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Chapter 10

MLL Gene Alterations in Acute Myeloid Leukaemia (11q23/*MLL*+ AML)

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Additional information is available at the end of the chapter

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1. Introduction

Acute myeloid leukemia (AML) is characterized by the malignant transformation of myeloid cells from myeloblasts to a pathological cell clone. These pathological cell clones lose their ability to differentiate and mature, are no longer subject to regulatory mechanisms and suppress other components of normal hemopoiesis. AML does not fall under a single nosological entity. The heterogeneity of AML is reflected by differences in morphology and immunophenotype, as well as cytogenetic and molecular genetic abnormalities. It includes a number of subtypes, which can be further classified according to the FAB and World Health Organization (WHO) criteria.

Acute myeloid leukemia represents 15% to 20% of all childhood leukemias, approximately 33% of adolescent leukemias, and approximately 50% of adult leukemias. After a peak during the first 2 years of life, the subsequent annual incidence of AML slowly increases after 9 years of age (incidence rate 5/1 million in 5 to 9-year-olds, 9/1 million in 15 to 19-year-olds). In general, the biological features, other than age, of pediatric and adult AML appear to be similar, but the differences have not been reviewed systematically [1].

The rate of *therapy-related AML* (that is, AML caused by previous chemotherapy) is rising; therapy-related disease currently accounts for approximately10–20% of all cases of AML [2]. The incidence of secondary leukemias is increasing because of aging of the population (MDS is more frequent in elderly people) and widespread of successful use of chemoradiotherapy in cancer patients (solid tumors or hematological malignancies). The majority of secondary leukemias resulting from the use of cytotoxic drugs. Therapy – related AML is heterogeneous collection of diseases characterised by distinct chromosomal abnormalities. One subset of therapy related AMLs is associated with exposure to alkylating agents. The chromosomal abnormalities 5q- and monosomy 7 are commonly observed in leukemic cells in this groups. The other major subset of therapy-induced leukemia is associated with

exposure to epipodophyloltoxin drugs teniposid and etoposid. A high proportion of epipodophyllotoxin - associated AMLs are of the M4 (monocytic) or M5 (myelomonocytic) subtype and have abnormalities at chromosome band 11q23 involving rearrangement of the MLL gene. The same genetic abnormality is also found in some secondary AMLs associated with exposure to antracyclines. These two classes of chemotherapeutic agents share a common mechanism of action that involves binding to and inhibition of DNA topoisomerase II.

Acute myeloid leukemia is a curable disease; the chance of cure for a specific patient depends on a number of prognostic factors. The current five-year survival rates of adult patients under age 60 range from 30% - 40%; for pediatric patients, five-year survival rates are up to 65% [3, 4].

The cure rates in pediatric AML have been achieved not only by the more effective use of anti-leukemic agents but also by improvements in supportive care and better risk-group stratification. Recurrent cytogenetic and genetic aberrations and early responses to treatment are important prognostic factors in AML and therefore are used for risk group stratification.

The prognostic value of cytogenetics is well established in all age groups. The biologic data differ considerably between infants and older age groups but only slightly between children, adolescents, and young adults. The distribution of cytogenetic aberrations in infants is different from that in older patients. Infants have almost no favorable aberrations but have frequent 11q23 aberrations and complex karyotypes, which is similar to older AML patients (>60 years)[1]. Schochet et al. [5] analyzed the effect of age and cytogenetics on clinical outcome in adult patients (>16 years). They found that both age and cytogenetics were independent prognostic parameters in AML; however, up to the age of 49 years, age had no major impact on prognosis, whereas the karyotype did. Therapy today consists of a limited number of intensive courses of chemotherapy based on cytarabine and an anthracycline. An important problem in the treatment of AML remains the high frequency of treatment-related deaths and long-term side effects [6,7].

This problem hampers further therapy-intensification, and most investigators therefore feel that we have reached a plateau in the number of patients that can be cured with current chemotherapy regimens. Our efforts should therefore focus on clarifying the biology of pediatric AML. This knowledge can be used for novel classification and risk-group stratification. In addition, it creates the potential for targeted, i.e., more leukemia-specific, therapy. It is anticipated that such therapies will increase the cure-rate and decrease the toxicity of treatment of patients with AML [4].

Leukemias bearing translocations involving chromosome 11q23 are of particular interest due to unique clinical and biological characteristics. The development of acute leukemias is associated with *MLL* gene alterations in about 10% of all leukemia cases of acute lymphoblastic leukemia and acute myeloid leukemia) [8].

***MLL* alterations** correlate with specific disease subtypes (acute myeloid and acute lymphocytic leukemias), a specific gene expression profile [9, 10], and outcomes (favorable or poor), depending on the particular *MLL* alteration [11]. Certain *MLL* alterations are independent unfavorable prognostic factors, and patients are usually treated according to high-risk protocols. For this reason, identifying *MLL* alterations has relevant implications for therapy decision-making. In pediatric AML, optimized intensive regimens for AML have also improved outcomes for *MLL*-rearranged AML. Patients have an intermediate outcome, with a 5 y OS (probability of overall survival at 5 years from diagnosis) ranging from 42-62% [12]. Therefore, further insights into the biology of *MLL*-rearranged AML, the development of reliable methods for screening in laboratory settings, and safe testing of new potential *MLL*-targeted therapies could have a significant impact on the overall outcomes for adult and pediatric patients.

The ***MLL* gene** was identified in 1991; a year later, it was completely characterized and cloned. The origin of the previous *ALL1* designation dates back to 1970, when van den Bergh described the reconstruction of the gene area for the first time in a patient with lymphoblastic leukemia [13]. Scientists later completed the characterization of the gene, and the gene was named *MLL* (mixed-lineage leukemia) based on the 11q23 translocation, which is observed in myelogenous and lymphoblastic types of leukemia. *MLL* has other synonyms as well, such as *HRX* (human trithorax) and *Htrx1*, which express its homology with the trithorax (*trx*) gene in *Drosophila melanogaster*. The ***MLL (ALL1, HRX, Htrx1)* gene** is located on the long arm of chromosome 11 (11q23), telomerically to the *PLFZ* gene and centromerically from the *RCK* gene (Figure 1).

Figure 1. Chromosome 11. The red mark indicates the position of the *MLL* gene.

The *MLL* gene consists of 36 exons over 100 kb. The product of the resulting 12 kb mRNA is a protein with 3968 amino acids and a molecular mass of approximately 430 kD. It is transcribed from centromere to telomere. Most, but not all, breaks in the *MLL* gene are localized in the 8.3 kb breakpoint region (bcr – breakpoint cluster region). The bcr region can be divided into a centromeric portion and a telomeric portion. Breaks in the *MLL* gene in infant leukemia and t-AML occur primarily in the telomeric part, while breaks in patients with *de novo* AML are closer to the centromere [1] (Figure 2).

Several translocation partners of *MLL* were found recently to coexist in a super elongation complex (SEC) that includes known transcription elongation factors such as eleven-nineteen lysine-rich leukemia (ELL) and positive transcription elongation factor b (P-TEFb). The SEC is required for *HOX* (homeobox domain gene) gene expression in leukemic cells, suggesting

that chromosomal translocations involving *MLL* could lead to the overexpression of *HOX* and other genes through the involvement of the SEC [14].

