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# Molecular Markers for Risk Stratification in Adult Acute Myeloid Leukemia with Normal Cytogenetics

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## 1. Introduction

Acute myeloid leukemia (AML) is a broad range of disorders that are all characterized by a block in the differentiation and by uncontrolled proliferation of hematopoietic progenitor cells. AML is the most frequent hematological malignancy in adults, with an annual incidence of three to four cases per 100 000 individuals. Specific recurrent chromosomal abnormalities can be identified in approximately 55% of cases by cytogenetic analysis. These detected chromosomal aberrations are the most important tool to classify patients at their initial diagnosis and to divide them into favorable, intermediate and unfavorable subgroups. The age of the patient is also an important prognostic factor (Juliusson et al., 2009; Szotkowski et al., 2010). However, approximately 45% of adult patients with AML have normal karyotype (cytogenetically normal /CN/-AML patients) and are usually classified as an intermediate risk group (Mrozek et al., 2007; Smith et al., 2011). These patients have a 5-year overall survival rate between 24% and 42%, but clinical outcome may vary greatly.

The prognosis of AML with normal cytogenetics may be further subdivided based on genetic lesions. Even though a growing number of genetic lesions have been identified in CN-AML, about 25% CN-AML patients do not carry any of the currently known mutations. Therefore, many research groups conducted retrospective studies to find some candidate molecular markers that could identify good and poor risk AML patients with normal karyotype. In recent years, a number of gene mutations (*NPM1* /nucleophosmin/, *FLT3* /Fms-like tyrosine kinase 3/, *MLL-PTD* /mixed lineage leukemia-partial tandem duplications/, *C/EBP $\alpha$*  /CCAAT/enhancer-binding protein alpha/CEBPA/, *WT1* /Wilms tumor 1/, *DNMT3A* /DNA methyltransferase 3A/, *IDH1* and *IDH2* /isocitrate dehydrogenase/, *CBL* /Casitas B-lineage lymphoma/and others), as well as deregulated expression of genes (*BAALC* /brain and acute leukemia cytoplasmic/, *ERG* /ETS-related gene/, *MN1* /meningioma 1/, *EVII* /ecotropic virus integration 1/, *AF1q* /ALL1-fused gene chromosome 1q/, *PRAME* /preferentially expressed antigen in melanoma/, *WT1*) have been found and further molecular markers (gene expression profiles and microRNA expression signatures) are studied and incorporated into clinical practice (Baldus & Bullinger, 2008; Bullinger & Valk, 2005; Bullinger 2006; Kohlmann et al, 2010; Marcucci et

al., 2009, 2011a,b; Motyckova & Stone, 2010; Ramsingh et al., 2010; Schlenk et al., 2008; Wouters et al., 2009). It is necessary to improve current classification systems in order to reflect better the molecular heterogeneity of CN-AML (Döhner et al., 2010; Vardiman et al., 2009).

In addition, some of the genetic abnormalities have also been found to be useful for minimal residual disease (MRD) monitoring and as potential therapeutic targets in the development of new agents for AML therapy. The aim of monitoring of MRD is the identification of cases with a very high risk of relapse who then can be treated much earlier and more effectively (Jaeger & Kainz, 2003; Schnittger et al., 2009; Shook et al., 2009).

Knowing the status of *FLT-3* and other molecular markers (mutations and gene expression described above together with gene expression profiling) in CN-AML patients has not only prognostic significance but is important in the treatment based on these molecular markers. For example, patients with *FLT3*-ITD (an internal tandem duplication of the *FLT3* receptor tyrosine kinase gene), who are not candidates for induction therapy and allogeneic stem cell transplantation may respond to an *FLT3* inhibitor used in combination with chemotherapy or without chemotherapy. Deregulation of microRNA (miR) in CN-AML patients may act as complementary hit in the multisteps mechanism of leukemogenesis and has been not only used to identify subsets of CN-AML patients with diversified outcome but will certainly play a role in the future of treatment in new therapeutic strategies. Thus, miR expression profiling has diagnostic and prognostic significance.

## 2. Methods for the detection of gene mutations and expression

### 2.1 Sample material

After informed consent patient- or healthy individual-derived mononuclear cells were isolated from bone marrow (BM) or peripheral blood cells by a ficoll (for example Ficoll-Paque PLUS, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient centrifugation. Total RNA and genomic DNA were isolated. Yield and quality of the RNA were measured by spectrophotometric analysis. Each sample was assessed for the integrity of RNA by discrimination of 18S and 28 S ribosomal RNA on 1% agarose gels using ethidium bromide for visualization. Complementary DNA was synthesised from total RNA using reverse transcriptase (for example SuperScript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA)).

### 2.2 Detection of gene mutations

Most mutation detection methods use PCR (polymerase chain reaction) to amplify the region of DNA of interest. Mutant DNA has a different secondary structure (conformation) compared with that of the normal DNA. Frequently, this difference in conformation results in altered gel electrophoresis mobility of the mutant DNA species. Single-strand conformation polymorphism (SSCP) method (Orita et al., 1989; Perry, 1999) based on this conformation change is a fast and efficient method for detecting many types of aberrations, including point mutations, insertions, deletions and rearrangements (Frayling, 2002; Kutach et al., 1999). SSCP has the sensitivity to identify single nucleotide changes in the DNA sequence. Thermally denaturated DNA is electrophoresed and mutation is detected as aberrantly migrating bands on the electrophoresis gel. Analyzed PCR products must be less than 300 bp and preferably less than 200 bp in size because the method is increasingly inefficient with increasing size of the PCR product. Another technique, related to SSCP is

DNA heteroduplex analysis (Frayling, 2002). Both SSCP and heteroduplex analysis can be carried on fluorescent DNA analysers at a controlled temperature. The related techniques DGGE (denaturing gradient gel electrophoresis) and TGGE (thermal gradient gel electrophoresis) are very efficient, but they require specially designed PCR primers of increased length (Frayling, 2002). Sequencing of the mutated PCR product is almost universally carried out using dideoxy terminator chemistry.

High resolution melting (HRM) analysis is rapidly becoming the most important initial screen procedure for potential mutations (Razga et al., 2009; Tan et al., 2008; Vaughn & Elenitoba-Johnson, 2004; Wittwer, 2009). PCR amplification and subsequent HRM analysis are sequentially performed in the one tube. HRM is more simple than denaturing high-performance liquid chromatography (DHPLC), which can be also used after PCR amplification for mutation detection (Bianchini et al., 2003; Kosaki et al., 2005; Roti et al., 2006). Real-time PCR machine with HRM capability, for example LightCycler 480 High-Resolution Melting Master (Roche Diagnostics) has been successfully used for PCR amplification and HRM analysis. PCR reactions must be designed in the manner to avoid primer dimers and non-specific amplification. DNA has to be prepared in a uniform fashion to avoid variation in salt concentration with effect on the melting. HRM significantly reduces the quantity of samples that is necessary for sequencing with consequent reduction of cost and labour and enables rapid detection of mutations. The melting curves are normalized and temperature shifted to allow samples to be directly compared. Difference plots are generated by selecting a negative (nonmutated) control as the base-line and the fluorescence of all other samples is plotted relative to this negative control. Significant differences in fluorescence are indicative of mutations which must be detected by sequencing. Noordermer et al. (2010) used HRM curve analysis for rapid identification of *IDH1* and *IDH2* mutations in AML.

