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Measurable Residual Disease Testing in Acute Leukemia: Technology and Clinical Significance

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Abstract: Measurable/minimal residual disease (MRD) is the strongest independent prognostic predictor in acute leukemia. Patients with undetectable MRD or good MRD response consistently demonstrate a lower risk of relapse and better survival outcomes compared with similarly treated patients with positive MRD or poor MRD response. MRD has already been used to guide risk-adapted therapies in routine care of patients with acute leukemia or in clinical trials in many countries. MRD can also be used as a surveillance biomarker with the potential to detect early relapse, and as a surrogate endpoint to speed up the testing and approval process for a new therapeutic agent. Multi-parametric flow cytometry and quantitative PCR are two methods commonly used for MRD detection. Recently, new techniques, such as digital PCR, next-generation sequencing, and next-generation flow cytometry, have also been applied in MRD detection and showed improved sensitivity and accuracy. These methods have

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their own advantages and limitations. Despite tremendous advances in this field, there are still issues and questions regarding MRD testing methods and how to translate MRD information accurately into clinical and therapeutic applications. This chapter gives an overview of the methods and the clinical implications of MRD testing in acute lymphoblastic leukemia and acute myeloid leukemia.

Keywords: acute lymphoblastic leukemia; acute myeloid leukemia; measurable residual disease; minimal residual disease; multiparameter flow cytometry

INTRODUCTION

Detection of malignant cells that remain in the body (residual disease) during and after treatment for acute leukemia is the best way to monitor therapeutic response and predict relapse (Figure 1). In general, achieving a deeper response is associated with a better prognosis. Although most of the patients achieve complete remission (CR) according to clinical and morphological criteria, a large proportion of patients eventually relapse. It is clear that not all malignant cells are killed in the patients defined as being in CR, and the level of residual disease is strongly associated with relapse risk and survival outcomes (Figure 1).

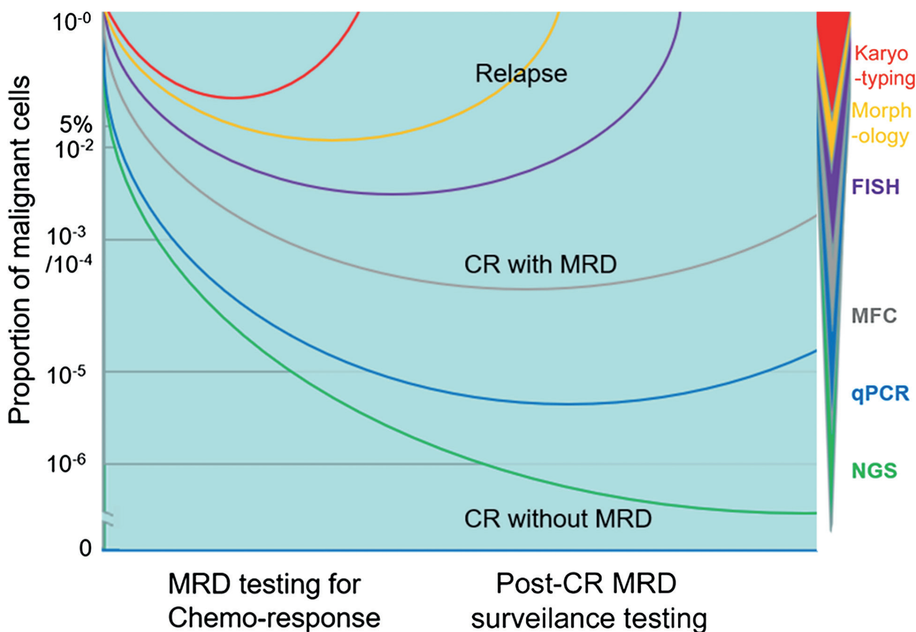

Figure 1. Diagram of the therapeutic response and relapse patterns of patients with hematologic malignancies based on MRD measured by different techniques with inconsistent sensitivities. CR, complete remission; FISH, fluorescence in situ hybridization; MFC, multiparametric flow cytometry; MRD, measurable residual disease; NGS, next-generation sequencing; qPCR, quantitative polymerase chain reaction.

Morphology has a very limited capacity of assessing treatment response since only patients with high levels of residual disease (>1–5%) can be identified. The residual disease below the detection limit of morphology is referred to as measurable residual disease (MRD, also known as minimal residual disease). The preferred change of name from minimal RD to measurable RD is to emphasize the clinical importance of MRD and to reflect the result variation with respect to testing methods applied. Conventional chromosomal analysis (karyotyping) and fluorescence in situ hybridization (FISH) have no or very little role in MRD testing due to their low sensitivity. The useful methods suitable to detect MRD include multiparametric flow cytometry (MFC) and quantitative polymerase chain reaction (qPCR). Recently, more innovative techniques, such as digital PCR (dPCR), next-generation sequencing (NGS) and next-generation flow cytometry (NGF), are also applied in MRD detection (1–5). These methods have their own advantages and limitations (Table 1). During the last three decades, numerous