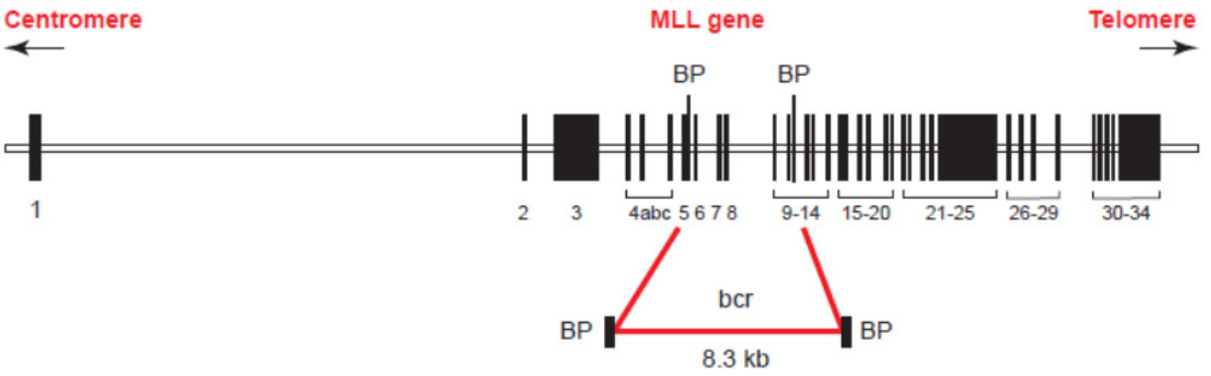

Figure 2. The structure of the *MLL* gene. Structure of the MLL gene: exon 1-34. Bcr (breakage region) region: exons 5-11, BP – break point.

The **MLL protein** is expressed in different tissues, such as the brain, colon, liver, spleen, thymus, kidneys, tonsils, heart, lungs, testes and thyroid. Genetic mutations of the *MLL* gene seem to occur preferentially in hematopoietic cells, suggesting that this system enables special permissivity, allowing for the survival and development of leukemic clones of different MLL fusion proteins. Specific signals are derived from stromal cells during fetal liver and definitive hematopoiesis. This enables the activation of anti-apoptotic pathways and stem cell maintenance necessary to receive survival signals caused by the presence of oncogenic MLL fusion proteins [15, 16].

The MLL protein is involved in chromatin regulation. It is specifically hydrolyzed by the endopeptidase Taspase1 and methylates histone core particles at histone H3 lysine 4 residues [17-19]. Therefore, MLL is part of an epigenetic system that co-regulates mitotic gene-expression signatures during embryonic development and tissue differentiation in mammalian organisms. The MLL complex binds to different promoters in various tissues. Recently, a genome-wide array study revealed that MLL was bound to more than 2000 different promoter regions [20]. This protein belongs to the group of Trithorax (trx-G) proteins, which are responsible for maintaining gene expression during growth. It is assumed that the MLL protein controls the expression of *HOX* genes. Several *HOX* genes are involved in the regulation of normal and leukemic hemopoiesis. The products of *HOX* genes are localized in the nucleus and represent a major class of transcription factors controlling cell proliferation/differentiation during early embryonic development [21].

2. Etiology and pathogenesis of causative *MLL* gene abnormalities in AML

The cause of 11q23/ *MLL*+ AML is unknown, but important factors include ionizing radiation (the highest incidence was observed five to seven years after radiation exposure), chemicals (such as benzene and various organic solvents), drugs (cytostatic drugs in particular), physical agents (such as electromagnetic fields), and environmental factors to

which the fetus is exposed in utero. In infant AML, a prenatal origin has been suggested by data from neonatal bloodspots on Guthrie cards [22, 23]. The 11q23 locus is particularly sensitive to cleavage after treatment with topoisomerase-II inhibitors. Because DNA topoisomerase II seems to be highly expressed in the developing fetus, exposure to inhibitors of DNA topoisomerase II could induce *MLL* AML in utero. A large case-control study of maternal diet and infant leukemia showed that the amount of maternal consumption of food containing DNA topoisomerase II inhibitors was correlated with the risk of developing MLL-rearranged AML [24].

The pathogenesis of AML is related to oncogenic fusion proteins, the formation of which results from chromosomal translocations or inversions [25] (Table 1).

Chromosomal aberation	FAB subtype AML	Frequency	Fusion gene
t(8;21) (q22;q22)	AML- M2	18% (30%)	*AML1- ETO*
t(15;17) (q21-q11-22)	AML- M3	10% (98%)	*PML- RARα*
t(11;17) (q23;q21)	AML- M3	rare	*PLZF- RARα*
Inv(16) or t(16;16)	AML- M4Eo	8% (~100%)	*CBFβ- MYH11*
t(9;11) (p22;q23)	AML- M4	11% (30%)	*MLL- AF9*
t(6;11) t(10;11) t(11;17) t(11;19) t(4;11)	AML- M5	~ 35% AML	*MLL-AF6/AF6q21* *MLL-AF10;CALM-AF10* *MLL-AF17/AF17q25* *MLL-ENL/ENL/EEN* *MLL-AF4*
t(6;9) (p23;q34)	AML- M1,M2,M4,M5	1%	*DEK- KAN*
t(16;21) (p11;q22)	AML	< 1%	*TLS(FUS)- ERG*
t(16;21) (q24;q22)	t-AML, MDS	< 1%	*AML1- MTG16*
t(3;21)	AML	< 1%	*AML1- EVI1, EAP, MDS1*
t(7;11) (p15;p15)	AML- M2, M4	< 1%	*NUP98- HOX49*
t(1;11) (q23;p15)	AML- M2	< 1%	*NUP98- PMX1*
t(8;16) (p11;p13)	AML- M4, M5	< 1%	*MOZ- CBP*
Inv(8) (p11;q13)	AML- M0, M1, M5	< 1%	*MOZ- TIF2*
t(8;22) (p11;p13)	AML- M5	< 1%	*MOZ- p300*
t(12;22) (p13;q23)	AML- M4, CML	< 1%	*TEL- MN1*
t(5;12) (q33;p12)	CMMol	2-5%	*TEL- PDGFRβ*
t(1;19) (q23;p13)	AML- M7	< 1%	*OTT- MAL*

* The percentage of translocation in AML subtypes. Values in brackets indicate the frequency within the morphological or immunological disease subtype.
** Percentage refers to the frequency of reciprocal translocation chromosome products resulting. AML - acute myelogenous leukemia, t-AML (therapy-related AML) - AML associated with therapy, MDS - myelodysplastic syndrome; CMMoL - chronic myelomonocytic leukemia

Table 1. Common gene fusions caused by chromosomal abnormalities and associated with acute myeloid leukemia.