Currently, identification of mutations by PCR and direct nucleotide sequencing is used as the gold standard. The great progress was achieved in DNA sequencing technology in last ten years after the end of Human Genome Project (Mardis, 2011). The Roche/454, Life Technologies SOLiD and Illumina instruments have been used to sequence the complete tumor and normal genomes in order to identify mutations that alter the protein-coding genes. Whole genome sequencing is now possible at a reasonable cost per tumor and normal genome of around \$ 30,000 and can be completed in about 8 days. A pilot study of high-throughput, sequence-based mutational profiling of primary AML cell genomes was done eight years ago (Ley et al., 2003). Whole genomic DNA sequencing of a cytogenetically normal FAB M1 AML patient was done three years ago and discovered ten genes with acquired mutations (Ley et al., 2008). Eight novel somatic mutations were described in genes not previously implicated in AML pathogenesis. Two well-known AML-associated mutations, including *FLT3-ITD*, which constitutively activates kinase signaling and a four base insertion in exon 12 of the *NPM1* gene were also detected. Mardis et al. (2009) identified twelve somatic mutations within the coding sequences of genes and 52 somatic point mutations in conserved or regulatory portions of the AML genome. Two known mutations in *NRAS* and *NPM1* and ten novel mutations were detected. One of these mutations (missense R132C mutation in *IDH1* gene) is today included in many mutational screening of AML patients. The more efficient, faster and cheaper approach will be sequencing of coding regions (the cDNA transcriptome) but the abundance of transcripts can vary and some mutations can be missed (Greif et al., 2011). The size of the transcriptome is about ten times shorter than a diploid human genome.

Mutations in molecular markers can be present in low abundance within a high background of wild type sequence that may only differ from mutant at a single nucleotide. Several methods exist for detection of somatic mutations by real-time PCR. These methods include use of allele-specific competitive blocker PCR (Orou et al., 1995), blocker-PCR (Seyama et al., 1992), real-time genotyping with locked nucleic acids (Ugozzoli et al., 2004), the amplification refractory mutation system (Newton et al., 1989), and fluorescent amplicon generation as a novel real-time PCR technology (Amicarelli et al., 2007).

Mutated NPM1 is localised in the cytoplasm and not in the nucleus (Bolli et al., 2007; Falini et al., 2006; Liso et al., 2008; Oelschlaegel et al., 2010). Immunohistochemical detection of cytoplasmic nucleophosmin is performed with monoclonal antibody or by Western blotting (Falini et al., 2006, 2009, 2010a; Martelli et al. 2008).

### 2.3 Technique of real-time PCR (RQ-PCR) for marker gene expression evaluation

RQ-PCR permits accurate quantification of PCR products during the exponential phase of the PCR amplification process. Three main types of this analysis are used: (1) RQ-PCR using the hydrolysis probe format ("TaqMan probe"); (2) RQ-PCR using the hybridization probe format; and (3) RQ-PCR using SYBR Green Dye (Kern et al., 2005). Analysis with TaqMan probe uses 5'-3' exonuclease activity of the *Thermus aquaticus* (Taq) polymerase to detect and quantify the PCR product. The hydrolysis probe is positioned within the target sequence and is conjugated with a reporter fluorochrome at the 5' end and a quencher fluorochrome at the 3' end. The quencher avoids the reporter from emission of a fluorescence signal as long as the probe is intact and both fluorochromes are in the close proximity. Upon amplification of the target sequence, the hydrolysis probe is displaced from the DNA strand by the Taq polymerase and subsequently hydrolysed by the 5'-3' exonuclease activity of the Taq polymerase. This results in displacement of the reporter from the quencher and the fluorescence of the reporter becomes detectable.

Generally two quantification types (relative or absolute) in RQ-PCR are possible. A relative quantification based on relative expression of a target gene versus a reference gene is adequate for the most purposes. For absolute quantification, based either on an internal or an external calibration curve (Bustin et al., 2005; Ptáfl, 2001, Ptáfl et al. 2002), the methodology must be highly validated and the identical LightCycler PCR amplification efficiencies for standard material and target cDNA must be confirmed.

### 2.4 Genes and microRNAs microarrays

RNA was extracted using Trizol reagent and processed for Affymetrix U133 plus 2.0 GeneChip (Affymetrix, Santa Clara, CA) hybridizations. Briefly, from 5 µg total RNA, double-stranded cDNA was prepared with the use of the T7-Oligo(dT) primer (Affymetrix). *In vitro* transcription for amplification and biotinylation of the RNA transcript was performed with the BioArray HighYield RNA Transcript Labeling Kit (T7; Enzo Life Science, Farmingdale, NY). Biotin-cRNA (10 µg) was fragmented and hybridized onto the U133 plus 2.0 GeneChip for 16 hours at 45°C and labelled with Cy-3-streptavidin conjugate according to manufacturer protocols. Scanned images were converted to CEL files using GCOS software (Affymetrix). For the miRNA microarray chips, biotinylated first-strand cDNA was synthesized in reverse transcription from 2.5 to 5.0 µg total RNA using biotin-labeled random octamer oligo primer from pretreatment BM and blood mononuclear cell samples and hybridized to miRNA microarray chip KCC/TJU containing 368 probes in triplicate,



corresponding to 245 human miRNA genes. After hybridization for 18 hours at 25°C and washing, direct detection of the biotin-containing transcripts by streptavidin-Alexa647 conjugate was done and processed slides were scanned. Expression profiles were analyzed in GENESPRING software (Silicon Genetics, Redwood City, CA).

### 3. Mutations in the molecular markers

#### 3.1 Mutations in the *NPM1* gene

Nucleophosmin (NPM1, also called nucleolar protein B23, numatrin or NO38) is a multifunctional phosphoprotein which contains 294 amino acids (Okuwaki et al., 2006). NPM1 is one of the three nucleophosmin isoforms which are generated through alternative splicing. NPM1 resides predominantly in the nucleoli, but also continuously shuttles between nucleus and cytoplasm (Frehlick et al., 2006). The *NPM1* gene is located on chromosome 5q35 in humans and is composed of 12 exons (Chan et al., 1989). NPM1 is essential for processing and transportation of ribosomal RNA and proteins, molecular chaperoning, and regulation of the stability of tumor suppressors, such as p53 and ARF (Borer et al., 1989; Colombo et al., 2002; Enomoto et al., 2006; Herrera et al., 1995; Li & Hann, 2009; Maggi et al., 2008; Savkur & Olson, 1998; Yu et al., 2006). The ARF tumor suppressor is a protein that is transcribed from an alternate reading frame of the inhibitor of cyclin-dependent kinase CDK4. NPM1 can affect DNA replication, repair and transcription by interacting with the components of chromatin such as histones and chromatin remodeling proteins (Amin et al., 2008a,b; Angelov et al., 2006; Koike et al., 2010). NPM1 plays important roles in cell cycle (Ugrinova et al., 2007; Xiao et al., 2009). NPM1 may preferentially promote ribosome biogenesis in G1, facilitate DNA replication during S-phase while supporting chromosome segregation in mitosis (Hisaoka et al., 2010).

Almost 40% of CN-AML patients have mutations in exon 12 of the *NPM1* gene which result in loss of tryptophan residues normally required for NPM1 binding to the nucleoli and in the generation of an additional nuclear export signal motif at the C-terminus of NPM1 which causes its abnormal cytoplasmic localization (Bolli et al., 2007; Falini et al., 2006; Liso et al., 2008; Oelschlaegel et al., 2010). These mutations are the most common genetic alterations in adult CN-AML patients and are associated with female sex, higher white blood count, increased blast percentage, and low or absent CD34 expression. Cytoplasmic nucleophosmin leukemic mutant is also rarely generated by a exon-11 NPM1 mutation (Albiero et al., 2007). Acute myeloid leukemias with mutated NPM1 (NPM1c+) have distinct characteristics, including a significant association with a normal karyotype, involvement of different hematopoietic lineages, a specific gene-expression profile and clinically, a better response to induction therapy and a favorable prognosis (Meani & Alcalay, 2009; Rau & Brown, 2009; Falini et al., 2010). NPM1c+ maintains the capacity of wild-type NPM to interact with a variety of cellular proteins, and impairs their activity by delocalizing them to the cytoplasm. NPM1c+ specifically inhibits the activities of the cell-death proteases, caspase-6 and -8, through direct interaction with their cleaved, active forms, but not the immature procaspases. NPM1c+ not only affords protection from death ligand-induced cell death but also suppresses caspase-6/-8-mediated myeloid differentiation (Leong et al., 2010).