TABLE 1 Comparison of methods for measurable residual disease detection

Method	Sensitivity	Advantages	Disadvantages
Flow cytometry (LAIP+DFN)	10^{-3} to 10^{-5}	Fast (within few hours) High applicability Relatively inexpensive Information at cellular level Potential detecting phenotypic shift	Requires fresh sample and viable cells Requires high level of expertise Limited standardization
RT-qPCR for gene fusions	10^{-4} to 10^{-5}	Sensitive Relatively simple Standardized No need of patient-specific (PS) primers	Limited applicability Risk of cross contamination Can't detect small subclones or clonal evolution
RT-qPCR for IG/TCR gene rearrangements	10^{-4} to 10^{-5}	Sensitive Standardized with consensus guidelines	Requires diagnostic sample and PS primers Time consuming, labor-intensive, expensive Can't detect small subclones or clonal evolution
Digital PCR	10^{-3} to 10^{-5}	Sensitive Absolute quantification No need of standard curve Not affected by PCR inhibitors	Lack of standardization May require PS design Can't detect small subclones or clonal evolution
Next generation sequencing	10^{-6}	Highly sensitive No need of PS primers Wide applicability Potential to track small subclones and clonal evolution	Requires pretreatment specimen No standardization Requires high degree of informatics expertise Expensive

DFN, different from normal; LAIP, leukemia/lymphoma associated immunophenotype; PS, patient specific; RT-qPCR, real-time quantitative PCR

studies have been conducted, regarding the MRD detection methodologies and the clinical significance of MRD. These studies have demonstrated that MRD is an independent and the most powerful predictor of relapse and survival outcome (6–8). MRD testing has already become a part of routine care for some patients with acute leukemia, and the treatments for these patients are modified based on the MRD status. MRD can also be used as a surrogate endpoint to speed up the testing and approval process of a novel therapy or a new therapeutic product (9, 10). For patients in remission with maintenance therapy or off therapy, MRD testing can serve as a surveillance tool with potential to detect early relapse. This chapter gives an overview of the MRD testing methods and the clinical implications of MRD testing in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML).

METHODS FOR MRD TESTING

The technological advances have kept improving MRD detection methods, which have become more and more sensitive, more and more accurate, and subsequently capable of evaluating therapeutic responses at deeper and deeper levels. Classic microscopy and karyotyping have little or no value in MRD detection due to their low sensitivity. FISH is not considered as a sensitive method for MRD detection, although it can detect certain level of MRD depending on the target and how many nucleated cells screened. The useful methods suitable for MRD detection include multiparameter flow cytometry (MFC), real-time quantitative PCR (RT-qPCR), digital PCR (dPCR) and next-generation sequencing (NGS), which have been used in various clinical studies with different advantages and limitations (Table 1).

Multiparameter flow cytometry

MFC assesses the antigens present on the surface, cytoplasm, or nuclei of cells by using fluorochrome-conjugated specific monoclonal antibodies, as well as the physical characters of cells (size and complexity) by light scatter (Figure 2A). Antigens are expressed by malignant cells with variable degrees of difference from their normal counterpart cells. There are two strategies for MFC MRD detection: "Leukemia-Associated ImmunoPhenotype" (LAIP) and "Different from Normal" (DFN). The LAIP is identified at diagnosis by comparing the antigen expression profile of malignant cells to reference cell counterparts, through a panel of monoclonal antibodies. DFN defines malignant cells by recognizing immunophenotypic deviation from their normal counterpart population through the evaluation of antigen expression patterns (11). LAIP method is simple and easy to perform, and it can use a very limited number of antibodies. However, it needs diagnostic specimen and carries the risk of false positivity due to background noise and false negativity due to phenotypic changes. DFN does not need a pre-treatment immunophenotype, but its interpretation is more subjective and requires a higher level of expertise. It is recommended that both LAIP and DFN strategies should be used together to allow tracking of diagnostic and shifted leukemia phenotypes, whenever it is possible. Both strategies require expertise in the recognition of aberrant populations and exclusion of normal /reactive cell populations or potential backgrounds (11, 12).