The WHO suggested characterizing 11q23/*MLL*+ AML within ALL as a separate entity with recurrent cytogenetic translocations in 1999. This hypothesis was supported by microarray analyses, which proved that 11q23/*MLL*+ AML has a unique profile of gene expression and that *MLL*+ leukemic blasts resemble very immature progenitor cells [9]. AML with *MLL* gene alterations is characterized by a high degree of clinical and immunological heterogeneity, resulting in immunophenotype variability. This variability originates in myeloid cells/monocytes [26]. The prognosis of AML is unfavorable and varies depending on the type of translocation and the phenotype and age of the patient. The prognostic effect of 11q23 aberrations may depend on *MLL* partner genes. Many studies have shown that the translocations of t(6;11)(q27;q23) and t(10;11)(p12;q23) are associated with an unfavorable prognosis; however, the t(9;11)(p22;q23) translocation is associated with a significantly longer patient survival rate [27, 28]. However, none of the 11q23 aberrations has a favorable prognosis. When different MLL fusion protein complexes were characterized, a novel cancer mechanism was uncovered. It has been known since 1999 that direct MLL fusion proteins are able to deregulate *HOX* genes, except when reciprocal MLL fusion proteins are present [29-31]. The leukemogenesis concept suggests that all MLL fusion proteins increase and maintain a high level of transcription of *MEIS1* (myeloid ecotropic viral integration site 1 homolog) and *HOXA* (homeobox A cluster) gene family members. The functional importance became the association of MEN1, LEDGF and MYB proteins at the N-terminal location of the MLL fusion [32-35]. Changes in the regulation of *HOXA* gene expression influence the function of the hematopoietic system during its development and therefore contribute to the initiation of leukemogenesis. *HOXA* genes are not deactivated, but a high expression of the *MEIS1* gene was observed [36]. Stam et al. [37] described low *HOXA* gene expression in pediatric leukemia patients with chromosomal translocation t(4;11), which is associated with a worse prognosis. Stumpel et al. [38] studied the methylation of promoter regions in samples with t(4;11), t(11;19) and t(9;11). His data indicated that different epigenetic mechanisms accompany the development of leukemia. Recent studies identified two mechanisms for leukemia development. One is the changing of epigenetic imprints, initiated by the presence of *MLL-MLLT3*, *MLL-MLLT10*, *MLL-MLLT1* or the reciprocal *AFF1-MLL* fusion protein through activation of P-TEFb kinase. The second function of the *MLL–AFF1 (*ALL1-*fused gene from chromosome 4 (*AF4*))* fusion protein is the ability to block apoptosis and to transcriptionally activate *HOXA* genes and *TERT* (telomerase reverse transcriptase) [39]. There is evidence that for all MLL-rearranged leukemias, this is the typical activation pattern of *HOXA* and *MEIS1* genes. Faber et al. [40] documented that overexpression of *HOXA9* (homeobox A9) in complex with *MEIS1* is able to drive the myeloid phenotype in mice.

3. Distribution of *MLL* gene alterations

AML with *MLL* gene alterations (11q23/*MLL*+AML) represents 3-4% of all AML cases and occurs most frequently in young people with *"de novo"* AML (5-7%) and in treatment-induced AML (t-AML) patients (10-15%). It is rarely seen in older patients (aged 60 and up) [5]. AML with *MLL* gene conversions occurs more frequently in infants than in adults and is

usually manifested through the AML M4/M5 phenotype [41] Overall, the incidence of *MLL* gene conversions in children with AML varies within the range of 35-50% [42]. The percentages of representation are slightly different between individual studies because the sensitivity of 11q23/ *MLL*+ AML detection depends on the method of testing. The *MLL* gene is also a relatively frequent target of cryptic alterations, which were not always identified in the past using conventional karyotyping [8]. According to a report by Marschalek, more than 70 different fusion partner genes have been characterized at the molecular level. The analyzed *MLL* fusion alleles were classified according to their occurrence in ALL and AML patients. Of all *MLL* rearrangements, 80% are caused by *AF4* (80%), *AF9* (16%), *ENL* (11%), *AF10* (7%) and *ELL* (4%). The remaining 20% of MLL-rearranged leukemia patients displayed 59 different fusion partners, most of which were identified in single patients [8]. Approximately 50% of pediatric AML cases with an MLL consist of t(9,11)(p22,q23). The other 50% primarily include t(6,11)(q27,q23), t(10,11)(p12,q23), t(11,19)(q23,p13.1) and t(1,11)(q21,q23) [43]. This distribution is almost identical with adult AML, with the exception of t(6,11)(q27,q23), which has a greater distribution in adult *MLL*-rearranged AML [5]. Although *MLL* rearrangements are predominantly found in AML, they are also detected in 6% of pediatric ALL cases.

4. Conversion mechanism from an *MLL* proto-oncogene to an oncogene

Extensive cytogenetic and molecular studies have revealed that 11q23/MLL is a highly promiscuous locus. Based on the results of research from the past 19 years, 71 different *MLL* translocation partner genes and their specific breakpoint regions have been characterized (published and unpublished data [8]). Of these, 43 (60.5%) are reciprocal chromosomal translocations, 4 (5.7%) are 11q23ter deletions and 8 (11.3%) are 11q inversions. In 13 (18.3%) *MLL* fusion partners, more than two DNA strand breaks and the insertion of 11q23 material into another chromosome were identified. A very rare situation of three different *MLL* fusion partners has been described in 4.2% of all cases [44]. The *MLL* "recombinome" currently consists of 104 different areas of fusion [45]. The question remains: How many of them do we not yet know?

The *MLL* proto-oncogene can be transformed into an oncogene via several mechanisms, such as:

- chromosomal translocations
- complex chromosomal alterations, such as deletions, inversions in the area of 11q, MLL gene insertions into other chromosomes or the insertion of chromatin material into the MLL gene
- partial tandem duplications
- amplifications and gains

A. Translocations

The *MLL* gene is frequently involved in chromosomal translocations with other genes, leading to a break within the *MLL* locus and a partner gene, resulting in the emergence of a