After the discovery of NPM1-mutated AML in 2005 and its subsequent inclusion as a provisional entity in the 2008 World Health Organization classification of myeloid neoplasms, several controversial issues remained to be clarified (Falini, 2011). It was unclear

whether the *NPM1* mutation was a primary genetic lesion and whether additional chromosomal aberrations and multilineage dysplasia (MLD) had any impact on the biologic and prognostic features of *NPM1*-mutated AML. Moreover, it was uncertain how to classify AML patients who were double-mutated for *NPM1* and *CEBPA*. Recent studies have shown that: (1) the *NPM1* mutant perturbs hemopoiesis in experimental models; (2) leukemic stem cells from *NPM1*-mutated AML patients carry the mutation; and (3) the *NPM1* mutation is usually mutually exclusive of biallelic *CEBPA* mutations. Moreover, the biologic and clinical features of *NPM1*-mutated AML do not seem to be significantly influenced by concomitant chromosomal aberrations or multilineage dysplasia. *NPM1*-mutated AML with and without MLD showed overlapping immunophenotype (CD34 negativity) and gene expression profile (CD34 down-regulation, homeobox (*HOX*) genes up-regulation). Altogether, these pieces of evidence point to *NPM1*-mutated AML as a founder genetic event that defines a distinct leukemia entity accounting for approximately one-third of all AML. Distinctive gene expression and microRNA signatures were found associated with AML bearing cytoplasmic mutated *NPM1* (Becker et al., 2010; Garzon et al., 2008; Verhaak et al., 2005).

Approximately 40% of patients with *NPM1* mutations also carry *FLT3* internal tandem duplications (*FLT3*-ITD). Patients with *NPM1* mutations, who did not also have *FLT3* mutation have generally more favorable prognosis (Gale et al., 2008; Scholl et al., 2008; Luo et al., 2009). The favourable prognosis of *NPM1*-mutated/*FLT3*-ITD negative patients might be explained by a higher *bax/bcl-2* ratio (Del Poeta et al., 2010). These patients respond to induction therapy and stay in remission more likely. These patients may be exempted from allogenic hematopoietic stem cell transplantation during the first complete remission because their outcome after conventional consolidation chemotherapy is the same as after allogenic transplantation. However, patients with *NPM1* mutations who also carry *FLT3* mutation have bad prognosis.

Moreover, *NPM1* mutations due to their frequency and stability, may be used for minimal residual disease monitoring in AML patients with a normal karyotype (Bacher et al., 2009; Schnittger et al., 2009; Dvorakova et al., 2010).

### 3.2 *FLT3* mutations

The feline *c-fms* proto-oncogene product is a 170 kd glycoprotein with associated tyrosine kinase activity. *Fms*-like tyrosine kinase 3 (*FLT3*) and its ligand (FL) are important in hematopoietic progenitor cell proliferation and differentiation (Gilliland & Griffin, 2002). As a result of ligand binding, *FLT3* receptor on the cell surface of hematopoietic progenitors dimerizes, resulting in activation of its tyrosine kinase domain, receptor autophosphorylation, and recruitment of downstream signaling molecules such as signal transducer and activator of transcription 5a (*STAT5a*), and the MAPK (mitogen activated protein kinases) pathways leading to proliferative and pro-survival effects.

Internal tandem duplication (ITD) of base pairs within the juxtamembrane coding portion or point mutations in the second kinase domain occur in approximately 30% of patients with newly diagnosed AML and result in constitutive activation of the *FLT3* gene on chromosome 13q12 ( Nakao et al., 1996; Naoe & Kiyoi, 2004; Yamamoto et al., 2001). *FLT3* mutations in the case of ITDs are associated with chemoresistance in the leukemic stem cells, shorter disease-free survival and overall survival and higher rate of relapse (Frohling et al., 2002; Ravandi et al., 2010; Whitman et al., 2010). Specific gene expression signature associated with *FLT3*-ITD was described (Bullinger et al., 2008; Whitman et al., 2010).

Overexpression of *FLT3*, homeobox genes and immunotherapeutics targets and decreased expression of erythropoiesis-associated genes is connected with *FLT3*-ITD. The prognostic significance of *FLT3* point mutations is less clear with conflicting results (Mead et al., 2008). In clinical practice, a frequent approach to patients with poor prognostic AML is to offer allogenic stem cell transplantation (SCT). Gale et al. (2005) found no benefit from any form of transplantation consolidation for patients with *FLT3*-ITD. Several inhibitors of *FLT3* have entered clinical trials and are studied alone or mainly in combination with chemotherapy (Kindler et al., 2010; Small, 2008; Weisberg et al., 2009; Wiernik, 2010).

3.3 CCAAT/enhancer binding protein alpha (CEBPA) mutations

The CCAAT/enhancer binding protein alpha (C/EBPα) is the founding member of a family of related leucine zipper transcription factors that play important roles in myeloid differentiation (Friedman et al., 2007; Keeshan et al., 2003; Pabst & Mueller, 2007; Suh et al, 2006; Tenen et al. 1997). Members of this family consist of N-terminal transactivation domains, a DNA-binding basic domain, and a C-terminal leucine rich dimerization region (Fig. 1).

