Contents lists available at Science-Gate

International Journal of Advanced and Applied Sciences

Journal homepage: http://www.science-gate.com/IJAAS.html

Minimal residual disease in acute leukemia based on the insight of molecular genetics monitoring

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ARTICLE INFO

Article history:
Received 16 November 2022
Received in revised form
10 March 2023
Accepted 14 March 2023

Keywords:
Minimal residual disease
Acute lymphoblastic leukemia
Acute myeloid leukemia
Polymerase chain reaction
Bone marrow

ABSTRACT

Patients with acute leukemia port 10 malignant cells at presentation. Following chemotherapy or stem cell transplant, patients in complete remission by conventional analyses may still harbor 106/108 malignant cells below the detection limit of standard clinical assessment. Minimal residual disease (MRD) monitoring is one of the most powerful predictors of disease-free and overall survival, particularly for children with acute lymphoblastic leukemia (cALL), the percent annual of cALL increase in the incidence of cALL in Saudi Arabia. Breakpoint fusion regions of chromosomal aberrations can be used as tumor-specific targets for MRD detection by polymerase chain reaction. Levels of MRD, measured at critical time points, significantly correlate with clinical outcomes. Previous works investigated the prognostic significance of leukemia-associated immunophenotypes (LAIPs) as an assessment of the index of MRD in 125 adult B-ALL patients by eight-colour flow cytometry. More advanced molecular and genetics studies are so necessary to identify the mechanisms and cellular structure of the minimal-level disease. Selecting molecular methods for minimal residual disease detection have a much higher sensitivity and precision (100-fold or more) than others. This review highlights the minimal residual disease molecular detection to demonstrate the characterization of the lymphoblastic leukemia gene. Precise MRD monitoring predicts disease relapse after chemotherapy or SCT, provides early intervention, and may result in *the rescue* of many patients and improvement in the probability of long-term disease-free survival.

1. Introduction

Patients with acute leukemia port 1012 malignant cells at presentation. Using chemotherapy has effects on destroying the cancer cells of most children and adults below the age of 65, therefore, they achieve complete clinical remission (CCR) following the first course of induction therapy. However, patients in CCR still harbor as many as 1010 malignant cells in the marrow, responsible for relapse. Thus, several studies have aimed to find out the correlation between the clonal composition and evolution of leukemic cell populations during chemotherapy treatment, and genetic, epigenetic, and gene expression changes associated with relapse (Foroni et al., 2005; Al-Mawali et al., 2009; Hackl et al., 2017). Hackl et al. (2017) mentioned the expression of some protein-coding and microRNA genes was reported to change between diagnosis and relapse in a statistically significant manner.

Minimal residual disease (MRD) in acute lymphoblastic leukemia (ALL) refers to the post-therapeutic of leukemia cells of the bone marrow and more rarely in peripheral blood circulation. MRD cells can be the remnants of pretreatment originator ALL cells or can be transformed into secondary ALL. Transformed secondary ALL cells are distinguished from pretreatment originator ALL cells by the rearrangement patterns, identifiable immunoglobulin (Ig), and T-cell receptor (TCR) gene variations (Szczepański et al., 2001a; Rosenberg et al., 2017). Demonstrating the molecular and genetic changes of MRD can be helpful for treatment response and the risk of leukemia relapse. Also, MRD levels are used to modify the intensity and duration of chemotherapy based on measured clearance of leukemic cells and post-treatment probability of disease (Kruse et al., 2020). MRD levels can be detected in CCR by using specific, sensitive,

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reproducible, and quantitative advanced techniques (van der Velden et al., 2003). Several molecular approaches have recently developed. Among these, polymerase chain reaction (PCR) enables accurate assessment of the number of leukemic cells. Real-time PCR technology can replace the complex and time-consuming standard PCR. Most molecular diagnostics are based on PCR technology, allowing the detection of small amounts of the genetic material of patient samples (Barragan et al., 2001; Buonamici et al., 2002; Willemse et al., 2002; Gotham et al., 2021). Acute lymphoblastic leukemia (ALL) is classified into genetic subtypes due to structural chromosomal aberrations which can cause expressed fusion genes with an increase of white blood cell count at diagnosis and minimal residual. Gene changes of ALL are detected by karyotyping (G-banding) by using fluorescent in situ hybridization (FISH), and/or PCR amplification (Iacobucci and Mullighan, 2017). All are more common cancer in children than in adults, this accounts for more than 80% percent of all cases of childhood leukemia. Childhood ALL originates in the T and B lymphoblasts in the bone marrow cells (Figs. 1 and 2). There is a trend towards higher complete clinical remission rates of 80-90% and leukemia-free survival (LFS) cell rates of 30-40% (Hoelzer and Gökbuget, 2000b). From above, it is valuable to highlight the molecular detection and gene alterations of MRD in acute leukemia using accurate technical advances compared to immunophenotyping (Table 1).