new fusion gene. *MLL* fusion proteins (the products of fusion genes) are often associated with the development of acute myelogenous types of leukemia, and their oncogenic characteristics have been extensively studied in vitro and *in vivo* in mouse models [28]. The *MLL* gene represents more complex rearrangements, with at least three or more DNA double-strand breaks. The reciprocal *MLL* fusion is cryptic in these cases; an *MLL* fusion gene cannot be detected. Complex rearrangements can be divided into three subgroups. The first group represents the participation of three independent chromosomes in complex translocation and results in three different fusion genes [45]. The most frequently fused genes are *AFF1/AF4*, *MLLT3/AF9*, *MLLT1/ENL*, *MLLT1/AF1Q* and *ELL*. The second group is built from reciprocal chromosomal translocations containing deletions on either of the involved chromosomes. The third group consists of chromosomal fragment insertions. In this type, a portion of chromosome 11 (including part of the *MLL* gene) is inserted into another chromosome. Translocations with transcription oriented toward the centromere belong to this subgroup. In these cases, three independent fusion genes are generated. There is also spliced fusion, generated by fusing the 5′- location of the *MLL* gene to the upstream region of a TPG (translocation partner gene). Approximately 50% of all recombination events are spliced fusions [44]. The final group of 3′*MLL* fusion represents head-to-head fusion. The transcriptional orientation of the fused transcriptional genes is opposite of the orientation of the *MLL* gene. This genetic situation often results in LOH. All known translocation participating genes (TPGs) were classified according to their cellular function. They can build membrane proteins and nuclear proteins. As membrane proteins, these TPGs function as extracellular proteins, cell adhesion proteins with functions in the organization of focal adhesion plaques, endocytotic proteins (EPS15 (epidermal growth factor receptor substrate 15) and PICALM (phosphatidylinositol binding clathrin assembly *protein)* proteins)), proteins involved in diverse signaling pathways (AF6 (actin-filament-binding*)*, ABI1(abl-interactor 1) , GPHN (gephyrin), KIAA0284 (centrosomal protein 170kDa) and MYO1F (myosin IF) proteins), the organization and regulation of the cytoskeleton, metabolic functions and pre-apoptotic proteins (MLLT11/AF1Q (myeloid/lymphoid or mixed-lineage leukemia fused to ALL1 fused gene from chromosome 1q) protein). As nuclear proteins, they can control the cell cycle and take part in the organization of the nuclear cytoskeleton during cytokinesis (SEPTINS (cytoskeletal GTP-binding) protein), nucleic acid binding (TNRC18- trinucleotide repeat containing 18), chromosome association (CASC5- cancer susceptibility candidate 5), chromatin regulation (CREBBP- CREB binding *protein*), transcription factors (AF17 (*ALL1*-fused gene from chromosome 17), FOXO3 forkhead box O3)., FOXO4 forkhead box O3 , FRYL (furry homolog-like), MAML2 (mastermind-like 2) and TET1 (tet methylcytosine dioxygenase 1)) and regulation factors. Recurrence of *MLL* rearrangements was observed in approximately 44% of all TPGs. The most frequent translocations within *11q23/MLL+ AML* are illustrated in Figure 3 and Figure 4.

B. Partial tandem duplication (PTD) and MLL gene amplification

Approximately 7.5% of AML patients with a normal karyotype are hiding a PTD of the *MLL* gene. An *MLL* gene PTD is uniquely distinguished from other *MLL* gene alterations that result in chimeric gene fusions. In the PTD of the *MLL* gene, all of the protein domains encoded by the *MLL* gene are retained [46]. *MLL* PTD is common in adult AML patients but

not in pediatric AML patients. It has also been identified at a low level in healthy humans [47]. The frequency of *MLL* gene PTD in infants with AML, as well as in older children with AML, is not well established. In adult patients with the *de novo* form of AML and a normal karyotype, the presence of an *MLL* gene PTD versus its absence is associated with poor prognosis (shorter remission time) [48, 38] (Figures 5 and 6).

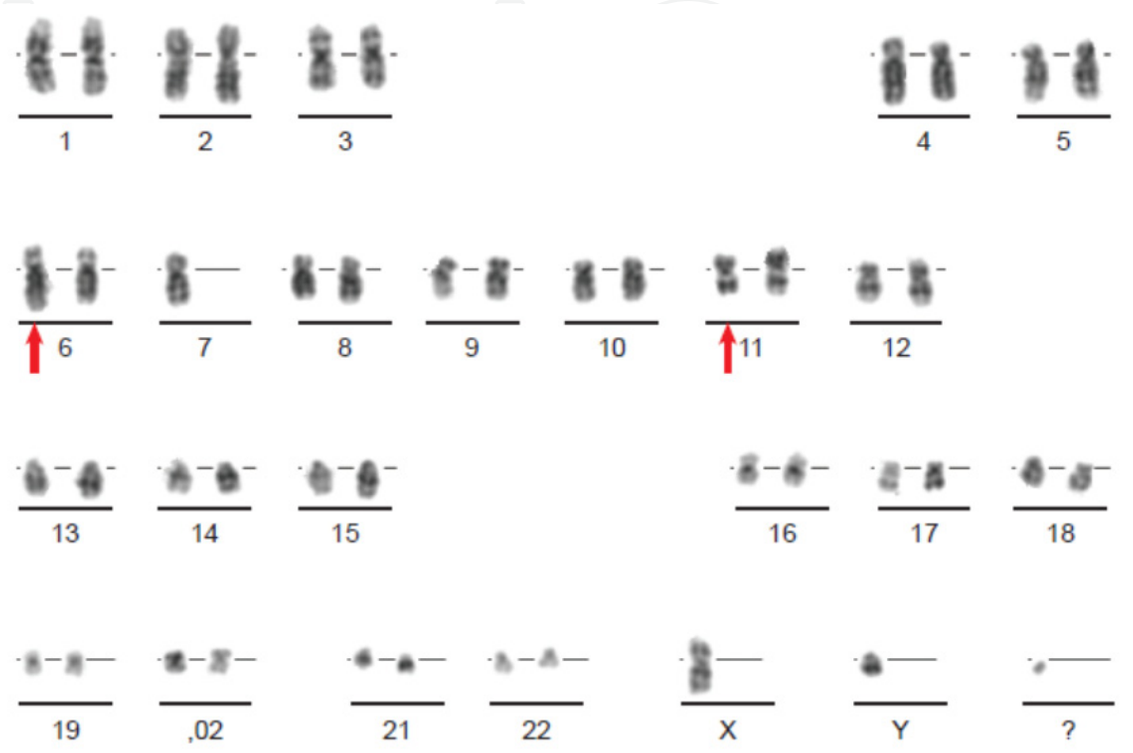

Figure 3. Complex karyotype with translocation *MLL*/*AF6*. Complex karyotype: 45,XY,-4,der(7)t(4;7)(q?12;q?11),+dimin/45,XY,idem,t(6;11)(q27;q23).

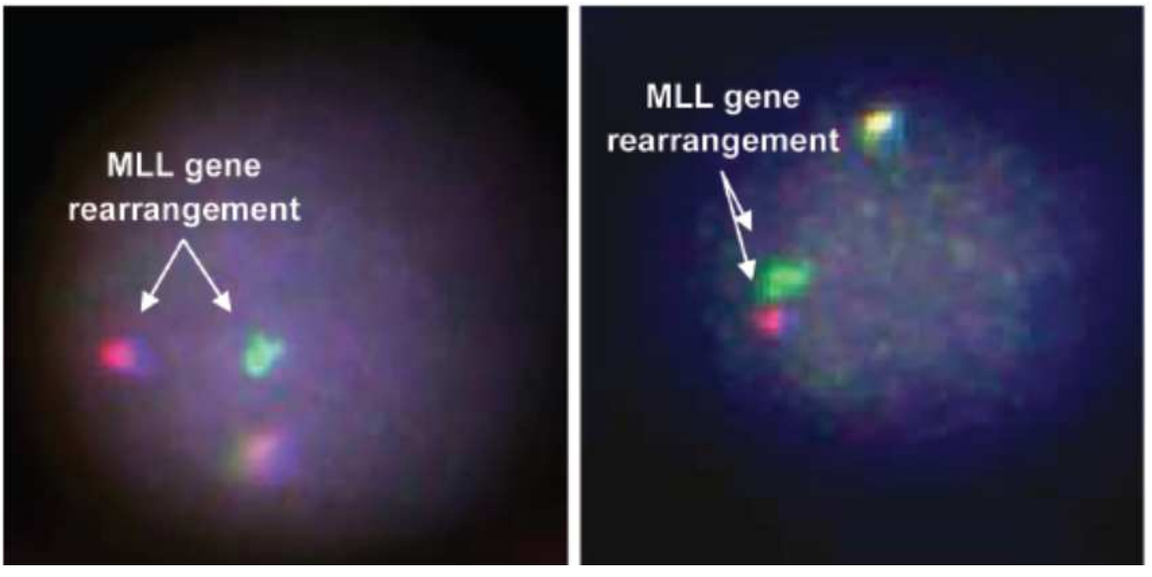

Figure 4. The results of fluorescence analysis of interphase nuclei obtained by taking a photograph with the CCD camera in a fluorescent microscope.