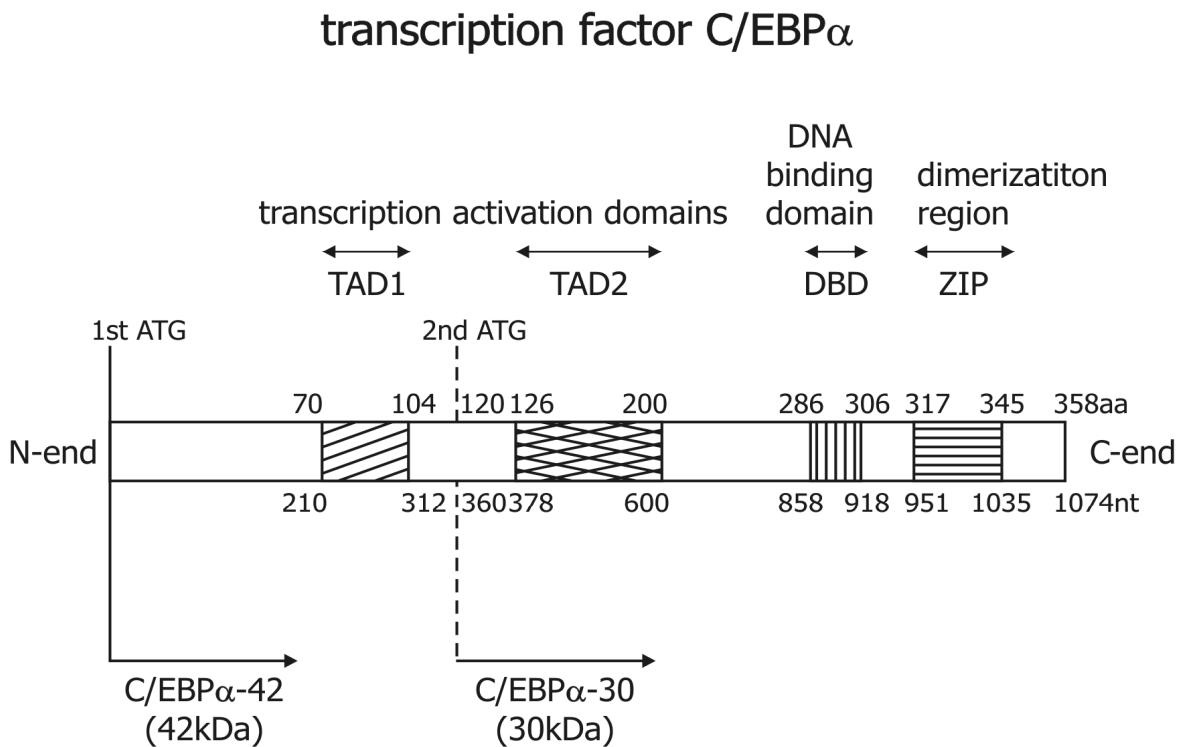


Fig. 1. The location of functional domains within the C/EBPα protein. Numbers directly above the schema indicate the amino acids of the human C/EBPα. Numbers directly under the schema indicate nucleotides (GenBank Accession No. NM\_004364.2). The full-length 42 kDa form of C/EBPα protein and the shorter, dominant negative 30 kDa form of this protein are also shown.

The dimerization domain, known as "leucine zipper", contains leucine repeats that intercalate with leucine repeats of the dimer partner forming a coiled coil of α helices in parallel orientation. C/EBPα mRNA is translated into two major proteins, C/EBPα p42 (42



kDa) and C/EBP $\alpha$  p30 (30 kDa) by ribosomal scanning mechanism in which a fraction of ribosomes ignore the first two AUG codons and initiate translation at the third AUG codon located 357 nucleotides downstream of the first one (Fig. 1). The 30 kDa protein lacks the transactivating domain TAD1 (Fig. 1) and was shown to inhibit DNA binding and transactivation by C/EBP $\alpha$  p42 (Pabst et al., 2001). C/EBP $\alpha$  p30 fails to induce myeloid differentiation (D'Alo' et al., 2003; Friedman et al., 2007). Targeted inactivation of C/EBP $\alpha$  in mice demonstrates its importance in the proper development and function of liver, adipose tissue, lung and hematopoietic tissues (Flodby et al., 2006; Wang et al., 1995; Zhang et al., 1997). C/EBP $\alpha$  is highly expressed in these differentiated tissues where it controls differentiation-dependent gene expression and inhibits cell proliferation (Fuchs 2007). Learning more about the precise molecular functions of the C/EBP $\alpha$  protein and how these are affected by leukemogenic mutations should lead to an improved understanding of the cellular functions that are disrupted in patients with AML.

*CEBPA* mutations were found in 10-19% of CN-AML patients (Gombart et al., 2002; Fröhling et al., 2004; Fuchs et al., 2008, 2009; Lin et al., 2005; Pabst et al., 2001; Preudhomme et al., 2002). Two kinds of mutations were mainly described: 1) truncating, frameshift mutations occurring near the N-terminus in one of the two transcription activations domains (TAD1 and TAD2) on one allele and 2) in-frame insertions or deletions clustering within the C-terminal basic domain- leucine zipper (DBD and ZIP) on the other allele. Often, CN-AML patients with *CEBPA* mutations belong to FAB (French –American –British) subtypes M1 or M2 and have one mutation towards N-end and one towards C-end but other cases of mutations were also detected. Kato et al. (2011) showed that a mutation of *CEBPA* in one allele was observed in AML after MDS while the two alleles are mutated in de novo AML. Favourable impact of *CEBPA* mutations was mainly observed in patients with biallelic mutation and with lack of *FLT3*-ITD (Dufour et al., 2010; Hou et al., 2009; Pabst et al., 2009; Radomska et al. 2006; Taskesen et al., 2011; Wouters et al., 2009).

### 3.4 Partial tandem duplications of the *MLL* gene

The mixed lineage leukemia gene (*MLL*, also known as *ALL-1* or *HRX*), located on chromosome 11q23, encodes a histone methyltransferase and is frequently rearranged in AML. Wild-type *MLL* is schematically presented in Fig. 2. To date, *MLL* has been found in more than sixty different translocations with different fusion partners (Basecke et al., 2006; De Braekeleer et al., 2005). Partial tandem duplications of the *MLL* gene were first observed in CN-AML by Caligury et al., 1994. These duplications consist of an in-frame repetition of *MLL* exons in a 5'-3' direction and lead to the change of the resulting transcript and protein. *MLL*-PTD are named according to the fused exons (mainly e9/e3, e10/e3, e11/e3).

*MLL*-PTD are detectable in 5%-11% of patients with CN-AML (Döhner et al., 2002; Döhner & Döhner 2008; Schnittger et al., 2000; Steudel et al., 2003). *MLL*-PTD have been found also in peripheral blood and bone marrow samples of healthy adults. However in contrast to the *MLL*-PTD in AML, *MLL*-PTD in healthy adults had often unusual exon fusions and showed an ladder on gel electrophoresis after the nested RT-PCR (Basecke et al., 2006; Marcucci et al., 1998; Schnittger et al., 1998). *MLL*-PTD cooperate with silencing of the *MLL* wild-type allele by epigenetic mechanisms. *MLL*-PTD contribute to leukemogenesis through hypermethylation of DNA and epigenetic silencing of tumor suppressor genes (Dorrance et al., 2006; Whitman et al., 2008a). Inhibitors of DNA methyltransferase and histone acetylase inhibitors and their combination can re-activate the wild-type allele in *MLL*-PTD-positive blasts (Whitman et al., 2005).

MLLWT

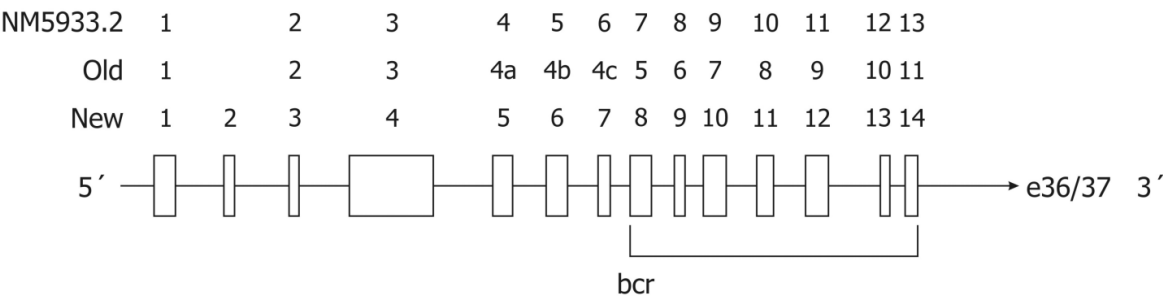


Fig. 2. Exon-intron structure of the wild-type *MLL* gene involved in tandem and nontandem duplications. The nomenclature is different in various studies (Nilson et al., 1996; Strout et al., 1998, Šárová et al., 2009). The *MLL* gene as a whole contains 36 or 37 exons according to the different nomenclatures and the resulting product of its expression contains 3969 amino acids. In the case of *MLL*-PTD e9/e3 are exons and introns between exons 3 and 9 inserted between exons 9 and 10 of the wild-type *MLL* and are duplicated by this way (the fusion of introns 2 and 9).

*MLL*-PTD are associated with shorter duration of the complete remission, shorter relapse-free survival and event-free survival, but *MLL*-PTD have no effect on overall survival Döhner & Döhner.