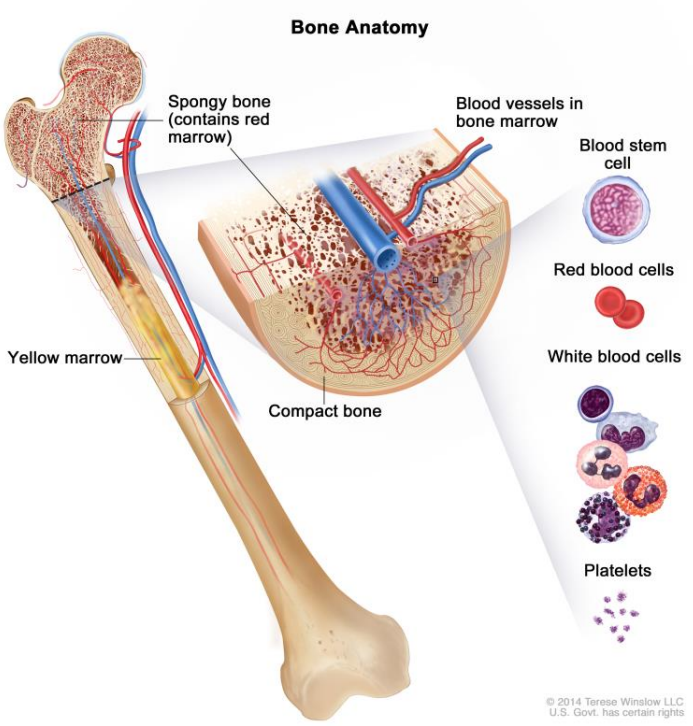

Fig. 1: Bone anatomy shows types of bone marrow and their components: Red marrow and yellow marrow and blood vessels. (Board, 2021)

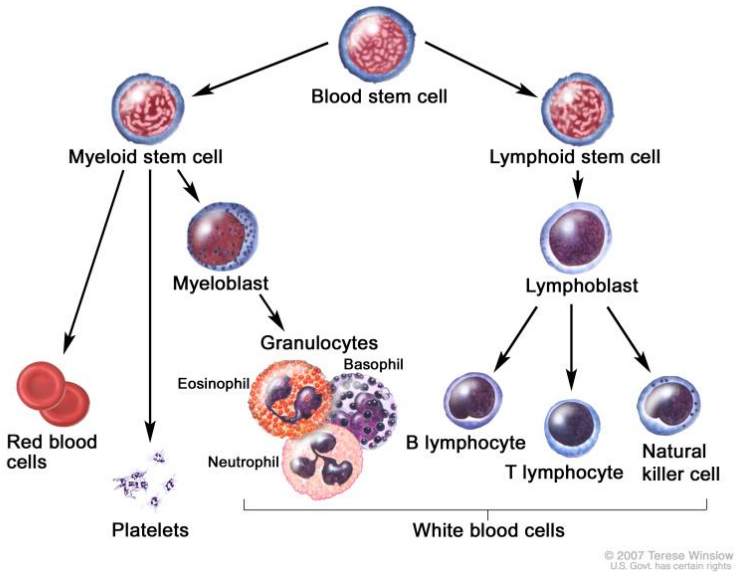

Fig. 2: A blood stem cell development to become red blood cells, platelets, and white blood cells (Board, 2021)

Table 1: Sensitivity of methods for MRD detection

Methods	Sensitivity (%)	Features
Standard morphology	1 – 5	Low sensitivity
Cytogenetics	5	Labor-intensive, slow, requires metaphase chromosome preparation.
FISH[a]	0.3 – 5	Labor-intensive, interphase FISH obviates the need for high-quality metaphases.
Immunophenotyping	10^{-4}	Lack specificity.
PCR[b]	10^{-4} – 10^{-6}	Sequence information required, false positive results
Southern blotting	1 – 5	
PCR[b]	10^{-4} – 10^{-6}	

a: FISH: Fluorescence in situ hybridization; b: PCR: polymerase chain reaction

2. Genetics of relapse

The clonal complexity of ALL dynamics during therapy and at relapse has been assessed by genomic sequencing and single-cell analysis (Anderson et al., 2011; De Bie et al., 2018). Alterations of pathway signaling lesions (FLT3, KRAS, NRAS) are sub-clonal and they have been lost or gained between diagnosis and relapse (Ma et al., 2015).

Dobson et al. (2020) mentioned that minor relapse-initiating subclones can exhibit inherent resistance to chemotherapy, even before secondary mutation acquisition. Further relapse-specific mutations in PRPS1, PRSP2, NT5C2, or MSH6, each influencing thiopurine metabolism, may emerge only during therapy, being motivated by selective therapeutic pressure (Meyer et al., 2013; Li et al., 2015; 2020; Waanders et al., 2020) (Fig. 3). Monitoring the dynamics of mutation clearance through therapy or monitoring relapse-associated mutations might identify patients who will benefit from early modification of therapy (Inaba and Mullighan, 2020).