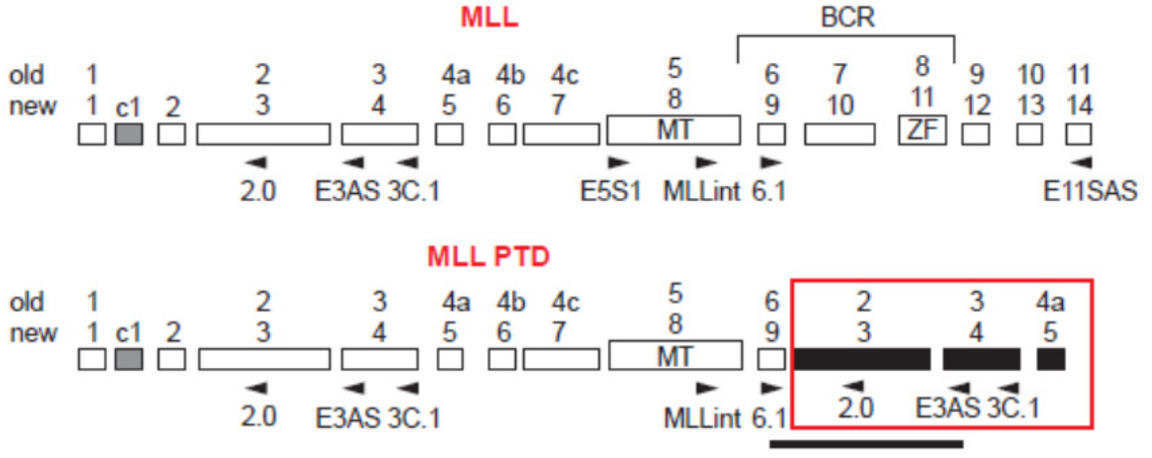

Figure 5. The *MLL* gene and *MLL* PTD. Above: the old nomenclature exon. Below: the new numbering.

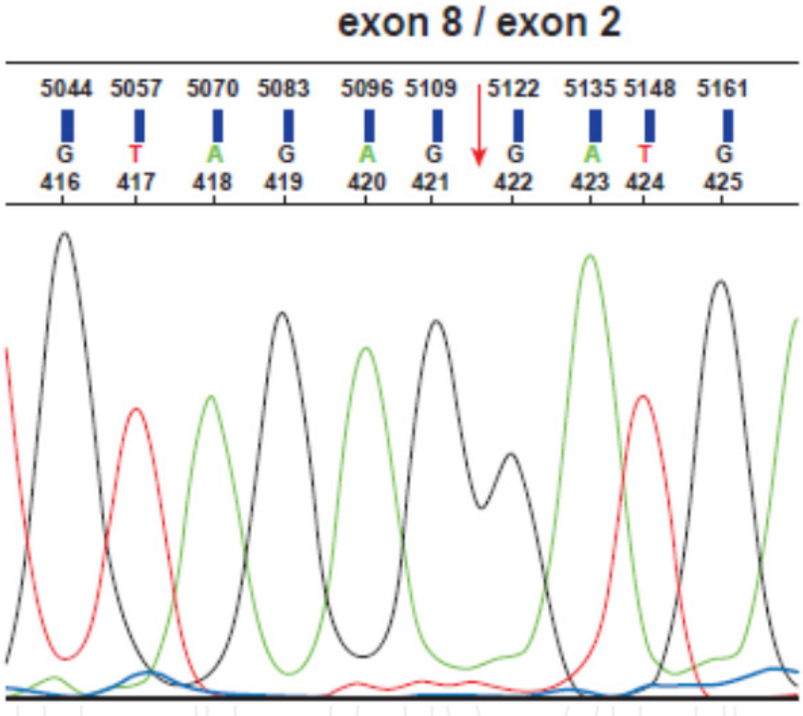

Figure 6. Sequence of *MLL* PTD from sequencing, showing a break in exon8/exon2 (exon3/exon11).

Previous studies have associated an *MLL* gene PTD with AML subtypes M1 and M2. Presently, according to FAB (the most widely used classification of AML, derived from the French-American-British group in 1976), the association of an *MLL* gene PTD with any specific FAB subtype of AML has not been confirmed. On the contrary, *MLL* translocations occur predominantly in the myelomonocytic (M4) and monocyte (M5) AML subtypes [49].

Some AML patients have an increased number of *MLL* gene copies in the form of double minute chromosomes, also called homogeneously staining regions. *MLL* gene amplification may occur through skipping translocations, in which the amplicon of chromosome 11 is integrated into one or more other chromosomes, creating multiple copies of the *MLL* gene.

In general, the amplification of the gene leads to an overgrowth of structurally normal copies of the gene, resulting in overproduction of the oncogene-stimulating protein. These extra proto-oncogene copies increase the amount of the gene product in a cell, thereby inducing unlimited cell proliferation. Gene amplification is usually manifested cytogenetically, either in an intra-chromosomal manner as a homogeneously staining region (HSR – homogeneously staining region) or in an extrachromosomal manner as double minute chromosomes (dmin). Identification of genes in the amplified region allows us to perform fluorescent in situ hybridization (Figure 7).

