenetic Mechanisms in AML

The epigenetic control of gene expression, mainly DNA methylation, plays an important role in determining the biology of the cell. The addition of methyl groups to cytosines at the palindromic CpG sites clustered in promoter regions may alter gene expression by creating new binding sites for methylation-dependent repressor molecules, or by impairing the affinity of TFs to their target sequences (Figueroa et al. 2010b). The alterations in gene methylation, i.e. hypermethylation resulting in the silencing of particular tumor suppressor genes, as well as hypomethylation reactivating stem cell phenotype, may contribute to carcinogenesis (Alvarez et al. 2010; Ko et al. 2010). In most cases, AML blasts show a very strong hypermethylation signature compared to normal bone marrow CD34+ cells; however, some genes are hypomethylated. As different cytogenetic and molecular AML subtypes were found to display a unique methylation profile, the epigenetic changes seem to represent an important step in leukemic transformation. Mutations of genes involved in DNA methylation, like *TET2*, *IDH1*, *IDH2* and *DNMT3A*, were recently identified in AML patients (Alvarez et al. 2010; Figueroa et al. 2010a). By microarray genome-wide screening, the number of aberrantly methylated genes in AML patients was found to increase at relapse (Kroeger et al. 2008).

Since the methylation status of some genes, such as:

- *E2F1* E2F transcription factor 1,
- *SMG6* smg-6 homolog, nonsense mediated mRNA decay factor (*C. elegans*),
- *CXCR5* chemokine (C-X-C motif) receptor 5; CD185,
- *LCK* lymphocyte-specific protein tyrosine kinase; p56lck, and
- *DBC1* deleted in bladder cancer 1

associates with OS of AML patients, the analysis of a methylation profile may be helpful in the design of clinical trials with demethylating agents (Alvarez et al. 2010; Figueroa et al. 2010a).

2.9 Clinical Implications of Molecular Genetics in AML

The detection of cytogenetic and molecular alterations may be used for personalized therapeutic approach, adjusting the mode and intensity of treatment for individual patients. This strategy includes three different aspects: (1) the stratification of patients according to the estimated risk of relapse based on initial findings, (2) the implementation of novel agents targeting particular molecular pathways, (3) making therapeutic decisions based on the monitoring of minimal residual disease (MRD).

2.9.1 Risk Stratification Based on Cytogenetic and Molecular Features of AML

The initial treatment of AML patients consists of remission induction followed by intensive consolidation. The postconsolidation approach may include either allogeneic or autologous hematopoietic stem cell transplantation (HSCT). Standard induction regimens based on the combination of anthracyclines and cytarabine result in up to 70 % CR rate. However, the majority of patients who achieved CR experience relapse, which potentially could be prevented by strongly invasive allogeneic HSCT.

Blast cell karyotype is considered the most important, clinically relevant prognostic parameter in AML. The Medical Research Council (MRC) and the Southwest Oncology Group (SWOG) proposed stratification systems distinguishing patients with favorable, intermediate, and adverse karyotype (Grimwade et al. 1998; Slovak et al. 2000). Both systems were validated in subsequent studies, leading to the implementation of classical cytogenetics in standard procedures in AML (Döhner et al. 2010). Unlike patients with favorable karyotype, those with adverse cytogenetic features become candidates for allogeneic HSCT in the first

CR. Unfortunately, two-thirds of AML patients belong to the intermediate group, mostly CN-AML, without clear treatment recommendations.

The identification of molecular alterations influencing AML outcome allowed for the modification of the stratification system. As proposed in 2010 by the ELN, CN-AML patients that are NPM1(+) and FLT3-ITD(−) as well as those CEBPA^{dm}(+) are now classified as the favorable group (Döhner et al. 2010). The intermediate group has been further divided into subgroups I and II. The intermediate-I group displays better, NPM1(+)FLT3(+) or worse, NPM1(−)FLT3(+), prognosis, but in both cases a good response to targeted treatment is anticipated. The intermediate-II group has generally worse outcome. The adverse risk group remains unchanged (Table 2.2). The classification does not include APL.

The new system allows for precise discrimination of patients ≤60 years with regard to the probability of relapse, relapse free survival, and OS. It must be emphasized that, according to current recommendations, examination of NPM1, CEBPA, and FLT3-ITD is obligatory within clinical trials and recommended in the routine treatment of CN-AML patients (Röllig et al. 2011).