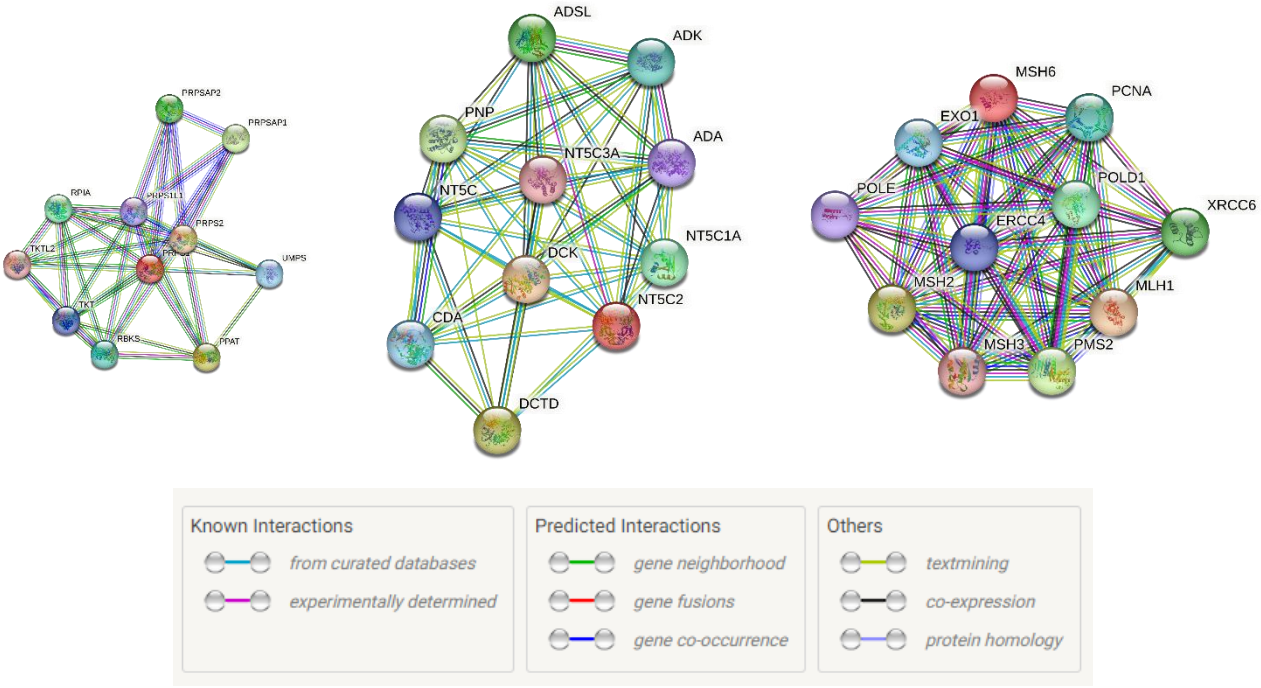

Fig. 3: Protein-protein network interactions contained the *PRPS1, PRSP2, NT5C2,* and *MSH6* genes created with STRING (https://string-db.org/), in which there are strong interactions between the *PRPS1, PRSP2,* and *NT5C2, MSH6* genes. On the Left side: More extended genes containing *PRPS1* and *PRSP2* gene families are shown

2.1. Molecular monitoring of MRD

MRD monitoring can be attained using advanced technologies, such as PCR, next-generation sequencing (NGS), microarray chip analyses, multiplex ligation probe amplification, digital PCR, and pyrosequencing (Roloff et al., 2017).

2.1.1. PCR-based MRD detection

Qualitative end-point RT-PCR and semi-quantitative PCR can be used for MRD monitoring, but recently, these methods have largely been swapped for quantitative techniques that allow the estimation of disease levels. Quantitative MRD can be done with real-time quantitative PCR (RQ-PCR) analysis of immunoglobulin and T-cell receptor gene rearrangements, breakpoint fusion regions of chromosome aberrations, fusion-gene transcripts, or aberrantly expressed genes; their application depends on the disease. RQ-PCR can be done using different instruments (e.g., Thermo Fisher, ABI's 7500/7900/ViiA™ Real-Time PCR systems, Roche's LightCycler, Bio-Rad's CFX96™ Real-Time PCR Detection System, Cepheid's SmartCycler and Qiagen's Rotor-Gene), using different fluorescent

chemistries (e.g., DNA binding dyes (Syber green), TaqMan hydrolysis probes, LightCycler dual hybridization probes, Molecular Beacons, Locked Nucleic Acid probes. According to the MRD-PCR target, different types of oligonucleotides can be used for specific detection, such as an allele-specific oligonucleotide (ASO) probe, an ASO forward primer, an ASO reverse primer, or germline probe and primers (van der Velden et al., 2003).

Real-Time monitoring simultaneously with amplification throughout thermocycling to determine the quantity of target nucleic acid before amplification using PCR, i.e., the amplification product detected as it accumulates. The threshold cycle (Ct value) is when the system begins to detect the increase in the signal associated with the exponential growth of the PCR product. Thus, two main molecular targets to identify leukemic cells: clonally rearranged antigen-receptor genes and gene fusions (DNA level) or their resulting aberrant mRNA transcripts.

PCR-based MRD detection using antigen receptor genes

Clonally rearranged antigen receptor genes can be used as tumor-specific targets for MRD detection. All cells of a lymphoid malignancy have a common clonal origin with identically rearranged Ig and/or TCR genes, their junctional regions are considered as unique-like sequences' DNA-fingerprints'. Patient-specific primers are designed complementary to the junctional sequences of the target. Heteroduplex analysis or fluorescent gene scanning can distinguish between leukemia-derived and polyclonal PCR products (Pongers-Willemse et al., 1999). PCR products were sequenced to design the junctional region-specific oligonucleotides (Pott et al., 2019).

Allele-specific oligonucleotide (ASO) is one of the most advanced techniques. ASO designed from the CD3 region (unique recombination product) increases the sensitivity of MRD detection up to 1: 104 or 1: 105 (Foroni et al., 2005). Detection of clonal Ig/TCR gene rearrangements without the need for patient-specific oligonucleotides relied on high-resolution electrophoreses, such as radioactive fingerprinting or fluorescent gene scanning; both techniques provide lower sensitivity up to 1: 103, and date interpretation may be difficult (Campana, 2004). More than 95% of childhood B-lineage ALL cases have immunoglobulin heavy chain (IgH) gene rearrangements, and most of them contain immunoglobulin κ gene rearrangements (30%) or deletions (50%); 20% of B-lineage ALL cases have immunoglobulin λ gene rearrangements. TCR gene rearrangements also occur in B-lineage ALL: TCRβ, TCRγ, and TCRδ gene rearrangements and/or deletions are found in 35%, 60%, and 90% of cases, respectively. More than 95% of childhood cases with T-ALL, TCRβ, TCRγ, and/or TCRδ gene rearrangements can be identified. Immunoglobulin gene rearrangements occur in approximately 20% of T-ALL cases and involve only IgH genes (Beishuizen et al., 1993). Immunoglobulin and TCR gene rearrangements also can be identified in approximately 10% of patients with AML, but because of their low prevalence, they are not suitable for routine MRD studies (Czyz and Nagler, 2019) (Table 2).