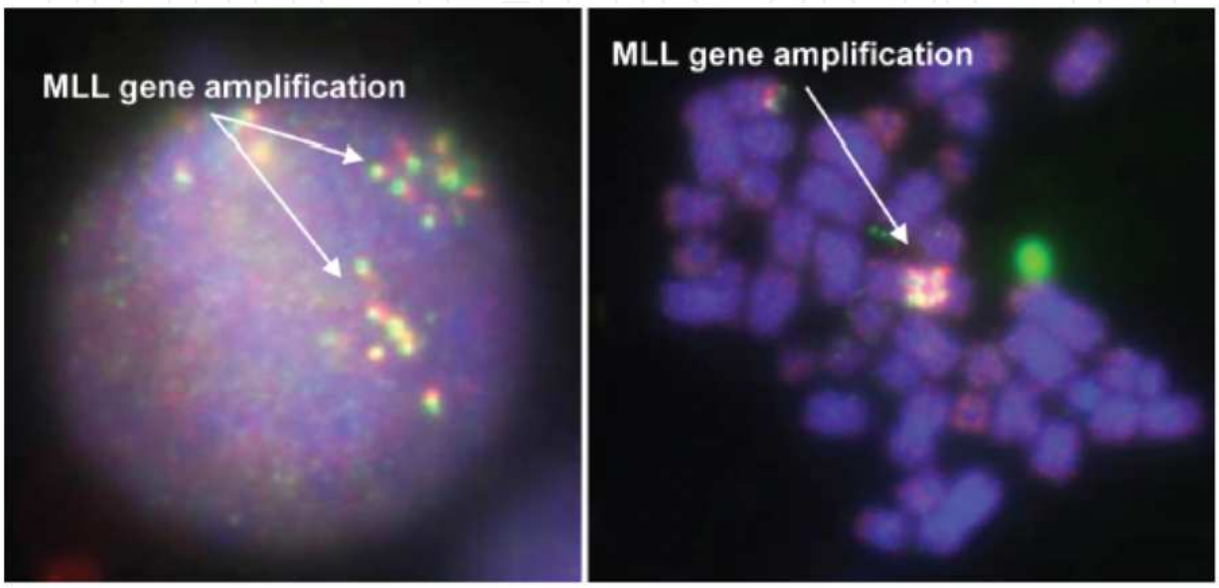

Figure 7. The results obtained from the analysis of a fluorescent CCD camera. Left: interphase nucleus. Right: mitosis. Amplification of MLL (9 copies) in a patient with karyotype 44, XY, -5, hsr (11) (q23), -18, +21, + mar (C).

The amplification of genes, a common occurrence in a wide range of tumors, is rarely observed in acute leukemia. Gene amplification is identified in approximately 1% of patients with AML by conducting a cytogenetic analysis in the form of dmin (the area of the *MLL* gene) [50].

It was found that patients with the *MLL* gene amplification share several common characteristics: they are older than 60 and have a *de novo* form of AML, a complex karyotype, and a short survival rate; 90% of them also have a 5q deletion.

5. Detection methods for *MLL* gene conversions

In diagnostic procedures, methods such as cytogenetic analysis, fluorescent in situ hybridization (FISH), and reverse transcriptase-polymerase chain reaction (RT-PCR) are routinely used for the identification of various regroupings within the *MLL* gene. Genomic molecular methods are also used, such as array comparative genomic hybridization (aCGH). Recently, the spectrum of diagnostics methods was expanded by long-distance inverse PCR (LDI-PCR), which detects rearrangements within the *MLL* gene at the molecular level [45].

Classical cytogenetics is used to determine a completed karyotype picture of the disease and to monitor the progress of the disease. It provides a full overview of qualitative and quantitative karyotype abnormalities and reveals primary and secondary clonal changes. Classical cytogenetics can reveal five most frequent *MLL* rearrangements include t(4;11)(q21;q23), *AFF1(AF4)/MLL*; t(6;11)(q27;q23), *MLLT4(AF6)/MLL*; t(9;11)(p22;q23), *MLLT3(AF9)/MLL*; t(11;19)(q23;p13.1), *MLL/ELL*; and t(11;19)(q23;p13.3), *MLL/MLLT1(ENL)*.Typically, conventional cytogenetics has been used to detect rearrangements involving the *MLL* gene. However, conventional cytogenetics may fail to detect nearly one-third of *MLL* rearrangements; therefore, fluorescence in situ hybridization (FISH) has emerged as the modality of choice for detection of such rearrangements. **Fluorescent in situ hybridization (FISH)** is a method of molecular cytogenetics that enables the detection of specific nucleotide sequences ranging from one to several hundred kilobases. The principle of this method lies in the ability of a single-strand DNA probe to bind with a complementary segment of single-stranded patient DNA. Using specific probes enables us to identify chromosome numbers and to identify specific chromosomal regions (loci). By running FISH on metaphase as well as interphase cells, one of the biggest problems of classical cytogenetic analysis has been overcome. The **LSI® MLL Dual Color, Break Apart Rearrangement Probe** (Vysis) is used for the detection of alterations, amplifications and deletions within the *MLL* gene.

Reverse transcriptase PCR (RT-PCR) is particularly useful if the internal organization of exons and introns within the gene is not known. The first step is isolating the mRNA of the respective gene from the tissue. Using a reverse transcriptase enzyme and an oligo dT primer, complementary DNA (cDNA) is created from an mRNA molecule, which then serves as a template for PCR. Using the appropriate primers allows for further amplification of a specific cDNA sequence. The resulting product is then visualized on an agarose gel**.**

Multiplexed reverse transcription PCR (MRT-PCR) Anderson et al., 2001, developed this quick and accurate method to identify the six most common *MLL* gene translocations: *MLL/AF4* (acute lymphoblastic leukemia), *MLL/AF6, MLL/AF9, MLL/AF10, MLL/ENL*, and *MLL/ELL*. MRT-PCR is based on two individual steps. The first step uses a mix of external (out) primers, and the second step uses a mix of internal (in) primers, which allows for the detection of six fusion genes in two multiplex PCR reactions. Thus, each sample is tested for the presence of the fusion gene twice. Primers are designed so that in the first step, there is a significantly greater amount of the product formed than in the second step, which increases the specificity of this method. If necessary, the MRT-PCR analysis can be extended by investigating other fusion genes [51].

5.1. Long-distance inverse PCR (LDI-PCR)

The different *MLL* translocation partner genes are identified by cytogenetic analyses, and only the most common *MLL* translocations are investigated by RT-PCR or by MRT-PCR analyses. However, the infrequent or unknown *MLL* translocations were excluded from further analyses. Therefore, it was a goal to establish a universal method that enables the

detection of *MLL* rearrangements with genomic DNA. Mayer et al. [52] designed a universal long-distance inverse-PCR approach for clinical use and verified it as a very suitable method for the identification of known and unknown translocation partner genes (TPGs) and the establishment of patient-specific *MLL* fusion sequences (Figure 8).