3.5 Wilms’ tumor 1 (WT1) mutations

The Wilms tumor 1 (*WT1*) gene is located on chromosome 11p13 and encodes a zinc-finger transcriptional regulator that can function as tumor suppressor in patients with the WAGR (Wilms’ tumor predisposition, aniridia, genitourinary abnormalities, and mental retardation) tumor predisposition syndrome (Haber et al., 1990) and as an oncogene in various leukemias, as well as other cancers (Ariyaratana & Loeb, 2007; King-Underwood et al., 1996; Miwa et al., 1992; Yang et al., 2007). Mutations in *WT1* gene were found in approximately 10% of AML patients (Hou et al., 2010; Gaidzik et al., 2009; Owen et al., 2010; Paschka et al., 2008; Virappane et al. 2008). Mutations are mainly localized in zinc-finger domains in exons 7 and 9 but can be also found in exons 1,2,3, and 8. The truncated WT1 protein is the result of frameshift mutations in exon 7. Truncated WT1 is without nuclear localization signal and does not bind to other interacting proteins as p53 and its homologue p73. Frameshift mutations in exon 9 are less frequent but there are also missense mutations. *WT1* mutations have been reported as an adverse prognostic factor in adult CN-AML and independently predict for poor outcome (Hou et al., 2010; Gaidzik et al., 2009; Owen et al., 2010; Paschka et al., 2008; Renneville et al., 2009; Virappane et al. 2008). *WT1* mutations lead to inferior rate of complete remission, higher incidence of relapse abd to shorter relapse-free survival and overall survival. A recent study demonstrated that a single nucleotide polymorphism SNP rs16754 in the WT1 mutational hotspot predicted favorable outcome in CN-AML (Damm et al. 2010).

3.6 Isocitrate dehydrogenase 1 and 2 (*IDH1*, *IDH2*) gene mutations

Mardis et al. (2009) found recurring mutations in codon 132 of the *IDH1* gene by sequencing a whole AML genome as described in paragraph 2.2. The protein encoded by this gene is the

enzyme that catalyzes the oxidative carboxylation of isocitrate to  $\alpha$ -ketoglutarate leading to nicotinamide adenine dinucleotide phosphate production in Krebs cycle and was found in the cytoplasm and peroxisomes (Geisbrecht & Gould, 1999). Three classes of IDH isoenzymes exist in mammalian cells (two forms of mitochondrial IDH and cytosolic IDH). *IDH1* gene is localized to chromosome band 2q33.3 and *IDH2* gene to chromosome band 15q26.1 (Narahara et al., 1985; Oh et al., 1996). *IDH2* encodes the mitochondrial isoform that uses nicotinamide adenine dinucleotide phosphate as a cofactor. The same cofactor is also used by *IDH1*.

Most cancer-associated enzyme mutations result in constitutive activation or inactivation of the mutated enzyme. *IDH1* and *IDH2* mutations result in the new enzyme activity, production of 2-hydroxyglutarate, not shared by wild type enzymes (Ward et al., 2010). This accumulation of 2-hydroxyglutarate induces global DNA hypermethylation, disrupts TET2 function because this enzyme is  $\alpha$ -ketoglutarate-dependent, and impairs hematopoietic differentiation (Figueroa et al., 2010). *TET2* is a homolog of the gene originally discovered at the chromosome ten-eleven translocation (TET) site in a subset of patients with AML. *TET2* catalyzes the conversion of methylcytosine to 5-hydroxymethylcytosine, suggesting a potential role for TET proteins in epigenetic regulation. Blocking the accumulation of 2-hydroxyglutarate through the inhibition of mutant IDH enzymes could represent a therapeutic target (Dang et al., 2010; Cazola 2010).

*IDH1* mutations at codon R132 occur in CN-AML patients with a frequency of 5.5% to 11% (Boissel et al., 2010; Gross et al., 2010; Patel et al., 2011; Schnittger et al., 2010; Wagner et al., 2010). A strong association between *IDH1* mutations and the *NPM1* mutation and M1 FAB subtype was observed. On the other hand, *IDH1* mutations are inversely associated with the M4 FAB subtype and expression of HLA-DR, CD13 and CD14 antigens. The prognostic impact of *IDH1* mutations in CN-AML is associated with a higher risk of relapse and a shorter overall survival (Abbas et al., 2010; Boissel et al., 2010; Marcucci et al., 2010; Paschka et al., 2010; Schnittger et al., 2010). Others (Chou et al., 2010a; Patel et al., 2011; Wagner et al., 2010), however, found no significant impact of *IDH1* mutations on CN-AML patients outcome. *IDH2* mutations in exon 4, including mainly codon R140 and in rare cases codon R172, had no prognostic impact (Thol et al., 2010). Recent study of Chou et al. (2011) showed high stability of *IDH2* mutations during disease evolution and their connection with favorable prognosis. Contrary to this observation, Boissel et al. (2010) found *IDH2* mutations independently associated with a higher risk of relapse and shorter overall survival. The prognostic impact of *IDH1* mutations and *IDH2* mutations needs further study as very controversial results were obtained. Green et al. (2010) observed no difference in outcome between *IDH1* mutated and nonmutated patients when the results were stratified by an *NPM1* mutation status but an adverse outcome for *IDH1* mutated patients when the results were correlated with *FLT3*-ITD mutation.

### 3.7 Mutations in gene for DNA methyltransferase 3A (*DNMT3A*)

About 22% of CN-AML patients have *DNMT3A* mutations. The most common *DNMT3A* mutation affects amino acid R882 but other parts of *DNMT3A* gene are also affected by mutations in CN-AML patients (Ley et al., 2010). Aberrant DNA methylation contributes to the pathogenesis of cancer (Rodríguez-Paredes & Esteller 2011; Taberlay & Jones, 2011; Watanabe & Maekawa, 2010). Clusters of CpG dinucleotides in promoters of tumor-suppressor genes are hypermethylated in cancer genomes and this hypermethylation

results in reduced expression of the downstream gene. However, inhibition of DNA methyltransferases is only one potential mechanism of function of demethylating agents (5-azacytidine and decitabine). *DNMT3A* mutations do not change 5-methylcytosine content in AML genomes but are associated with poor survival. *DNMT3A* mutations are in many cases found together with *FLT3* mutations, *NPM1* mutations and *IDH1* mutations. All these combinations of mutations have a significantly worse outcome.

### 3.8 RAS mutations in CN-AML

Ras-signaling cascade contributes to the molecular pathogenesis of myeloproliferative disorders (Chan et al., 2004). Ras oncogenes (small GTPases) regulate mechanism of proliferation, differentiation, and apoptosis. *NRAS* (neuroblastoma RAS) mutations were detected in 9% of adult CN-AML patients and 14% of CN-AML patients younger than 56 or 60 years (Bacher et al., 2006; Bowen et al., 2005). There was no prognostic impact of these mutations in most studies (Gaidzik & Döhner, 2008; Ritter et al., 2004; Schlenk & Döhner, 2009). Mutations in other members of *Ras* family are rare in CN-AML and there was also no consistent effect on prognosis but the presence of *Ras* mutations appears to sensitize AML blasts to high dose cytarabine *in vivo* (Motyckova & Stone 2010).

### 3.9 Other gene mutations in CN-AML

Mutations in *RUNX1* have been shown in approximately 10%-13% of CN-AML (Döhner & Döhner, 2008, Tang et al., 2009). These mutations were positively associated with *MLL*-PTD and negatively associated with *NPM1* and *CEBPA* mutations. They predicted a lower complete remission rate and shorter disease-free and overall survival.