Table 2: Frequency of Ig and TCR gene rearrangement in precursor B and T lineage ALL (Foroni et al., 2005)

Gene rearrangement	Precursor B-ALL% Adult childhood		Precursor T-ALL% Adult childhood	
IgH	70-80	90-95	5-10*	5-10*
DH-JH	20		10	
VH-DH-JH	>95		~2	
Igk	40-50		0	
VK-JK	30			
VK-Kde	50			
Igλ	20		0	
TCRβ	10		50	
TCRγ	70		60-70	>90
TCRδ	50**		~70#	

*: Lineage IgH gene rearrangement in T-ALL is mainly incomplete DH-JH rearrangement and occurs frequently in CD3- T-ALL and TCR γδ+T-ALL compared with TCR-αβT-ALL (<5%); **: Predominantly Vδ2-Vδ3 and Dδ2-Dδ3 rearrangement; #: Predominantly Vδ1-Jδ1 complete rearrangement and Dδ2-Dδ1 rearrangement

Chromosome aberrations with breakpoint fusion genes

Breakpoint fusion regions of chromosomal aberrations can be used as tumor-specific targets for MRD detection by PCR. Amplifying these sequences with PCR using DNA as a starting material is used when the breakpoints cluster in relatively small breakpoint areas, e.g., less than 2kb. The development of long-distance PCR allows larger breakpoint fusion genes and fusion genes to become feasible MRD molecular targets. For most translocations, the breakpoints are scattered over much larger segments. In these cases, chimeric messenger RNA and the resulting complementary DNA after reverse transcription (RT) are the preferred targets for PCR analysis (Szczepański et al., 2001b; Campana et al., 2008; Vellichirammal et al., 2021). European standardized protocols for measuring fusion gene transcripts in acute leukemia by RQ-PCR were published in 2003 and are still widely used (Gabert et al., 2003) (Table 3).

Amplicon fusion site-PCR

Weber et al. (2012) reported that; quantification of MRD by Amplicon fusion site-PCR (AFS-PCR) was directly comparable to IgH/TCR-based real-time quantitative PCR and fluorescence-activated cell sorting (FACS) analysis in consecutive bone marrow

specimens. AFS-PCR detects ampGR in the primary tumor or bone marrow specimens, even with low tumor cell content. Designing AFS-PCR must consider all possible orientations of the subsequent ampGR like head-to-tail or head-to-head and tail-to-tail Amplicon fusion site-PCR was a sensitive technique that detected one tumor cell per 106-107 control cells. Although AFS-PCR based on iAMP21 is present in 1-2% of cases of B-ALL. It is applied as an alternative MRD diagnostic when poor or qualitatively IG/TCR is not available.

Table 3: Most used breakpoint fusion genes for PCR for MRD

Disease	Abnormality	Susceptible genes	Frequency
B-ALL	t(9:22)(q34; q11)	BCR- ABL (p190)	Adults: 15-30% Children: 3-5%
	t(12;21)(p13;q22)	ETV6-AML1	Adults: <2% Children: 20-25%
	t(4;11)(q21;q23)	MLL- AF4	Adults: 3-4% Children: 3-5%*
	t(1;19)(q23;p13)	E2A- PBX1	Adults: 3-4% Children: 5-8%
T-ALL	Del(1)(p32 ; q32)	SIL-TAL1	Adults: ~10% Children10-25%
	t(5;14)(q35;q32)	HoxIIIL2 TCRβ	Adults: 13% Children 20%
	t(9:22)(q34;q11)	BCR ABL(p190)	Adults: <1% Children: <%
AML	t(8 ;21)(q22 ; q22)M2	AML1-ETO	Adults: 6-8% Children: 10-14%
	t(5 ;17)(q22 ;q21)M3	PML-RARα	Adults: 5-15% Children: 8-10%
	inv(16)(p13 ;q22)M4E0	CBFβ-MYHII	Adults: 5-6% Children: 5-7%
	t(9 ;22)(q34;q11)M0/1	BCR/ABL	Adults: rare Children: rare
	t(9 ;11)(p22 ;q23)M4 /5a/b t(6 ;9)(p23 ;q34)M1/2/4/7	MLL-AF9 DEK-CAN	

2.1.2. Next-generation sequencing

Wu et al. (2012) reported that next-generation sequencing (NGS) of lymphoid receptor gene repertoire might improve clinical diagnosis and subsequent MRD monitoring of lymphoproliferative disorders. Their studies applied high throughput sequencing (HTS) to the diagnosis of T-ALL/lymphoma using 43 paired patient samples. They found that TCRB and TCRG HTS not only identified clonality at diagnosis in most cases (31 of 43 for *TCRB* and 27 of 43 for *TCRG*) but also detected subsequent MRD. They conclude that HTS of *TCRB* and *TCRG* identified MRD that was not detected by flow cytometry in a subset of cases (25 of 35 HTS compared with 13 of 35, respectively), which highlights the potential of this technology to define lower detection thresholds for MRD that could affect clinical treatment decisions.

2.1.3. Microarray chip-based MRD detection

Microarray chip analysis that allows a genome-wide gene expression analysis offers new opportunities to identify markers for MRD studies. The study compared the gene profile of ALL cells with that of purified normal B-cell progenitors and showed that CD58 overexpressed consistently in ALL cells compared with normal CD19+CD10+ normal B-cell progenitors. These results suggest that comparing the gene profiles of normal and leukemic cells will identify new, widely applicable markers for MRD studies in ALL and AML (Chen et al., 2001; Coustan-Smith et al., 2011; Juárez-Avendaño et al., 2021).