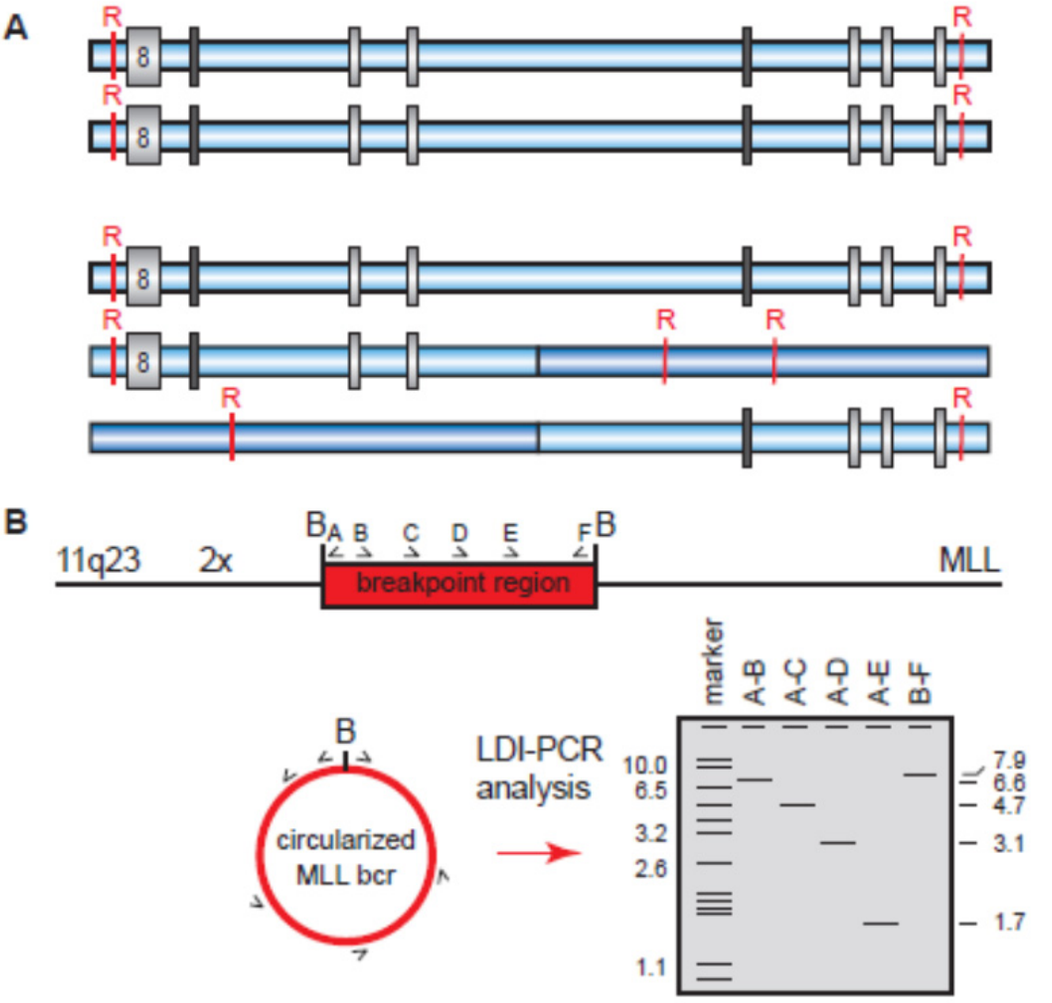

Figure 8. Principles of the LDI-PCR method. **A**. The schema summarizes how the genomic DNA is first restricted using distinct combinations of restriction enzymes (R: restriction site), then re-ligated to form two DNA circles that can be amplified with a specific set of oligonucleotides (A-B, A-C, A-D, and A-E). The primer combination B-F serves as internal control. **B.** *Bam*HI restriction recognition site. Translocation-bearing cells yield both wild-type and derivative templates, differing in size and detectible on the gel. PCR amplimers can be analyzed by sequence analysis using oligonucleotides.

LDI-PCR allows for the identification of a new class of *MLL* recombinations and for the discovery of new fusion genes, providing new insight into the origination mechanisms of *MLL* rearrangements. In this method, 1 μg of genomic DNA from the patient is isolated and digested with the restriction enzyme *BamHI.* The residual enzymatic activity is removed by phenol extraction and ethanol precipitation. After digestion, the DNA samples are re-ligated to form DNA circles (at 16ºC overnight in the presence of T4 DNA ligase). All ligation reactions are terminated at 65ºC for 10 minutes. *MLL* gene-specific oligonucleotides are

designed according to GenBank accession no. AJ235379 DNA sequences. For digestion and re-ligation of DNA, the five oligonucleotides (A-E) are used in four combinations (A-B, A-C, A-D and A-E). A positive control containing the oligonucleotides B and F is included in each analysis to amplify a 7.9 kb DNA fragment of the *MLL* breakpoint cluster region. All LDI-PCR reactions are performed using the TripleMaster PCR system. PCR amplimers are separated on 0.8% agarose gels. Non-germline PCR amplimers are isolated from the gel and subjected to DNA sequence analyses to obtain the patient-specific fusion sequences. Annotation of fused *MLL* sequences is carried out by blasting the human genome database (Genomic BLAST, www.ncbi.nlm.nih.gov/genome/seq/Blast). The presence of a rearranged *MLL* allele can be identified by digestion and re-ligation of the two *MLL* alleles. Three different DNA circles are formed (der(11) and der(TP), TP presets translocation partner) that can be amplified by the designated primer combinations A-L. The DNA sequences of oligonucleotides A-L are available at www.biozentrum.unifrankfurt.de/PharmBiol/ Mitarbeiter/Marschalek/ download.html. In some cases, is necessary to analyze the cDNA to validate an *MLL* spliced fusion or to investigate alternative splice products from an *MLL* fusion gene. Because these identified fusion gene sequences are patient-specific and exist in only one copy per leukemic cell, they can be used as reliable markers for minimal residual disease studies and for minimal residual disease monitoring by quantitative PCR techniques.

Analyses of novel identified *MLL* fusion genes provide a rich source for future analyses of oncogenic MLL protein variants. These MRD markers contribute to stratification and improved treatment and outcomes of leukemia patients.

6. Outcomes of these methods

Translocations of the Mixed Lineage Leukemia (MLL) gene at 11q23 are found in both acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML). The *MLL* gene contains an 8 kb breakpoint cluster region in which virtually all rearrangements occur. To date, more than 70 different fusion partners have been identified, although some of them have been observed only as a single case. The majority of *MLL* gene rearrangements are associated with infant ALL. Acute lymphoblastic leukemia (ALL) diagnosed within the first 12 months of life accounts for 2.5% to 5% of pediatric ALL cases and displays unique biologic, clinical, and prognostic features that are different from those of older children with ALL. Approximately 80% of infant cases harbor rearrangements of the *MLL* (chromosome band 11q23) [53]. Infants with ALL are treated with an intensive regimen of ALL- and AML-like chemotherapy, with the proportion of *MLL*-rearranged cases being responsible for the poor outcome in this age group [54].In contrast, in 75% of the *MLL*-rearranged pediatric AML cases, 4 partners are involved: *AF9*/*MLLT3* on chromosome 9p21, *AF10*/*MLLT10* on 10p12, *ELL* on 19p13.1, and *AF6*/*MLLT4* on 6q27 [55]. In infant AML, *AF9* and *AF10* are among the most frequent *MLL* fusion partners [9]. New translocation partners are still being reported, adding to the diversity of *MLL*-rearranged leukemia. Recently, *ABI2* on chromosome 2q33.2 has been identified as a new *MLL* translocation partner in an infant with

AML-M5 leukemia refractory to standard induction chemotherapy. This important c-Abl regulator is a functional homologue of *ABI1*, a recurrent *MLL*-translocation partner located on chromosome 10p11.2, and is implicated as a tumor suppressor by its inhibitory function in c-Abl signaling [56, 57].