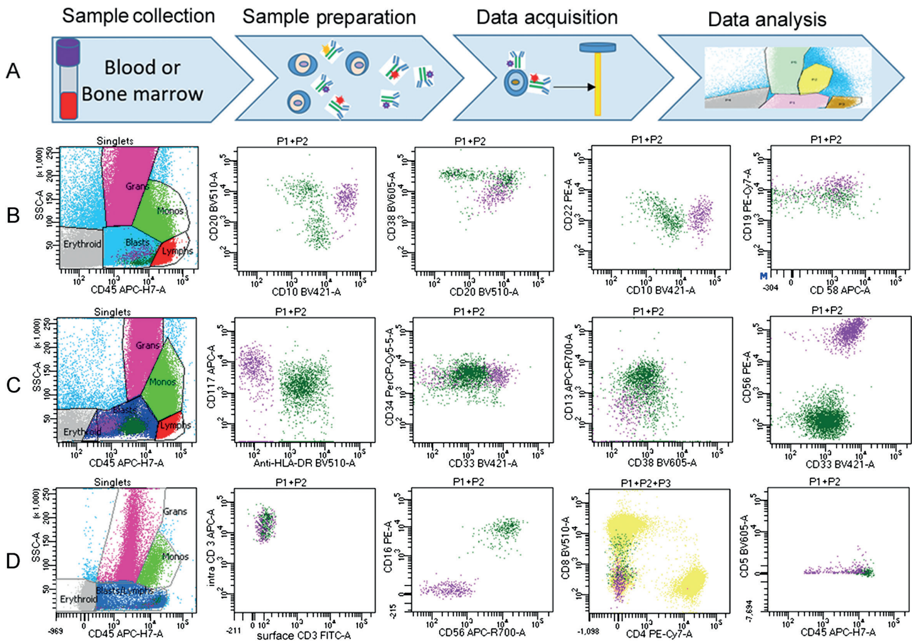

Figure 2. Measurable residual disease (MRD) detection by multi-parametric flow cytometry (MFC). A. The workflow of the MFC study is demonstrated. The nucleated cells of collected bone marrow or peripheral blood sample are incubated with fluorochrome-conjugated monoclonal antibodies, then run in a flow cytometer through the laser beam, and the signals of fluorescence and light scatters are collected and stored in the computer for analysis. **B.** Representative dot plots of MFC study on the bone marrow specimen (BM) of a precursor B-cell acute lymphoblastic leukemia (ALL) patient at the end of induction (EOI). The cell popution in dark green (P1, 0.45%) shows a normal antigen expression pattern, and is consistent with regenerating B-cell precursors (hematogones). The cell population in purple (P2, 0.07%) shows increased expression of CD10, CD19, CD20 and CD58, and decreased CD38, and is consistent with MRD. **C.** Representative dot plots of MFC study on the BM of an acute myeloid leukemia patient at the EOI. The cell popution in dark green (P1, 0.8%) shows a normal antigen expression pattern, and is consistent with regenerating myeloid precursors. The cell population in purple (P2, 0.2%) shows increased expression of CD33, CD117, decreased CD38, CD45, absent HLA-DR, and aberrant expression of CD56, and is consistent with MRD. **D.** Representative dot plots of MFC study on the BM of a T-cell ALL patient at the EOI. The cell popution in dark green (P1, 0.15%) is NK cells showing expression of intracellular CD3. The cell population in purple (P2, 0.1%) is positive for intracellular CD3, but is negative for surface CD3, CD4, CD5, CD8, CD16 and CD56, and is consistent with MRD. The cells in yellow (P3) are normal T cells.

MFC can be successfully applied to MRD testing for the majority of acute leukemia cases. MFC-MRD assay is relatively sensitive, and it can reach a sensitivity varying from 10^{-3} to 10^{-5} based on the disease and the panel used. The biggest advantage of MFC method is that it is fast and MRD results can be available within a few hours. The DFN strategy allows for the detection of phenotypic changes of MRD. Since the MFC assay measures proteins at the cellular level, the results can provide useful target information for monoclonal antibody treatment. However, this method has several limitations (Table 1). First, the test should be performed

shortly after sample collection, which could be an issue for the reference lab if the samples are received late due to delivery problems. Second, the assay requires a high level of technical expertise, and the interpretation can be very challenging, especially in cases with phenotypic changes (13, 14), although there are consensus guidelines published by the EuroFlow Consortium group (15, 16). Third, the assay is not fully standardized, and the interpretation and gating of relevant cell populations are quite subjective and time-consuming.

NGF takes advantage of innovative tools and procedures developed by the EuroFlow Consortium (17) and standardizes every step of the process from sample preparation to expert-guided automated reporting. NGF has overcome most of the obstacles mentioned above, and it has the advantages of objective expert-based panel design, fully standardized methodology, high reproducibility between laboratories, high sensitivity, and objective data analysis. EuroFlow-NGF MRD testing can reach a sensitivity of 10^{-6}, comparable to NGS, in multiple myeloma (MM), and it is applicable in 99% of MM cases and feasible in most laboratories (18). NGF and NGS showed good concordance in a recent comparison study (4). EuroFlow-NGF uses an automatic population separator, allowing to eliminate the inter-operator variability, and has a quality assessment of bone marrow (BM) cellularity by simultaneous detection of hematogones, erythroblasts, myeloid precursors, and/or mast cells (18). This information is important to ensure sample quality and identify significant hemodilution, which can lead to falsely low or negative results. The ability to analyze a complete immune profile, including T, B, and NK cells, as well as monocytes and other myeloid cell populations, at the time of MRD assessment, could also help to evaluate patients' immune system, which is likely another prognostic factor for survival.

The sample for MFC MRD detection can be peripheral blood (PB) or BM. In AML and B-cell ALL (B-ALL), MRD levels tend to be one or more logs higher in BM aspirate than in PB, whereas, in T-cell ALL (T-ALL), MRD levels are comparable in BM and PB (11, 19). In general, BM aspirate is typically the preferred specimen for MRD testing. However, since PB is an easily obtainable specimen without invasive procedures like BM aspiration, it can be used at an early time point of treatment to assess the kinetics of leukemia cell clearance, e.g., day 8 post-induction PB MRD assessment for B-ALL per the Children's Oncology Group (COG) protocol (20, 21). The BM aspirate sample for MRD assessment should be the first pull and less than 5ml to minimize hemodilution. BM samples should be anticoagulated with ethylenediaminetetraacetic acid (EDTA) or sodium heparin, transported at room temperature, and processed as soon as possible (ideally within 48 hours of collection). Sample preparation can be performed using two accepted techniques: (i) bulk lysis, followed by wash/stain/wash; or (ii) stain/lyse/wash or no-wash (22).

Acute lymphoblastic leukemia (ALL)

ALL is characterized by aggressive proliferation of lymphoblasts of either B-cell (B-ALL) or T-cell (T-ALL) in BM. MFC is a commonly used method for ALL MRD detection. In B-ALL, the specific challenge for MFC-MRD assay is to phenotypically distinguish leukemic cells from normal, regenerative B-cell precursors (also known as hematogones), which are commonly present in regenerative BM with consistent antigen expression patterns (Figure 2B, P1-green cell population).