2.2. Flow cytometry-based MRD monitoring

The general prognostic value of cellular MRD counts at the cutoff level of 0.01% MRD cells (10−4): Meaning 1 MRD cell in 10,000 cells out of all bone marrow mononuclear cells within a specimen. The limitation of prognostic at 0.01% is based on the immunohistochemical detection of 3–4-color flow cytometers. The clinical significance of the 0.01% MRD cutoff level refers to a patient has cellular MRD levels ≥0.01% in a bone marrow sample at important measurement time points during therapy, if MRD levels are less than 0.01% means the patient will have a significantly higher risk for leukemia relapse (Szczepański, 2007; Campana, 2010; Short and Jabbour, 2017). Immunophenotypes characteristic of leukemic cells are distinguished from normal cells by flow cytometry (FC).

Currently, multi-marker flow cytometry offers a sensitivity of around 0.01% in almost all patients with ALL (Della Starza et al., 2019). There are three main categories of leukemia-associated immunophenotypes: a) Expression of fusion genes such as BCR-ABL1, ETV6-RUNX1, and TCF3-PBX1. In addition to fusion genes, cooperative mutations exist within the chromosomal structure which is required to alter the progenitor cells to cause the leukemia condition. However, suitable antibodies for reliable flow-cytometric analysis of these proteins are lacking, b) Immunophenotyping of T-lineage ALL cells (T-ALL), which are normally expressed by a

subset of thymocytes, and c) Immunophenotyping of B-lineage ALL cells (B-ALL) (Tables 4 and 5).

Table 4: Genetic classification by prognosis of B-cell Acute Lymphoblastic Leukemia (Kruse et al., 2020)

Good prognosis	Intermediate prognosis	Poor prognosis	Undetermined prognosis
Hyperdiploid karyotypes	t(1;19); TCF3-PBX1	Hypodiploid karyotypes	t(5;14); IL3-IGH*
t(12;21);ETV6-RUNX1 (TEL-AML1)		t(9;22); BCR-ABL	
		Philadelphia-like ALL	
		11q23 MLL rearrangements	

* t(5;14); IL3-IGH is a World Health Organization-classified acute leukemia and prognosis data has not been determined

Table 5: Major marker combination to study MRD in childhood ALL and AML (Foroni et al., 2005)

Cell lineage	Marker combination	Applicability (%)
T-lineage	TDT/CD5/CD3	90-95
	CD34/CD5/CD3	20-25
B-lineage	CD19/CD34/CD10/CD38	40-60
	CD19/CD34/CD10/CD58	40-60
	CD19/CD34/CD10/CD45	40-60
	CD19/CD34/CD10/TDT	40-50
	CD19/CD34/CD10/CD66c	30-40
	CD19/CD34/TdT/IgM	10-20
	CD19/CD34/CD10/CD22	10-15
	CD19/CD34/CD10/CD13	10-15
	CD19/CD34/CD10/CD15	10-15
	CD19/CD34/CD10/NG-2	5-10
AML	CD33/CD34/CD117/CD15	20-40
	CD33/CD34/CD117/CD13	20-40
	CD13/CD33/CD34/CD56	20-30
	CD13/CD33/CD34/CD133	20-30
	CD13/CD33/CD34/CD7	20-30
	CD13/CD33/CD34/CD38	15-20
	CD33/CD34/CD117/HLA-Dr	15-20
	CD13/CD33/CD34/CD15	15-20
	CD33/CD34/CD117/CD11b	10-15
	CD13/CD33/CD34/CD19	5-10

2.3. Literature research of MRD in acute leukemia: An overview

2.3.1. MRD detection in acute lymphoblastic leukemia

Many studies in childhood ALL demonstrated that MRD is an independent prognostic factor and confirmed by many studies in adult ALL. The predictive value of MRD evaluation depends on the technical quality, such as sensitivity, number of targets, and frequency of evaluations (Hoelzer and Gökbuget, 2000a; Sommer et al., 2021) (Table 6).

In Germany, every year, four in every 100,000 children aged up to 15 develop ALL. Almost all patients in Germany were treated according to the valid treatment algorithms developed by the two collaborative study groups ALL-BFM (Berlin–Frankfurt–Münster) and COALL (cooperative study group for childhood acute lymphoblastic leukemia), subdivided into three treatment phases (induction and consolidation of remission, re-intensification, maintenance treatment) and covered two years. Remarkable improvements in the treatment of childhood ALL "in the past four decades" (Möricke et al., 2010); compared with the late 1960s, when 30% of cases were cured, nowadays, 80% of patients remain in their first remission even after 10 years, the remaining 20% of children have recurrences, and the cure rate falls to 25-40%. Contrariwise, the proportion of cured patients is currently overtreated and would benefit from a reduction in the treatment for lower toxicity and fewer long-term sequelae (Reiter et al., 1994).

In a retrospective study of GMALL (German Multicenter Studies for adult ALL), a broad spectrum of target genes (IgH, IgK, and TCR rearrangement measured quantitatively with high sensitivity 10-4, based on their results; two MRD risk groups, low risk defined as MRD negative at all-time points after induction therapy confirmed by two markers and a sensitivity >10-4 and MRD high risk, MRD above 10-4 at two points after induction therapy without decrease with intermediate-risk group in-between due to several reasons; lack of second markers, insufficient sensitivity and inconclusive course of MRD (Hoelzer and Gökbuget, 2000a).