*TET2* (ten-eleven-translocation) first described in 2008, include frameshift, nonsense and missense mutations lying across several of its 12 exons located on chromosome 4q24 (Abdel-Wahab et al., 2009; Bacher et al., 2010; Mohr et al., 2011; Nibourel et al., 2010). The direct influence of mutations in *TET2* on patient survival in CN-AML remains a disputable issue. *TET2* mutations were revealed in 10%-25% of CN-AML patients. Abdel-Wahab et al. (2009) showed a decreased survival rate in mutated *TET2* in comparison with wild-type *TET2* group of CN-AML. However, Nibourel et al. (2010) did not find significant impact of *TET2* mutation on clinical outcome of CN-AML patients but they observed mutated *TET2* strongly associated with mutated *NPM1*. Recently, Metzeler et al. (2011) have found *TET2* mutations in 23% of CN-AML patients and these mutations were associated with older age. In favorable-risk group of CN-AML patients with *CEBPA* mutation and/or mutated *NPM1* without *FLT3*-ITD, *TET2*-mutated patients had shorter event-free survival, lower complete remission rate and shorter disease-free and overall survival. In CN-AML patients with intermediate risk with wild-type *CEBPA* and wild-type *NPM1* without *FLT3*-ITD, *TET2* mutations were not associated with outcomes.

*CBL* (Casitas B-cell lymphoma) mutations were identified in rare cases of CN-AML (Bacher et al., 2010; Makishima et al., 2009; Reindl et al., 2009). Cbl is E3 ubiquitin ligase involved in degradation of activated receptor tyrosine kinases, including Src kinases (Makishima et al., 2009). Presence of these mutations was suggested to be involved in aberrant *FLT3* expression. *FLT3* ligand-dependent hyperproliferation of *CBL* mutant cells could be abrogated by treatment with the specific inhibitor, midostaurin (PKC412).

Mutations in the additional sex comb-like 1 (*ASXL1*) gene were analyzed in exon 12 in CN-AML patients and 8.9% mutations were detected (Chou et al., 2010b). This mutation was



closely associated with older age, male sex, *RUNX1* mutation and expression of human leukocyte-antigen-DR and CD34 (Chou et al., 2010b; Rocquain et al., 2010). Association with *FLT3*-ITD, *NPM1* mutation, *WT1* mutation, and expression of CD33 and CD15 was not detected. *ASXL1* mutated patients had a shorter overall survival than patients without this mutation, but the mutation was not an independent adverse prognostic factor in multivariate analysis.

Phosphoinositide phospholipase C $\beta$ 1 (*PI-PLC $\beta$ 1*) gene mutations are very rare in CN-AML (Damm et al., 2010). Follo et al. (2009) described greater representation of these mutations (monoallelic deletions) in AML and their association with a worse clinical outcome.

#### 4. Overexpression of marker genes with prognostic relevance

Alterations in the expression of genes belonging to signal transduction pathways as well as transcription factors are known to play a functional role in the pathogenesis of AML. Therefore, these marker genes are implicated in the process of leukemogenesis and their overexpression may be useful to predict outcome in CN-AML patients.

##### 4.1 *WT1* gene expression

The *WT1* gene overexpression was found in several leukemias, including AML (Cilloni et al., 2009). *WT1* mRNA levels in the peripheral blood can predict relapse after achieving complete remission, and its levels after consolidation therapy are closely correlated with disease-free and overall survival, and with early relapse (Cilloni et al., 2009; Gianfaldoni et al., 2010; Miyawaki et al., 2010). Monitoring of *WT1* expression is significant predictor of relapse in AML patients after hematopoietic cell transplantation (Lange et al., 2011).

##### 4.2 *BAALC* (brain and acute leukemia, cytoplasmic) expression

The *BAALC* gene, located on chromosome 8q22.3, is primarily expressed in neuroectoderm-derived tissues and in hematopoietic precursors and encodes a protein with unknown function (Baldus et al., 2003, 2006; Langer et al., 2008; Santamaria et al., 2010). High level of *BAALC* expression showed a higher refractoriness to induction treatment, lower complete remission rate after salvage therapy and lower overall survival and relapse-free survival in intermediate-risk AML (Santamaria et al., 2010). The *BAALC* expression is considered an independent prognostic factor in CN-AML. High *BAALC* expression was associated with *FLT3*-ITD, and high *ERG* expression in multivariable analysis (Baldus et al., 2006). High *BAALC* expression is also connected with overexpression of genes involved in drug resistance (*MDR1*) and stem cell markers (*CD133*, *CD34*, *KIT*). In low *BAALC* expressers, genes associated with undifferentiated hematopoietic precursors and unfavorable outcome predictors were downregulated, while *HOX*-genes and *HOX*-gene-embedded-miR were upregulated (Schwind et al., 2010). Global miR expression analysis did not reveal significant differences between different rate *BAALC* expression groups (Langer et al., 2008). Inverse association between the expression of *miR148a* and *BAALC* was revealed.

##### 4.3 *ERG* (v-ets erythroblastosis virus E26 oncogene homolog) expression

*ETS*-related gene (*ERG*), located at chromosome band 21q22, is downstream effector of signaling transduction pathways involved in the regulation of cell proliferation, differentiation, and apoptosis (Marcucci et al., 2005, 2007; Mrózek et al., 2007; Metzeler et al., 2009; Schwind et al., 2010). CN-AML patients with overexpression of *ERG* have been



reported to have a poor clinical outcome. When combined with other known prognostic markers, *ERG* expression can improve the molecular risk-based stratification of patients with CN-AML. Low *ERG* expression is associated with downregulation of genes involved in the DNA-methylation machinery, upregulation of *miR148a*, which targets DNA methyltransferase 3B (*DNMT3B*) and with better outcome (Schwind et al., 2010).

#### 4.4 *MN1* (meningioma 1) expression

*MN1* is located at 22q11 and its overexpression is associated with lower response rate after first course of induction therapy and poor clinical outcome for CN-AML patients. Moreover, high *MN1* expression was connected with a higher relapse rate and worse relapse-free and overall survival (Grosveld, 2007; Heuser et al., 2006; Langer et al., 2009). *MN1* expression levels were directly correlated with *BAALC* expression levels and with the expression of genes reported as associated with a *BAALC* expression signature, specifically with expression of *CD34* and *ABCB1* (*MDR1*) and several other genes (Langer et al., 2008, 2009). *MN1* expression levels were negatively connected with expression of *HOX* genes and with *NPM1* mutated CN-AML (Langer et al., 2009). *MN1*-associated miR-expression signature comprises 15 miR, expression of 8 miR (*hsa-miR-126* family) was positively correlated and expression of 7 miR (*hsa-miR-16*, *hsa-miR-19a* and *hsa-miR-20a*, all members of *miR-17-92* polycistron) negatively correlated with *MN1* expression (Langer et al., 2009). *MN1* overexpression conferred resistance to the differentiation activity of all-trans-retinoic acid (ATRA) in AML (Heuser et al., 2007).

#### 4.5 *EVII* (ecotropic viral integration site 1) expression

Human *EVII* is localized to chromosome 3 band q26, spans 60kb, and contains 16 exons (Goyama & Kurokawa, 2009). High *EVII* expression occurs in approximately 8% of patients with *de novo* AML (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2003). High *EVII* expression was observed not only in AML carrying the chromosome 3 abnormalities, but also in CN-AML (Gröschel et al., 2010; Lugthart et al., 2008; Santamaria et al., 2009) and is in both groups connected with poor treatment response.

#### 4.6 Other molecular marker genes expression

The *PRAME* (preferentially expressed antigen of melanoma) gene was shown to be expressed in high levels in AML. *PRAME* mRNA was observed in about one-third of AML cases and there was a good correlation between *PRAME* mRNA level and hematological remission and relapse. It may be also useful marker to detect minimal residual disease after allogeneic transplantation (Paydas et al., 2005; Qin et al., 2009). Epping et al. (2005) showed that *PRAME* is a repressor of retinoic acid signaling but Steinbach et al. (2007) did not confirm this mechanism in the pathogenesis of AML. Specific immunotherapies for patients with AML using leukemia-associated antigens (LAA) as target structures might be a therapeutic option. Expression of genes for these antigens have prognostic importance (Greiner et al., 2008).