The hematogones express CD34, CD10, and CD19 at early stage, gradually lose CD34, decrease CD10, gain CD20, and increase CD45 expression during maturation. CD38 is expressed at high level till the end stage. Leukemic B-lymphoblasts almost always show a certain deviation from normal antigen expression profile (Figure 2B, P2-purple cell population). The 6 or more-color panel including the backbone set (CD34, CD19, CD10, CD20, CD38 and CD45) can be used for most cases and reach a low limit of detection at 0.01% (23). Along with these six antigens, additional valuable markers for B-ALL MRD detection include CD13/CD33, CD9, CD58, CD22, and CD81, while other B-ALL diagnostic markers, such as CD79a, TdT, CD24, and surface immunoglobulins (IGs), are not very informative. The COG has a standardized 3-tube 6-color panel for B-ALL MRD detection, which is used for patients in COG studies. This panel includes CD34, CD19, CD10, CD20, CD38, CD45, CD9, CD58, CD13/33, CD71, CD3, and Syto16. Although it has limitations such as being not able to detect CD19-negative MRD due to its gating strategy and consuming more samples than a higher number panel, it is highly standardized with a good sensitivity (10^{-4}) for most of the cases. A highly sensitive standardized MFC B-ALL MRD assay has also been designed by the EuroFlow group (15), and it demonstrates that the application of a fully standardized bulk lysis protocol and two stepwise designed 8-color tubes (including the backbone panel plus CD81, and either CD66c/CD123 or CD73/CD304) allows highly sensitive MRD detection (up to 10^{-5}) in 99% of the B-ALL patients, when large numbers of events are acquired (>4 million cells).

The inclusion of other B-cell markers other than CD19 has been emphasized in recent years due to the increasing clinical use of immunotherapies targeting CD19 (i.e., blinatumomab and CAR-T 19). CD22 and CD24 are expressed in B lymphoblasts and can be essential for tracking B-ALL leukemic cells with downregulation of CD19 as well as for the identification of CD22+ cases eligible for inotuzumab ozogamicin therapy (24). For the cases with immunotherapies, caution should always be exercised when interpreting the results, and appropriate modifications should be made for the panels.

Normal immature T-cells (thymocytes) are not present in BM and PB. Theoretically, it is easy to detect T-ALL MRD since any immature T cells detected in BM or PB will be considered MRD. T-ALL MRD backbone panels are set up to evaluate both the aberrant expression of mature T/NK-cell antigens (i.e., surface/cytoplasmic CD3, CD5, CD7, CD2, CD4, CD8, CD45, CD16, CD56) and immature markers (CD34, CD1a, TdT, CD99) (25). Based on our experience, the detection of T-ALL MRD is more challenging than B-ALL. T-cell immature markers (CD1a, CD34, CD99, TdT) are frequently absent or very dim, and CD45 is commonly quite bright in the residual leukemic cells after treatment. It is hard to tell the immature nature of residual T-ALL. We must rely on the DFN strategy to identify a T-cell population different from normal T cells. T cells are heterogenous and contain many subsets, which are phenotypically different to some extent. Knowing all these subsets is critical to accurately identify T-ALL MRD and avoid erroneous interpretation (12, 26). The strategies useful for most cases include looking for abnormal CD4/8 double-negative (DN), CD4/8 double-positive (DP), and/or sCD3- cCD3+ cells. Our T-ALL MRD panel studies all the cells in blast and lymphocyte regions (Figure 2D). For CD4/8 DN and sCD3- cases, NK cells could be an MRD mimicker, especially when they express cytoplasmic CD3 epsilon chain (Figure 2D, P1-green cell population). NK cells are present in almost all the

specimens and are usually easy to recognize with their expression of NK cell markers. For CD4/8 DN, sCD3+ cases, normal/reactive CD4/8 DN T cells (gamma delta T cells, NKT cells, or reactive T cells) could be a mimicker. The antigen expression level of other markers in these cells is normal, which helps to make the distinction. For CD4/8 DP cases, the reactive CD4/8 DP T cells could be a mimicker occasionally. Most of the reactive CD4/8 DP cells show the characteristic right angle and smear pattern on CD4 vs CD8 dot plot, and thus are easily recognizable.

Acute myeloid leukemia (AML)

AML is a heterogeneous group of malignant myeloid neoplasms characterized by the proliferation of abnormal myeloid progenitors, which differ in morphology and immunophenotype among the different subsets. It is not possible to have one MRD panel to fit all. According to ELN recommendations, the MFC-MRD assay should use a combined "LAIP-based DFN approach", by which the specific LAIP tracking is integrated into broad immunophenotypic profiling of BM cells. The panels should include core AML markers CD34, CD117, CD45, CD33, CD13, CD56, CD7, HLA-DR, and other selected or LAIP-related markers. Normal regenerative myeloid precursors have consistent and typical antigen expression patterns (Figure 2C, P1-green cell population), whereas leukemic myeloblasts almost always show some abnormal differentiation/maturation patterns (Figure 2C, P2-purple cell population). In cases with monocytic differentiation or a monocytic component, additional markers (e.g., CD64, CD14, CD11b, CD4) should be added (27, 28). Assessing MRD in monocytic AML is generally more challenging than in non-monocytic AML. This is mainly because neoplastic immature monocytes often do not express immature progenitor markers, such as CD34 and CD117, and lack expression of the monocytic marker CD14. They typically maintain the expression of CD15, CD33, CD36, and CD64 at levels close to normal mature monocytes with some degree of deviation. In a significant subset of monocytic AML cases, the MRD is present as a population of immature monocytes (usually CD14 low to negative) with immunophenotypic abnormalities in the expression of CD4, CD15, CD64, and/or HLA-DR, and aberrant CD56 expression at a moderate to high level (29). It should be noted that regenerating monocytes commonly express CD56, which usually shows a smear pattern and should not be considered residual leukemia (12). In cases with megakaryoblast differentiation, one or more megakaryoblastic markers (CD41, CD42, and CD61) should be added. It should be aware that adhesion of platelets to the cell surface can cause false positivity of these markers.