Genetic monitoring of MRD in acute lymphoblastic leukemia

The MRD cellular in diagnostic leukemia relapse samples is the primary varying and prognostic indicator of the treatment decisions and consequences. Chemotherapy agents (including steroids) help to eliminate leukemic cells and give rise to cause epigenetic mutations in remaining leukemia cells. Treatment agents may leave small populations of leukemic MRD cells, which may either be clones of pretreatment leukemia progenitor cells or populations of mutated leukemia cells which are different cellular markers from the original diagnostic leukemia cells or have mutated genotypes that show differential expression of Ig and TCR gene patterns (Kruse et al., 2020). Molecular detection methods for MRD identify cells either through

patterns of phenotypic markers or differential gene expression through analysis by FCM, PCR, or next-generation sequencing (NGS) Chances for molecular monitoring are significantly better in ALL. About a quarter of patients in childhood all show cytogenetically cryptic TEL/AML1 transcripts. A similar percentage of patients in adult ALL include BCR/ABL transcripts. Other patients include MLL gene aberrations. In addition, clonally rearranged antigen receptor genes (Liang and Pui, 2000; Boldeanu et al., 2011). Multiple targets were identified in most of ALL cases that allow the detection of MRD with high sensitivity (e.g., 0.01%) (van der Burg et al., 2002; van der Velden et al., 2003). Studying the E2A-PBX1 fusion gene in childhood ALL by RT-real-time PCR they found it useful for monitoring MRD, prediction of relapse, and individual treatment. The expression of the E2A-PBX1 gene on day 33 during induction of remission can be used for prognosis evaluation (Zhang et al., 2013a).

Table 6: Characteristics of the techniques used for MRD detection in ALL (Schrappe, 2012)

Parameter	PCR analysis of Ig and TCR gene rearrangements	PCR analysis of BCR-ABL transcripts
Sensitivity	RQ-PCR: 10^{-4} and 10^{-5}	10^{-4} and 10^{-6}
Quantitative range	RQ-PCR: 10^{-2} and 10^{-4}	Not yet defined
Applicability	pcB-ALL: 90-95% T-ALL: 90-95%	Ph+ ALL (5-8% of children and 30-35% of adults with pcB-ALL.
Advantages	High sensitivity A high degree of standardization reached Well-established stratification tools in various clinical protocols Applicable for almost all ALL patients Stability of DNA (multicenter setting)	High sensitivity Stability of target during treatment Fast Relatively easy/cheap
Disadvantages	Time-consuming marker characterization Pretreatment sample required to sequence the patient-specific diagnostic clone Potential instability of targets (clonal evolution phenomena) Extensive knowledge and experience needed Relatively expensive	Applicable only in Ph+ patients Instability of RNA Differences in expression levels possible Standardization necessary Risk of false positivity due to contamination

Multiple targets were identified in most of ALL cases that allow the detection of MRD with high sensitivity (e.g., 0.01%) (van der Burg et al., 2002; van der Velden et al., 2003). Studying the E2A-PBX1 fusion gene in childhood ALL by RT-real-time PCR they found it useful for monitoring MRD, prediction of relapse, and individual treatment. The expression of the E2A-PBX1 gene on day 33 during induction of remission can be used for prognosis evaluation (Zhang et al., 2013b).

Another study in acute lymphoblastic leukemia patients measuring Ig/TCR by Real-time PCR found that risk stratification of precursor B-ALL, not T-ALL, could be improved by using MRD measurement on day 15 and day 33 instead of day 33 and day 79 in similar BFM protocols for patients with these diseases. According to Assumpção et al. (2013), measuring Ig/TCR by PCR followed by homo/heteroduplex clonality analysis, found MRD independent prognostic factor for leukemia-free survival and overall survival even when based on a non-quantitative technique but longer follow-up always needed.

Flow cytometric monitoring of MRD in acute lymphoblastic leukemia

Foroni et al. (2005) reported that flow cytometry in childhood T-ALL is a favorable prognostic marker in peripheral blood and bone marrow samples. In contrast, in B-ALL, distinguishing leukemic cells from normal regenerative post-chemotherapy cells is more difficult and varies with the stages of treatment. Samra et al. (2013) used 4-color flow cytometry in precursor acute lymphoblastic leukemia patients and stated that MRD by FCM is a strong independent predictor of outcome in terms of DFS and OS and is a powerful, informative parameter in guiding individual treatment in ALL.

Weng et al. (2013) investigated the prognostic significance of leukemia-associated immunophenotypes (LAIPs) as an assessment of the index of MRD in 125 adult B-ALL patients by eight-colour flow cytometry. The LAIPs could be identified in 96% and 81.6% of patients with a sensitivity of 10−4 and 10−5, respectively. The MRD-negative status could predict a favorable 2-year relapse-free survival (RFS) and overall survival (OS) at the end of induction of complete remission and one cycle of consolidation treatment. Moreover, they identified a group of cases with MRD of 0.001% to <0.01%, which showed a significantly higher 2-year relapse rate than those with undetectable ones.

Cheng et al. (2013) confirmed that MRD detection by flow cytometry is useful for prognostic evaluation in their Chinese Cohort of childhood ALL after treatment. Moreover, peripheral blood plasma DNA MRD can be an alternative where bone marrow specimen is unavailable and a less invasive method, which allows close monitoring. In contrast, Sun et al. (2013) stated that MRD detected in ALL patients by FCM has a large range of 10-2 to 10-8, which cannot be used as a single indicator of complete remission when MRD>1 after induction therapy and first consolidation therapy, the relapse rate significantly increases. MRD can be used as a sensitive indicator for prognosis (Tables 7 and 8).