*AF1q* (*ALL1* fused gene from chromosome 1q) gene overexpression in CN-AML patients is associated with a significantly greater incidence of concurrent *FLT3*-ITD and with a poor outcome (Strunk et al., 2009). CN-AML patients with low *AF1q* expression had better overall survival and complete remission rate than patients with high *AF1q* mRNA level.

High *MLL5* (mixed lineage leukemia 5) expression is associated with a favorable outcome of CN-AML patients and enables identification of a significant proportion of patients with favorable prognosis that are not identified by other markers analyses (Damm et al., 2011).

Increased expression of the phosphoinositide phospholipase C $\beta$ 1 (*PI-PLC  $\beta$ 1*) gene is an independent prognostic factor in CN-AML and is associated with a significantly shorter overall survival but with no difference for relapse-free survival (Damm et al., 2010).

The Rho family of small GTPases, including Rho, Rac and Cdc42, functions as critical mediators of signaling pathways from plasma membrane regulating actin assembly, migration, proliferation and survival in hematopoietic cells. *RhoH* gene, also known as Translocation Three Four (*TTF*), encodes a 191-amino acid protein belonging to the Rho family (Gu et al., 2005; Iwasaki et al., 2008). Rho H functions as a negative regulator for interleukin 3 (IL3) - induced signals through modulation of the JAK-STAT (Janus Kinase-Signal Transducer and Activator of Transcription)- signaling pathway (Gündogdu et al., 2010). Low RhoH levels are connected with an upregulation of IL3- dependent cell growth, STAT5 activity and an increase of CD123 surface expression that has been described in AML patients (Gündogdu et al., 2010). Multivariate analysis demonstrated that low expression of *RhoH* was an independent unfavorable prognostic factor for both overall and disease-free survival of AML in the intermediate risk group (Iwasaki et al., 2008).

Activation of Notch signal pathway (expression of *Notch1*, *Jagged1* and *Delta1* as members of this pathway) is associated with a poorer prognosis for AML patients with intermediate risk (Xu et al., 2010).

The Forkhead transcription factors (FOXO) are direct target of the PI3K/AKT (protein kinase B) signaling and they integrate the signals of several other transduction pathways at the transcriptional level. The PI3K/AKT/FOXO signaling pathway is up-regulated in AML. High *FOXO3a* expression is associated with a poorer prognosis in CN-AML (Santamaria et al., 2009) and the increased levels of both total and of highly phosphorylated FOXO3a correlate with higher proliferation and blood blasts and these high levels of FOXO3a are an adverse prognostic factor in AML (Kornblau et al., 2010).

Bone marrow neoangiogenesis plays an important pathogenetic and possible prognostic role in AML (Hou et al., 2008; Lee et al., 2007; Loges et al., 2005; Mourah et al. 2009). Multivariable analysis showed that the levels of vascular endothelial growth factor (VEGF) transcript isoform 121 (VEGF121) remained an independent prognostic factor for either event-free survival or overall survival (Mourah et al., 2009). High levels of VEGF121 were significantly related to a worse prognosis. Angiopoietin-2 (*Ang2*) gene expression represents also an independent prognostic factor in AML with intermediate risk and high *Ang2* expression is associated with an unfavorable prognosis (Hou et al., 2008; Lee et al., 2007; Loges et al., 2005). High *VEGFC* expression appeared strongly associated with reduced complete remission rate, reduced overall and event-free survival in adult AML independent of cytogenetic risk and white blood cell count (de Jonge et al., 2010). High *VEGFC* expression was related to enhanced chemoresistance and predicted adverse long-term prognosis.

TGF $\beta$  (transforming growth factor beta) superfamily receptors ALK-1 (activin receptor like kinase) and ALK-5 have an important role in endothelial cells behavior and might be involved in the pathogenesis of AML. *ALK-1* and *ALK-5* are both expressed by the majority of AML patients. *ALK-5* expression has a significant negative impact on complete remission achievement and overall survival of AML patients (Otten et al., 2011).

Dysregulation of the Wnt/ $\beta$ -catenin pathway has been observed in various malignancies, including AML. Overexpression of  $\beta$ -catenin is an independent adverse prognostic factor in AML (Chen et al., 2009; Ysebaert et al., 2006).

Chemokine (C-X-C motif) receptor 4 (CXCR4) retains hematopoietic progenitors and leukemia cells within the marrow microenvironment. Multivariate analysis revealed CXCR4 expression as an independent prognostic factor for disease relapse and survival (Konoplev et al., 2007; Spoo et al., 2007; Tavernier-Tardy et al., 2009). Low CXCR4 expression correlated with a better prognosis, resulting in a longer relapse-free and overall survival.

Many studies of AML have linked the overexpression of ABCB1 (also named permeability glycoprotein, Pgp), a member of ATP-binding proteins coded by the multi-drug resistance gene (*MDR1*), to poor prognosis (Leith et al., 1997; Steinbach & Legrand, 2007; Trnkova et al., 2007). Other drug-resistance proteins BCRP (breast cancer resistance protein, also named ABCG2) and LRP (lung resistance protein) have also an adverse impact (Dimiani et al., 2010; Huh et al., 2006).

## 5. Gene expression profiling in CN-AML

Gene expression profiling (GEP) was described twelve years ago by Golub et al. (1999). GEP analyses on the basis of microarrays allow the simultaneous characterization of thousands of genes. GEP is useful for the classification of leukemias. In CN-AML, microarray GEP has been applied to identify expression signatures in order to predict clinical outcome within this very heterogeneous group of patients.

Bullinger et al. (2004) and Radmacher et al. (2006) defined by GEP two novel molecular subclasses of CN-AML with significant differences in survival times with respect to the presence or absence of *FLT3* mutations and the FAB subtypes.

*NPM1* gene mutations are connected with specific gene expression pattern in CN-AML (Alcalay et al., 2005; Becker et al., 2010; Garzon et al., 2008; Verhaak et al., 2005; Wilson et al., 2006). This specific gene expression signature was characterised by the activation of homeobox (*HOX*) genes including a particular subset of homeobox *TALE* (three amino acid loop extension) genes distinguish themselves from typical homeodomains containing genes. Downregulated in the *NPM1* mutations group were genes whose low expression is associated with better prognosis in CN-AML as *BAALC*, *MN1*, *ERG*, and multidrug resistance genes.

Comparison of gene expression between biallelic *CEBPA* mutation and monoallelic *CEBPA* mutation AML was described by Dufour et al. (2010). Expression of multiple members of the homeobox gene family (*HOXA5*, *HOXA9*, *HOXA10*, *HOXB2*, and *HOXB6*), *CD34*, and lymphoid markers *CD6*, *CD52*, and *TSPO* (gene for translocator protein, benzodiazepine receptor) is downregulated in CN-AML patients with biallelic *CEBPA* mutation.

Specific gene expression signatures associated with *FLT3*-ITD and *FLT3*-TKD (mutations in the tyrosine kinase domain) were described (Bullinger et al., 2008; Neben et al., 2005; Whitman et al., 2008b, 2010). Overexpression of *FLT3*, homeobox genes (*HOXB3*, *HOXB5*, *PBX3*, *MEIS1*), and immunotherapeutic targets (*WT1*, *CD33*) and underexpression of leukemia associated (*MLLT3*, *TAL1*) and erythropoiesis-associated genes (*GATA3*, *EPOR*, *ANK1*, *HEMGN*) is typical for *FLT3*-ITD, whereas overexpression of gene for transcription factor *FOXA1* containing forkhead box was observed in *FLT3*-TKD (Neben et al., 2005, Whitman et al., 2010). Whereas the predictive value for *FLT3*-ITD was relatively high (77%), the high number of false predictions eliminates GEP as an investigational tool for research

studies waiting on an entrance to clinical practice and decision making (Marcucci et al., 2011a; Verhaak et al., 2009; Wouters et al., 2009). GEP technique seems not to be in future a primary diagnostic tool but will be used in many cases as a confirmative method.