Overall, MFC MRD assays for AML are less sensitive than those for ALL, which usually shows more homogeneous and specific phenotypic aberrancies. In most AML cases, a low limit of detection of 0.1% can be achieved by the MFC method and this is the clinical decision-making point for most clinical studies. A lower level of MRD can be detected with an increased number of cells acquired and in cases with LAIP more significantly different from normal.

Leukemic stem cells (LSC) have been experimentally defined as leukemia-initiating cells, which are therapy-resistant and are thought to be the cellular reservoir of relapse in AML (30). Some studies have demonstrated the association of high LSC frequencies at the time of diagnosis with the presence of MRD and

subsequent poor prognosis (31–33). Therefore, LSC measurements are warranted to facilitate accurate risk stratification. LSCs can be immunophenotypically defined as CD34+/CD38- cells combined with an aberrant marker such as CD45RA, CLL-1, or CD123 (34). A significant advantage of LSC testing over MRD testing is that the former delivers a prognostic value both at diagnosis and after treatment, and in contrast to MRD, does not require comprehensive knowledge of normal hematopoietic cell differentiation patterns. A disadvantage is that the frequencies of CD34+CD38- LSCs are very low at follow-up, so LSC detection requires ideally 4 million events, likely best achieved with a one-tube assay (34, 35).

Polymerase chain reaction (PCR)

PCR is a technique that can quickly make copies of a piece of DNA and is the basis of many molecular tests. PCR-based MRD testing can target gene rearrangements, fusion genes resulting from chromosomal translocations, or gene mutations. RT-qPCR for antigen-receptor gene rearrangements or fusion genes has been well developed as an MRD detection method in acute leukemia. Recently, a more innovative PCR technique, droplet digital PCR (dPCR), has also been applied to MRD detection in hematologic malignancies.

Real-time quantitative PCR (RT-qPCR) for antigen-receptor gene rearrangements

IG and T-cell receptor (TCR) gene rearrangements are important physiological events for the development of normal B cells and T cells, respectively. The unique gene sequences of antigen receptors for each B or T cell result from a somatic rearrangement of separated gene segments (V, D and J) and random deletion or insertion of nucleotides at the junction between gene segments (36). Lymphoid malignancy is derived from the clonal proliferation of a single transformed lymphoid cell, and therefore all malignant cells will contain the same rearranged clonal IG and/or TCR genes. Although IG rearrangements are mostly found in B cells and TCR rearrangements in T cells, both B-lineage and T-lineage malignant cells can display cross-lineage rearrangements (37, 38). For example, up to 90% of B-ALL cases may exhibit TCR gene rearrangements (38), while 20% of T-ALL cases may have IG rearrangements (39). To identify these molecular markers at diagnosis, genomic DNA extracted from malignant lymphoid cells is amplified by PCR and subsequently sequenced. Allele-specific oligonucleotide (ASO)-primers are designed based on the sequence and used for RT-qPCR MRD testing (40, 41). Amplification conditions and sensitivity for each ASO-primer set are established on the diagnostic material serially diluted with normal mononuclear cells. This RT-qPCR protocol combined with fluorescence-labeled probes allows the detection of MRD at a sensitivity of up to 10^{-5} (42). This method of MRD detection is applicable for over 90% of ALL, CLL and other lymphoid malignancy cases (41, 43, 44).

As one of the most commonly used methods for MRD detection in lymphoid malignancies, RT-qPCR for IG/TCR gene rearrangements had been extensively standardized within the EuroMRD Consortium, and guidelines for the analysis and the interpretation of the results had been established (43). This method is

sensitive and widely applicable. It may fail in about 5–10% of the cases due to the absence of IG/TCR gene rearrangements or technical issues. The big drawback of this method is the requirement for establishing a patient-specific RT-qPCR assay, which is time-consuming and laborious. An adequate diagnostic sample is critical for the success of this assay. Occasionally the diagnostic DNA may be insufficient since diagnostic DNA is not only needed for the initial testing and establishment of the patient-specific assay, but also needed for generation of the standard curve of each MRD testing run. Another limitation of this method is the lack of ability to detect subclone or clonal evolution (45).

RT-qPCR for fusion genes

Gene fusion transcripts are other targets for MRD assessment by qPCR method. More than 40% of ALL cases and about a quarter of AML cases carry chromosomal translocations that generate chimeric transcripts. These abnormal gene rearrangements are the main driver events, likely expressed in all leukemic cells, and are stable during the disease. Therefore, they are potentially ideal targets for MRD testing (46–48). The most common fusion gene detected in adult B-ALL cases is *BCR-ABL1*, accounting for 25–30% of all cases. The most common fusion gene in pediatric B-ALL patients is *ETV6-RUNX1*, accounting for 25–30% of all cases. *KMT2A* gene rearrangement is the most common cytogenetic change in infant leukemia (occurring in about 80% of cases). *PML-RARA*, *RUNX1-RUNX1T1*, and *CBFB-MYH11* are the most common fusion genes detected in AML. These cytogenetic abnormalities have prognostic value, and their detection must be performed at diagnosis so that each patient can be monitored for MRD using a predefined marker throughout the disease. In the cases carrying the same translocation with different breakpoints, the RNA splicing process may produce the same fusion transcript or few splicing variants. Therefore, RNA is the optimal material to detect these lesions, since it allows the use of a small number of qPCR assays and offers the opportunity to apply the same primer set to all cases bearing the same translocation (46). Quantification of the gene fusion transcript using RNA samples is achieved by comparing the amplified product to a standard curve derived from the amplification of serial dilutions of a cell line or plasmid DNA.