Rearrangements of the *MLL* gene are found in most cases of infant AML and, regardless of age, confer an intermediate risk. The treatment of *MLL*-rearranged ALL in children includes intensified chemotherapy. *MLL*-rearranged AML is a heterogeneous disease in both biology and outcome. In addition to translocation partners, other variables such as hyperleucocytosis, age (older than 10), additional cytogenetic aberrations and early response to treatment have prognostic relevance and are independent prognostic factors. In general, certain *MLL* rearrangements are associated with poor outcomes in pediatric and adult acute myeloid leukemia. However, patients with *MLL*-rearranged AML have intermediate outcomes when treated with the optimized treatment regimens, with a 5-year event-free survival probability (5y-EFS) ranging from 32-54%. Currently, hematopoietic stem cell transplantation is no longer advised during the first remission for favorable MLL rearrangements. Recently, there have been new prognostic subgroups identified within 11q23. A favorable example is t(1,11)(q21,q23). It has an excellent clinical outcome (5y-EFS of 92% and 5y-OS of 100%) [58]. In contrast, subgroups t(10,11)(p12,q23) and t(6,11)(q27,q23) have poor prognoses, with 5y-EFS rates of 31 and 11%, and 5y-OS rates of 45 and 22% [12]. Adults with t(6,11)(q27,q23) also have poor outcomes [59]. Within the most common subgroup t(9,11)(p22,q23), the prognosis appears to be related to morphology, as a group with acute monoblastic leukemia (AML FAB M5) had a significantly better outcome than groups with other FAB subtypes [12].

As with other types of leukemia, the cause of MLL-rearranged AML is unknown. The pathogenesis of AML requires both type-I and type-II mutations. *MLL* rearrangements belong to type-II mutations [60] and lead to the impaired differentiation of hematopoietic cells. Type-I mutations mainly reflect molecular mutation hotspots in specific genes (*FLT3*, *KIT*, *NRAS*, *KRAS*, *PTPN11*), which are involved in the proliferation of hematopoietic cells [12]. Although *MLL*-rearranged AML harbored one of the lowest frequencies of type-I aberrations (43%), mutations in the RAS-signaling pathway interestingly represented the vast majority in *MLL*-rearranged AML. Cases are routinely screened for *MLL* rearrangements by conventional cytogenetics and FISH; however, these techniques do not guarantee 100% sensitivity. Thus, GEP (gene expression profiling) and LDI-PCR (long-distance inverse PCR) could be used to identify cases not detected with FISH, although these techniques are currently used only in research settings. As the outcome of AML is also dependent on translocation partners, Balgobind et al. [12] suggest that for the next risk group stratification, all *MLL*-rearranged cases should be screened for the favorable prognostic subgroups t(1,11)(q21,q23) and t(9,11)(p22,q23) with FAB5 and the poor prognostic subgroups t(10,11)(p12,q23) and t(6,11)(q27,q23). It can be assumed that a systematic analysis of the *MLL* recombinome will allow conclusions on certain aspects of the mechanisms of leukemogenesis to be drawn by identifying the MLL fusion proteins. This points to the translocation partner as having a role in the disease phenotype and functional heterogeneity of MLL fusions, but the molecular details of these associations are unclear.

The monitoring of MRD by RT-PCR detection of leukemia-specific targets (e.g., gene fusions, gene mutations, overexpressed genes) or by multi-parameter flow cytometry identifying leukemia-associated aberrant phenotypes remains an active field of investigation. Despite technical developments, there is still a paucity of large prospective trials demonstrating its clinical utility, except for APL (acute promyelocytic leukemia). Potentially useful applications of MRD monitoring include early assessment of response to therapy to improve risk stratification and guide post-remission therapy and post-treatment monitoring to detect impending relapse and to guide preemptive therapy. Real-time quantitative (RQ)–PCR assays have been developed for other fusion gene targets such as MLLT3-MLL and DEK-NUP214, but the data are very scarce due to the low frequencies of these leukemias [61]. In AML, there is a need for new agents that target specific biological markers with crucial roles in the development of leukemia and that are related to outcome. Benefits from specific treatments have been shown for specific AML FAB 3 - APL with ATRA and for CML and Ph+ ALL, imatinib mesylate.

There are several recently developed agents that may target the *MLL* complex or downstream targets, such as *FLT3*, tyrosine kinase, which is highly expressed in MLL-rearranged AML. FLT3 inhibitors such as PKC412 showed potential in phase I/II trials of adult AML. Other targets include Glycogen synthase kinase 3–*GSK3* inhibitors, *RAS* pathways, and inhibitors of *MEK*. Some of these new agents likely will not fully block leukemic transformation, but may have an additive effect with current treatment strategies by targeting the proliferative advantages of these leukemic cells [62]. Another possibility is to directly target the *MLL* complex or proteins recruited by the *MLL* complex. However, further safety studies are warranted because genetic disruptions in mice resulted in embryonic lethality [63]. Another possibility could be downstream targets of *MLL*-rearranged AML, such as the upregulation of *HOX* genes. Recent studies suggest that MLL-rearranged leukemias are largely driven by epigenetic dysregulation. Several epigenetic regulators that modify DNA or histones have been implicated in *MLL*-fusion driven leukemogenesis, including DNA methylation, histone acetylation, and histone methylation. The histone methyltransferase DOT1L has emerged as an important mediator of MLL-fusion–mediated leukemic transformation. The clinical development of targeted inhibitors of these epigenetic regulators may therefore hold promise for the treatment of MLL-rearranged leukemia [64].

7. Conclusion

Acute myeloid leukemia is a heterogeneous group of leukemias that result from the clonal transformation of hematopoietic precursors through the acquisition of many chromosomal rearrangements and multiple gene mutations. The cytogenetic aberrations are commonly used as diagnostic and prognostic markers for specific subgroups; in addition, they also have important impacts on achieving complete remission, risk of relapse and overall survival of patients.

Among these aberrations is a subgroup of *MLL* aberrations that have a heterogeneous impact on prognosis, predicting good, intermediate or poor outcomes. This outcome is

dependent on different factors, such as translocation partner, age, WBC, and additional cytogenetic aberrations. For this reason, identifying the translocation partner with the methods discussed above has a crucial significance for the stratification of clinical risk groups, tailoring the intensity of treatment strategy and the overall outcome. Cases with favorable prognosis (t(1,11)(q21,q23)) may benefit from less intensive treatment, and cases with poor prognosis (t(6,11)(q27,q23) and t(10,11)(p21,q23)) need adjustment and alternative treatment approaches to improve outcome. It is commonly accepted that the AML phenotype results from multiple genetic/epigenetic lesions affecting differentiation, proliferation, and apoptosis. Distinguishing a particular gene signature for *MLL*-rearranged leukemias sheds light on the molecular mechanisms and potential therapeutic targets of these leukemias. It may also prove to have a useful role in both diagnosis and prognosis. Further investigation into the genetic aberrations of AML cells may provide the knowledge needed to develop new compounds directed against leukemia-specific targets. Consequently, the targeting of a single aberrant protein is unlikely to eradicate the leukemic clone. Although several molecularly-targeted therapies have been shown to be active in AML, it is clear from early clinical studies that most of these novel agents will need to be used in combination with conventional cytotoxic therapy.

However, although subgroup-directed and rationally targeted therapy offers possibilities for the improved care of patients with AML, it will also have implications for the design of clinical trials. In the long term, this may require large randomized trials with international subgroup-specific protocols.

The relationship of outcome with specific translocation partners requires that partners be searched for in the diagnostic work-up of AML and followed-up during treatment. However, to achieve further improvements in survival, unraveling the biology of AML is warranted.

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