2.9.2 Targeted Therapy of AML

Although knowledge concerning the molecular background of AML is rapidly increasing; until now, the only subtype with established indications for targeted treatment has been APL. Before the introduction of ATRA, this subtype was associated with high risk of therapy-related mortality. The use of standard cyto-statics causes a rapid destruction of leukemic cells that release procoagulation factors, leading to the development of disseminated intravascular coagulation (DIC) syndrome. Administration of ATRA results in rapid differentiation of leukemic promyelocytes, allowing achievement of CR while avoiding the risk of DIC. However, ATRA does not destroy leukemic stem cells, which makes it insufficient as a single agent in APL treatment. Current strategies are based on the combinations of ATRA with chemotherapy during remission induction, followed by consolidation, and maintenance (Sanz et al. 2009). Results are excellent, with the cure rate approaching 90 %; patients who relapse may be offered second-line protocols incorporating ATO.

The use of targeted therapies in other AML subtypes is under intense investigation. Of particular interest are patients with FLT3-ITD, associated with decreased DFS, and OS rates. Several FLT3-selective tyrosine kinase inhibitors have been developed, including midostaurin, lestaurtinib, and sunitinib (Weisberg et al. 2002; Levis et al. 2002; O'Farrell et al. 2003). All these agents have been shown to reduce the tumor burden in relapsed or refractory AML. However, responses were short term, suggesting that targeting FLT3 as a sole therapy is not sufficient (Zarrinkar et al. 2009).

AML with leukemic cells harboring MLL gene rearrangement is associated with shorter remission duration and shorter survival. It has been recently suggested that

reversing complex epigenetic processes with demethylating agents such as aza-citidine and decitabine or with histone deacetylase inhibitors may play a role in *MLL*(+) AML treatment (Altucci and Minucci 2009). This hypothesis requires verification in clinical trials.

Since AML is a heterogeneous disease with a complex molecular background, it is unlikely that targeting a single molecular pathway can be sufficiently effective. Thus, targeted treatment, adjusted for individual patient genetic profile would be a preferred part of broader therapeutic strategy.

2.9.3 Monitoring of Minimal Residual Disease

Conventional cytogenetic methods allow for the evaluation of remission status at the level of 5 % of blasts in bone marrow. The application of more sensitive methods, including multiparametric flow cytometry, or RT-RQ-PCR enables the detection of MRD with the sensitivity of $<10^{-4}$.

Molecular monitoring of *PML–RARA* is widely used in APL. While the detection of this fusion gene after remission induction is not of clinical relevance, the achievement of molecular remission after consolidation is considered a major treatment objective (Sanz et al. 2009). It is well documented that patients with the disease persistent or recurrent at the molecular level, confirmed by two consecutive assays after completion of consolidation, will invariably relapse, unless additional therapy is given (Breccia et al. 2004). The suggested therapy for patients with documented molecular relapse may include ATO, which prevents hematological relapse in the majority of patients (Grimwade et al. 2009).

More recently, RQ-PCR has been utilized in the monitoring of *NPM1* mutations. It has been shown that $NPM1^{mut}$ transcript levels were significantly associated with prognosis after each treatment cycle and that its higher level after consolidation therapy was a significant factor for an increased risk of relapse and death. Serial posttreatment assessments of MRD allowed for early detection of relapse (Krönke et al. 2011).

2.10 Conclusions

Due to its cytogenetic and molecular complexity of prognostic significance, AML is nowadays the focus of intensive research. Apart from class I and II mutations resulting in increased proliferation/survival and impaired differentiation of AML blasts, respectively, alterations of genes regulating DNA repair, transcription, translation, and protein turnover were also shown to contribute to leukemogenesis. While chromosome aberrations are mainly large enough to be detected by conventional cytogenetics, the identification of cryptic changes requires sophisticated methods of molecular biology. Some of the aforementioned alterations are

well-known prognostic or predictive factors in AML; others, such as *IDH1/2* mutations or *KIT* and *RAS* aberrations, have to be further examined to definitively establish their prognostic significance. miRNA and epigenetic changes were proven to be important for AML leukemogenesis, but their prognostic value similarly requires further investigation.

The knowledge about molecular pathogenesis of AML coupled with novel, genome-wide screening methods, has significant clinical implications for more precise risk stratification, response to therapy and MRD monitoring, as well as targeted therapies development.

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