A similar assay targeting mutated NPM1 has also been developed for MRD assessment in AML patients with *NPM1* mutations (49). This sensitive MRD assay can detect up to 1 malignant cell within 100,000 (10^{-5}) nucleated cells. The assays targeting the common fusion genes present in ALL and AML have been standardized by Europe Against Cancer (EAC) consortium and are widely used by clinical laboratories worldwide (46). This MRD detection assay is not patient-specific, which means that it does not need to design patient-specific primers, so it is relatively easy to perform and is not expensive. The limitation of this method is its limited applicability. In addition, the accuracy of MRD results by this assay may be affected by the variability in the number of RNA transcripts per malignant cell from patient to patient, and among different cells within the same patient.

Digital PCR (dPCR)

The dPCR technology is a newly developed technique based on sample splitting and Poisson statistics (50), and it has the potential to overcome the limitations of

conventional qPCR. The sample (RNA or DNA) is fractionated into thousands of droplets, where PCR amplification of the target gene occurs. The dPCR technique has been studied as an MRD detection method in multiple hematologic malignancies, and the data show that the dPCR assay has sensitivity and reproducibility at least comparable to the conventional qPCR method (1, 3, 51, 52). Recently, the clinical significance of dPCR MRD results has been reported in a study of pediatric ALL patients (53). The results showed that among "slow early responder" patients, most relapses occurred in cases with quantifiable dPCR MRD at day78, while patients with a negative or positive-not-quantified (PNQ) MRD by dPCR at day78 had a better outcome, indicating that MRD by dPCR can provide further risk stratification.

The dPCR method appears more accurate than RT-qPCR, and shows higher amplification efficiency, being less affected by the presence of inhibitors (54, 55). It is a high-throughput technology that produces an absolute quantification by amplifying target genes without a reference standard curve required. Hence, it has a lower chance of contamination. Compared with NGS, dPCR tends to show an inferior error rate, and it is faster and does not require a bioinformatics expert to analyze the results. The limitations of this method include the need for validation and the challenges of having to design an experiment for each assay. No guidelines for dPCR MRD assays have been established so far. A major standardization effort is underway within the EuroMRD Consortium.

Next-generation sequencing (NGS)

NGS is a high-throughput parallel sequencing technique that can produce millions of short-read sequences in a moment. The current available NGS platforms apply different approaches to achieve high-throughput sequencing. The general steps for a typical NGS run include DNA or RNA extraction from the samples, library preparation (DNA or cDNA fragmentation and adapter ligation, or PCR amplification), cluster generation, and finally sequencing (Figure 3A) (56). In a single experiment, NGS can provide accurate information on a DNA sequence and its alterations, such as mutations, insertions, deletions, or rearrangements. NGS is potentially applicable to all acute leukemia cases, but the interpretation of the data requires highly specialized bioinformatic approaches. There are three main types of NGS: whole genome sequencing (WGS), whole exome sequencing (WES), and targeted-gene sequencing. Targeted-gene sequencing method can provide profiling of several genes of interest simultaneously, and thus it is applicable to evaluate the mutations of several genes as potential targets for MRD assessment, and as measurable biomarkers for treatment (57).

Comparable to conventional qPCR methods, the first step of NGS MRD testing is also the identification of leukemia-specific clones using the diagnostic sample (Figure 3B, the first time point). However, in contrast to qPCR methods, the laborious design and testing of patient-specific assays are not needed since the same multiplex approach is applied to follow-up samples, with the index sequence(s) re-identified and quantified (Figure 3B, 2nd and 3rd time points). Moreover, the readout is more specific than qPCR testing, where false-positive results may occur due to nonspecific binding of the ASO primers. NGS MRD method targeting IG/TCR gene rearrangements can also detect clonal evolution and provide insight

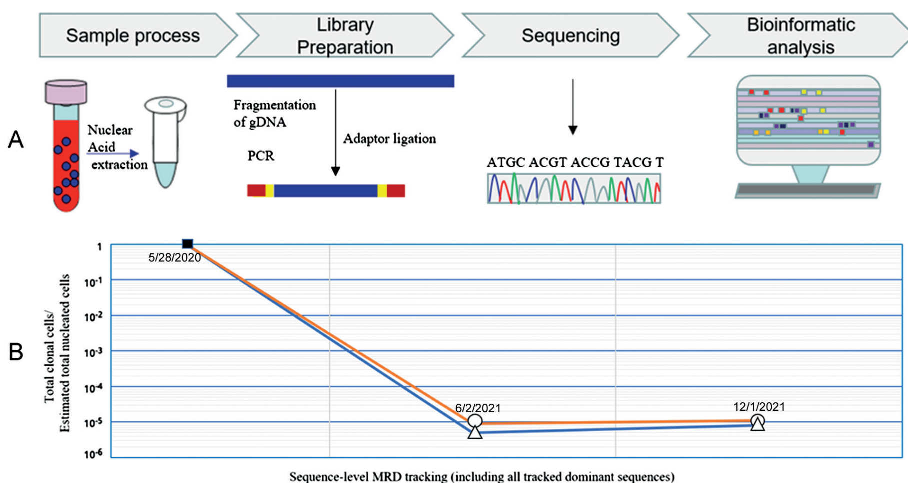

Figure 3. Next-generation sequencing (NGS) analysis for measurable residual disease (MRD). **A,** The workflow of NGS. **B,** An example of NGS MRD assay targeting T-cell receptor gamma (TCRG) gene rearrangements for a patient with T-cell lymphoblastic leukemia. NGS testing on the diagnostic bone marrow sample (5/28/2020) identified two major TCRG sequences. The follow-up bone marrow specimens showed 0.0005% MRD based on sequence A and 0.0009% MRD based on sequence B on 6/2/2021; 0.0008% MRD based on sequence A and 0.0011% MRD based on sequence B on 12/1/2021. ■, clonality test; Δ, tracking test: TCRG-sequence A; O, tracking test: TCRG-sequence B.