Table 7: Characteristics of the common features of flow cytometry for MRD detection in ALL (Schrappe, 2012)

Parameter	PCR analysis of Ig and TCR rearrangements	PCR analysis of BCR-ABL transcripts
Sensitivity	RQ-PCR: 10^{-4} and 10^{-5}	10^{-4}and10^{-6}
Quantitative range	RQ-PCR:10^{-2} and 10^{-4}	Not yet defined
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Disadvantages	Time-consuming marker characterization Pretreatment sample is required to sequence the patient-specific diagnostic clone The potential instability of targets (clonal evolution phenomena) Extensive knowledge and experience needed Relatively expensive	Applicable only in Ph+ patients Instability of RNA Differences in expression levels are possible Standardization necessary Risk of false positivity due to contamination

Table 8: The sensitivity and frequency of PCR-based methods target in ALL (van der Velden et al., 2003)

Leukemia type	Ig/TCR gene rearrangement	Fusion gene transcript
Sensitivity	10^{-4}-10^{-5}	10^{-4}-10^{-6}
B-Lineage		
Prec-BALL children	95	40-50
Prec-BALL Adult	90	35-45
T-Lineage		
T-ALL	> 95 (%)	10-25
T-ALL adult	90	5-10

2.4. MRD detection in acute myeloid leukemia

2.4.1. Molecular monitoring of MRD in acute myeloid leukemia

About half of AML patients lack a molecular target suitable for MRD monitoring. So, AML is lagging ALL regarding applying MRD criteria for guidance during therapy (Guerrasio et al., 2002; Sievers et al., 2003; Paietta, 2012). Both Boeckx et al. (2002) and Kim (2020) reported that fusion gene transcripts and Ig/TCR rearrangements are infrequent but complementary MRD-PCR targets in AML.

Zhang et al. (2013a) reported that both qualitative and quantitative detection of AML1/ETO has a prognostic value in MRD monitoring in AML patients. Negative or low expression of AML1/ETO indicates disease-free survival. In addition, Hoyos et al. (2013) stated that age, leucocyte count, Bal1 and *MN*1 gene expression, and high copy number of RUNX1 or CBFB-MYH11 after induction chemotherapy are useful tools to predict the outcome and should be considered for risk-adapted therapy (Tables 9 and 10).

2.4.2. Flow cytometric monitoring of MRD in acute myeloid leukemia

Schuurhuis et al. (2013) reported that FCM-MRD and molecular-MRD might complement. Their study stated that FCM-MRD best predicts outcome and molecular MRD did not add to the prognostic value of MRD in FCM-MRD negative AML patients. The argument against PCR as the method of choice for MRD analysis seems mostly based on the persistence of PCR positivity in patients who remain in remission and are negative with FCM-MRD. The authors show that FCM-MRD is a powerful tool to define new risk groups and is used as a guide for therapeutic intervention.

However, FCM-MRD still needs considerable experience in recognizing aberrant immunophenotypes at diagnosis and bone marrow remission. For that reason, further optimization of the techniques and targets available for MRD assessment by both FCM-MRD and molecular-MRD approaches is still merited.

Table 9: MRD Methods in acute myeloid leukemia (Raanani and Ben-Bassat, 2004)

Leukemia type	MRD methods	Sensitivity
Core binding factor t(8; 21)(q22; q22) AML	RT-PCR Nested Q-RT-PCR	1 step 10^{-2}-10^{-3} 10^{-5}-10^{-6} 10^{-4}-10^{-5}
Inv(16)(p13;q22) T(16; 16)(p13; q22)	RT-PCR Nested Q-RT-PCR	1 step 10^{-2}-10^{-3} 10^{-5}-10^{-6} $10^{-4}10^{-5}$
AML 11q23 and partners	Flow cytometry	10^{-1}-10^{-4}
AML PRAME	Q-RT-PCR	10^{-4}-10^{-5}
AML New expression markers	Microarray analysis	

Table 10: New molecular markers in AML and utility in MRD (Iñigo et al., 2011)

Involvement of transcription	Prevalence	Prognostic value	Associated mutation	Utility in MRD
CBF-leukemias Inv16/t (16;16) CBFB/MYH11 T(8;21)RUNX1T1	15 (%)	Favorable (poor with KIT in normal karyotype leukemia)	FLT3 NRAS KIT	Possible
PML-RARA	10-15 (%)	Favorable	FLT3(40%)	Yes,
MLL mutation	10-30 (%)	Poor	-	Yes,
CEBPA mutation	15-20 (%)	Favorable if biallelic	-	Yes,
AML1 mutation	1-20 (%)	Poor	FLT3 (IN20%M0)	Yes,
		Activating mutation		
FLT3-ITD mutation	28-34 (%)	Poor		Possible
FLT3-TKD mutation	20-30 (%)	Controversial	CBF, NPM1	
c-KIT mutation	6-48 (%)	Poor	CBF-leukemias	Yes,
RAS mutation	NRAS 11% KRAS 5%	Not influenced	FLT3-ITD (24-26 (%))	-
		Other genes alteration		
NPM1 mutation	35 (%)	Favorable (without FLT3)	FLT3	Yes,
BAALC over-expression	65 (%)	Poor	-	Possible
EVI-1 overexpression	10-22 (%)	Poor	-	Possible
WT1 overexpression	10-15 (%)	Poor	-	Yes,
DNMT3A	20 (%)	Poor	FLT3-ITD	-
IDH1-2 mutation	15 (%)	Controversial	NPM1, FLT3-ITD	-

Feller et al. (2013) also reported that immunophenotypic MRD assessment in AML is a complex process that requires knowledge and experience that is not covered by standard diagnostic immunophenotyping. The quality of MRD assessment can only be guaranteed after a substantial period of training under the supervision of highly experienced centers).