## 6. MicroRNA expression profiling

MicroRNAs (miRs) are small noncoding RNAs of 19 to 25 nucleotides which function as negative regulators of gene expression by causing target mRNA cleavage or by interfering with target mRNA translation. Dysregulation of miRs plays an important role in the pathogenesis of many cancers based on their involvement in basic cellular functions (Nana-Sinkam & Croce, 2010). In addition, miRs have the capacity to target tens to hundreds of genes simultaneously. Thus, they are attractive candidates as prognostic biomarkers and therapeutic targets in cancer.

MiR expression signatures have been correlated with recurrent molecular aberrations in AML. *NPM1* mutations associate with upregulation of *miR10a*, *miR10b*, and *miR196a*, all lying in the genomic cluster of *HOX* genes that are overexpressed (Becker et al., 2010, Garzon et al., 2008). Upregulation of *miR181a* and *miR181b* expression is associated with *CEBPA* mutations in CN-AML (Marcucci et al., 2008; 2009; 2011b). *FLT3*-ITD was observed to be associated with *miR155* upregulation and *miR144* and *miR451* downregulation (Whitman et al., 2010). Genome-wide profiling identified aberrantly expressed miR associated with R172 *IDH2* mutated CN-AML patients (Marcucci et al., 2010). The most upregulated miR genes were genes of *miR125* family (*miR125a* and *miR125b*), *miR1* and *miR133*. The most downregulated miR genes were *miR194-1*, *miR526*, *miR520a-3p*, and *miR548b*.

Recent studies have also shown that clinical outcome in CN-AML is affected by changes in miR expression. Overexpression of *miR20a*, *miR25*, *miR191*, *miR199a* and *miR199b* adversely affected overall survival (Garzon et al., 2008).

## 7. DNA methylation arrays

DNA cytosine methylation in CpG islands regulates gene expression. Aberrant methylation of specific genes was observed in cancer including leukemia, although little is known about the mechanisms of this specific gene sets methylation. Genome-wide promoter DNA methylation profiling revealed unique AML subgroups and methylation patterns that are associated with clinical outcome (Bullinger & Armstrong, 2010; Figueroa et al., 2010). DNA methylation profiles segregates patients with *CEBPA* mutations from other subtypes of leukemia and defined four epigenetically distinct forms of AML with *NPM1* mutations. Epigenetic modification of the *CEBPA* promoter regions was also described and *CEBPA* hypermethylation appeared to be favorable prognostic marker in addition to *NPM1* mutation with lack of *FLT3*-ITD and *CEBPA* bi-allelic, double mutations (Hackanson et al., 2008; Lin et al., 2010; Szankasi et al., 2011). Lugthart et al. (2011) found that the promoter DNA methylation signature of *EVI1* AML blast cells differed from normal bone marrow cells and other AMLs and contained many hypermethylated genes. *EVI1* was observed to physically interact with DNA methyltransferases 3A and 3B and colocalize with them in nuclei and complex is involved in *EVI1*-mediated transcriptional repression. Cases with the significantly higher levels of *EVI1* are associated with many more methylated genes (Lugthart et al., 2011).



## 8. Conclusion and future directions

CN-AML is very heterogeneous on the molecular level and harbours many genetic alterations that define new molecular subgroups. This molecular heterogeneity of CN-AML is not fully reflected in current classification systems (Vardiman et al. 2008, Döhner et al., 2010). Molecular markers with prognostic significance are very important for future therapies. Decision over whether to allograft a patient in first complete remission depends on the evaluation in a risk/benefit analysis in prognostic scoring system (Smith et al., 2011). The favorable cytogenetic risk group is now supplemented by CN-AML with mutant *NPM1* or biallelic *CEBPA* mutations in the absence of *FLT3*-ITD (Döhner et al., 2010). These CN-AML patients may not need to be referred for allogenic stem cell transplantation in first complete remission (Burnett et al., 2011). Low expression of *BAALC* is also associated with favorable outcome in CN-AML (Santamaria et al., 2010), but not in association with *FLT3*, *NPM1*, and *CEBPA* mutations and may not be prognostic in older patients (Langer et al., 2008). Low *BAALC* expression is an important factor for complete remission achievement and longer disease-free survival. Even better overall survival is reached in CN-AML patients who had low *ERG* expression in addition to low *BAALC* expression (Burnett et al., 2011). The similarity of *BAALC* and *ERG* expression signatures between younger and older CN-AML patients and the fact that these molecular markers affect similarly outcomes in the group of younger and older than 60 years CN-AML patients suggest that older patients with favorable molecular risk factors, such as low *BAALC* and *ERG* expression, if treated more intensively, might have outcomes comparable with those of younger CN-AML patients with the same molecular markers (Schwind et al., 2010). Patients with low *ERG*, low *EV11*, and high *PRAME* expression levels were also shown to have a good prognosis (Santamaria et al., 2009). Recently, Damm et al. (2011) proposed an integrative prognostic risk score (IPRS) for CN-AML patients based on clinical and molecular markers. Nine clinical, hematological and molecular factors including age, white blood cell count, mutation status of *NPM1*, *FLT3*-ITD, *CEBPA*, *WT1* single nucleotide polymorphism SNP rs16754, and expression levels of *BAALC*, *ERG*, *MN1*, and *WT1* (Damm et al., 2011). Other molecular markers like *NRAS*, *MLL*-PTD, *WT1*, *IDH1*, or *IDH2* mutations were not significant and thus not included in the IPRS.

Genomewide search and new technologies will help to subcategorize CN-AML. Gene and microRNA signatures and DNA methylation signatures obtained in these studies may detect potential targets for new therapies.

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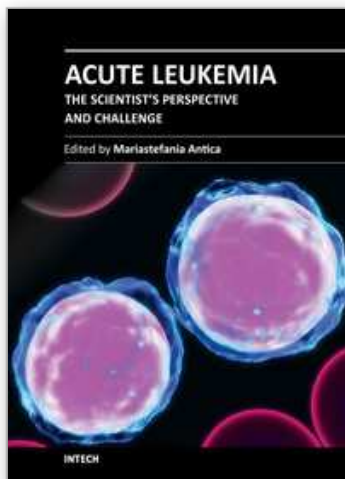
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## **Acute Leukemia - The Scientist's Perspective and Challenge**

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This book provides a comprehensive overview of the basic mechanisms underlying areas of acute leukemia, current advances, and future directions in management of this disease. The first section discusses the classification of acute leukemia, taking into account diagnoses dependent on techniques that are essential, and thankfully readily available, in the laboratory. The second section concerns recent advances in molecular biology, markers, receptors, and signaling molecules responsible for disease progression, diagnostics based on biochips and other molecular genetic analysis. These advances provide clinicians with important understanding and improved decision making towards the most suitable therapy for acute leukemia. Biochemical, structural, and genetic studies may bring a new era of epigenetic based drugs along with additional molecular targets that will form the basis for novel treatment strategies. Later in the book, pediatric acute leukemia is covered, emphasizing that children are not small adults when it comes to drug development. The last section is a collection of chapters about treatment, as chemotherapy-induced toxicity is still a significant clinical concern. The present challenge lies in reducing the frequency and seriousness of adverse effects while maintaining efficacy and avoiding over-treatment of patients.

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