into the background repertoire of B and/or T cells. One disadvantage of NGS that had limited its use for MRD assessment was the sequencing error rate and its impact on the sensitivity of this technique compared to the methods discussed above (58). The introduction of error-corrected read technologies has overcome this limitation and greatly improved its sensitivity (59, 60). Another improvement is the use of Bayesian analytical techniques for mutation calling informed by site-specific error rates and prior clinical data regarding mutation frequencies (61). Overall, the current NGS MRD detection method reaches a higher sensitivity ($\leq 10^{-6}$) than other methods (2, 4, 62).

Besides IG/TCR gene rearrangements, gene mutations are also molecular markers commonly targeted by NGS MRD testing, especially in AML (62–64). Some of the mutations are not stable during the disease and can disappear or emerge at the time of relapse, as reported in the AML case with FLT3-ITD (65). Some of the persisting mutations such as *DNMT3A*, *ASXL1*, and *TET2* (64), collectively termed as DTA, known to be frequent in clonal hematopoiesis of indeterminate potential (CHIP) (66), do not have a prognostic value. Therefore, these mutations cannot be used as the target molecular markers for MRD detection in AML patients. Other limitations of this method include the requirement for a high-quality diagnostic DNA sample, and a high level of informatics expertise which is not widely available. In addition, currently, there are no uniform analytic and reporting standards for NGS MRD testing, which has complicated the comparison of different studies.

CLINICAL SIGNIFICANCE OF MRD DETECTION

There are many published studies investigating the clinical significance of MRD assessment in acute leukemia. Overall, MRD has consistently been demonstrated as one of the most powerful prognostic factors, and patients benefit from MRD-based risk-stratified management and therapy (6–8), although the clinical impact of MRD varies according to the diseases studied, patient age groups selected, MRD detection methods applied, chemotherapy regimen used, relative rates of allogeneic hematopoietic stem cell transplantation (allo-SCT), the timing of MRD assessment, and other factors. MRD testing can also serve as a surveillance tool with the potential to detect early relapse. In addition, MRD can be used as a surrogate endpoint to assess the therapeutic effect of a novel treatment or a new therapeutic product to speed up the testing and approval process (9, 10).

Prognostic prediction

The clinical significance of MRD has been extensively studied in ALL by either MFC or molecular methods for IG/TCR gene rearrangements (6). The early study of the COG measured the impact of different MFC MRD levels at the end of induction (EOI) on event-free survival (EFS) and showed that those with <0.01% MRD had an EFS of 88%, in contrast to an EFS below 60% for all other groups (20). Unfortunately, approximately two thirds of relapses occur in EOI MRD negative patients. To better explore the impact of MRD kinetics on the outcome, the COG measured MRD at an earlier time point (day 8 PB) by MFC. Patients with day 8 MRD > 1% had inferior outcomes compared to those with MRD ≤1% even if they cleared MRD by EOI (day 8 MRD-positive: 5-year EFS of 79% vs. 90%, if MRD-negative) (20). The AIEOP-BFM ALL 2000 trial evaluated the prognostic impact of molecular MRD at two different time points, day 33 and day 78 (equivalent to EOI and end of consolidation across different protocols worldwide), and the B-ALL patients (n = 3184) were classified by MRD status: MRD-standard risk if negative at a level of $<10^{-3}$ at day 33, MRD-intermediate risk if positive at day 33 but negative at day 78 and MRD-high risk if persistently positive at both time points. They found that MRD significantly correlated with outcome (5-year EFS of >90%, 78%, and 50% in the standard, intermediate and high-risk groups, respectively) (67). A later COG study on 7430 children with the National Cancer Institute (NCI) standard or high-risk B-ALL demonstrated a better 5-year disease-free survival (DFS) in a subset of patients with 0.01–0.1% MRD treated with augmented Berlin–Frankfurt–Munster therapy (ABFM) plus two interim maintenance and delayed intensification phases (68). This data suggest that intensification based on MRD response can rescue some unfavorable risk patients. Investigators at St. Jude Children's Research Hospital (SJCRH) investigated the impact of lower MRD thresholds based on the PCR MRD detection method in a cohort of 455 children with B-ALL and demonstrated that a persistent low-level disease of 0.001% to <0.01% was associated with a cumulate relapse risk of 12.7% compared to 5.0% for those with undetectable MRD (<0.001%) (69).

The prognostic values of MRD detected by IG/TCR gene qPCR in different genetic subtypes of B-ALL have also been studied (70). It was found that the risk

of relapse was strongly associated with MRD in all genetic subgroups. However, the relapse risk associated with a single MRD value varied significantly between genetic subgroups. These results suggest that a single threshold may not be appropriate for all subgroups, and individualized thresholds for different subtypes should be a better practice. For patients who receive allo-SCT, the detection of MRD (>0.01%) is associated with early post-transplant relapse and worse prognosis (71, 72).