3. Molecular methods versus immunophenotyping

It is a matter of arguing what the best method to measure MRD. Each method of MRD has advantages and disadvantages.

3.1. Molecular monitoring

Real-time PCR is a powerful method of monitoring MRD levels (van der Reijden et al., 2002; Yin et al., 2012). Fluorescence-based real-time PCR is one of the most widely used quantification methods because it has a high dynamic range, is extremely sensitive and specific, and requires no post-amplification processing. Although RQ-PCR can precisely quantify PCR products, this method has some limitations concerning the precise assessment of MRD levels. The sensitivity of RQ-PCR analysis depends on; the total number of cells investigated, the total amount of DNA/RNA used/PCR conditions, and the type of PCR (single or nested).

3.1.1. PCR-based methods detecting chromosomal aberration

PCR-based methods detecting chromosomal aberration with fusion gene and their mRNA expression have advantages and disadvantages. One potential advantage of using fusion transcripts to monitor MRD is stability, and it might be possible to detect pre-leukemic cells, but the clinical significance of such a finding needs to be investigated. A clear disadvantage of using fusion transcripts as targets is that it is difficult to estimate the number of leukemic cells present in the sample. The transcript: Cell ratio may vary among leukemia of the same genetic subtype and perhaps between cells collected during diagnosis and therapy and between cells at different maturation stages within the leukemic clone (Campana, 2004). Transcript PCR products are not patient-specific and might be affected by the cytotoxic treatment, potentially resulting in transcript levels that differ per treatment phase, and RNA-based MRD results are often reported as gene expression levels and not as tumor load (Coustan-Smith et al., 1998).

Although PCR products obtained from breakpoint fusion regions at the DNA level are stable and can be identified using patient-specific oligonucleotide probes, sometimes we cannot use a DNA template because of the enormous size of the target being amplified, e.g., t (15;17), in this translocation, the primer binding sites are so far that the amplification being difficult. However, mRNA transcribed from this translocation makes the product much smaller than DNA (Foroni et al., 2005). RNA degradation can cause false-negative results, so the quality of RNA preparation should be assessed by evaluating ribosomal RNA bands, and the RT step should be checked by parallel amplification of appropriate housekeeping genes.

3.1.2. PCR-based methods detecting Ig and TCR gene

The sensitivity of Ig/TCR gene rearrangement as an MRD target depends on the junctional region (extensive or small) and the number of inserted nucleotides and the background of normal cells. PCR methods detecting Ig and TCR genes may also be affected by subclone formation; the problem of oligoclonality at diagnosis is the uncertainty of which clone is going to emerge at relapse and should be monitored with MRD-PCR techniques. In addition to secondary rearrangements, resulting in the loss of specific MRD targets can be the source of false-

negative results (van der Burg et al., 2002). The identification of Ig/TCR gene rearrangement and design of patents-specific oligo is time-consuming.

3.2. Flow cytometry-based MRD monitoring

Flow cytometry is successful in most patients with ALL or varying proportions of AML (Mukda et al., 2011; Peters and Ansari, 2011; Appelbaum, 2013; Samra et al., 2013; Terwijn et al., 2013). Flow cytometry's advantage includes accurately quantifying residual leukemic cells distinguishing viable from apoptotic cells. Multiple antibodies sets maximize the chances of detecting the residual leukemic cells even when partial changes in phenotype have occurred, so false-negative results rarely occur. A major disadvantage of flow multicolor cytometry-based MRD assays relies on the interpretative abilities of the investigator (Shook et al., 2009).

4. Conclusion

Sensitive, quantitative detection of MRD provides unique prospects for therapeutic intervention. Flow cytometry is successful in most patients with ALL or varying proportions of patients with AML. Real-time PCR is a powerful method of monitoring the MRD level. Both methods have some limitations regarding the precise assessment of MRD levels. Combined flow cytometry and molecular monitoring of MRD and strict laboratory control are essential to achieve a better result for the benefit of patients. Further optimization of the techniques and targets available by both FCM-MRD and molecular-MRD approaches is warranted. Despite the high sensitivity of most MRD techniques, negativity does not exclude the presence of leukemic cells because the test screens only a minor fraction of all bone marrow and peripheral blood leukocytes.

5. Future prospective

Evaluation of gene mutations and minimal residual disease (MRD) in patients with core-binding factor acute myeloid leukemia is of interest (Jourdan et al., 2013). Monitoring NPM1 transcript levels independent prognostic factors for relapse and survival in AML should be incorporated in future clinical trials to guide therapeutic decisions (Shayegi et al., 2013). Plasma DNA integrity is increased in acute leukemia and may be a potential biomarker for monitoring MRD (Gao et al., 2010). Plasma DNA integrity is a potential marker for screening and monitoring in cancer patients (Cirmena et al., 2022). Peripheral blood samples could replace bone marrow samples for close observation and peace of patients.

Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
CCR	Complete clinical remission
FACS	Fluorescence-activated cell sorting
FC	Flow cytometry
HST	High throughput sequencing
Ig	Immunoglobulin
LFS	Leukemia free survival
MRD	Minimal residual disease
NGS	Next-generation sequencing
OS	Overall survival
PCR	Polymerase chain reaction
RFS	Relapse free survival
RQ-PCR	Real-time quantitative PCR
RT	Reverse transcription
TCR	T-cell receptor

Compliance with ethical standards

Conflict of interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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