The prognostic value of MRD in AML has also been well-demonstrated by many studies (7, 8, 27, 64, 73). Those with detectable MRD have higher cumulative incidence rates of relapse and shorter relapse-free survival (RFS) and/or OS than similarly treated patients without MRD. The strong association between detectable MRD and inferior clinical outcomes has been demonstrated at several timepoints throughout intensive AML therapy, which include early or mid-induction, after completion of one or two cycles of induction chemotherapy, after post-remission therapy, both before and after SCT, and after salvage chemotherapy for relapsed/refractory disease. Furthermore, the negative prognostic impact of a positive MRD test on outcomes has been found irrespective of MRD testing methods. Overall, the available data indicate that patients tested positive for MRD at any given timepoint, regardless of the detection methods used, have a higher risk of experiencing a relapse. On an individual patient level, results from MRD testing refine the prediction of RFS and OS to some degree, but the ability to accurately predict these outcomes remains limited (74). While different MRD detection methods can be used to provide prognostic information, it is important to note that the concordance between these assays is not 100%. Therefore, it may be most valuable to use different MRD assays in a complementary, rather than isolated manner. Retrospective studies have shown that when both MFC and NGS assays are used, patients without MRD by both methods have particularly good outcomes, patients with MRD by both methods have particularly poor outcomes, and patients with MRD by one method but not by the other have intermediate outcomes (64, 75).

Therapeutic implications of MRD

Given the strong prognostic value, MRD can be used to guide risk-adapted therapies. By tailoring therapies according to MRD response, patients with a high risk of relapse can selectively receive more aggressive therapy, such as allo-SCT in first CR, intensification of chemotherapy, or the introduction of novel therapeutic agents; while the patients with a low risk of relapse can receive reduced therapy to minimize therapy-related morbidity and mortality (76–78). Allo-SCT is associated with a reduced likelihood of relapse compared with nontransplant post-remission therapy but bears considerable risks of non-relapse-related morbidity and mortality. Allo-SCT has been shown to improve outcomes of patients with ALL and suboptimal MRD response to frontline chemotherapy (76). The study (79) also indicates that allo-SCT may be safely avoided in adolescent and adult ALL patients with good MRD response. A report from the Dutch COG has suggested that reduced chemotherapy in pediatric ALL patients who achieve MRD negativity is safe, and intensification of chemotherapy with or without allo-SCT can improve the outcomes of patients with suboptimal MRD response (77).

In addition to making decisions on whether to pursue allo-SCT in the first CR, MRD assessment can also identify patients who may benefit from novel therapies. This is especially important for those who may not be candidates for allo-SCT due to old age or significant comorbidities, as well as for those without an adequate donor. Inotuzumab and blinatumomab have shown significant promise in the management of relapsed/refractory ALL (80, 81). The apparently improved survival observed with these immunotherapies may be in part mediated through the higher MRD negativity rates achieved with these agents as compared to standard cytotoxic chemotherapy. The use of CD19-directed chimeric antigen receptor T cells (CAR T) in patients with relapsed/refractory ALL has also resulted in high rates of MRD negativity (82). Given the known significant impact of MRD response on long-term outcomes, these regimens leading to deeper remissions will ultimately translate into improved survival.

MRD also serves as a decision-making factor to identify AML patients for allo-SCT. The study on *NPM1*-mutated AML showed that DFS and OS were significantly improved by allo-SCT in those with suboptimal PB-MRD response, and allo-SCT provided no significant benefit to patients with *NPM1*-mutant AML who did not have detectable MRD or had good MRD response before allo-SCT (83). These data suggest that allo-SCT in the first CR might be a good option for patients with suboptimal MRD response, as is also supported by the study on t(8;21) AML (84). The GIMEMA AML1310 trial prospectively used MRD to guide SCT strategy in young adults with newly diagnosed AML (85). In this study, patients with intermediate-risk (IR) cytogenetic/molecular findings and detectable MRD after consolidation underwent allo-SCT and those without detectable MRD underwent autologous SCT (auto-SCT). Among these two groups of IR patients, there was no statistically significant difference in either 2-year OS (79% in MRD-negative vs. 70% in MRD-positive) or DFS (61% in MRD-negative vs. 67% in MRD-positive). These findings suggest that an MRD-directed selection of SCT consolidation may overcome the negative prognosis of MRD positivity in IR patients. It is also suggested that MRD status can reasonably be used to guide pre-SCT conditioning intensity (86). Recently, there have been quite a few studies evaluating MRD-directed approaches with the aim to eradicate MRD in patients with persistent or recurrent MRD after conventional therapy (8). These new approaches include the use of hypomethylating agents, FLT3 inhibitors, Venetoclax-based combinations, etc.

MRD monitoring and detection of early relapse

Since conversion from a negative to a positive MRD test result or an increase in MRD level over time is associated with overt disease recurrence, it is reasonable to consider MRD as a monitoring biomarker for routine surveillance and care of patients with acute leukemia following the completion of therapy. For example, the change from negative to detectable MRD by the RT-qPCR method in the cases of acute promyelocytic leukemia (APL) is almost always followed by hematologic relapse, although the interval between the MRD conversion and overt relapse can span more than one year (87). Change from negative to a

positive RT-qPCR test for *RUNX1/RUNXT1* transcripts in patients with t(8;21) AML is strongly indicative of disease recurrence, often with a very short latency from molecular to morphologic relapse (88). However, optimal timing for monitoring MRD and interval between tests are not well defined. More data are needed regarding the thresholds best suited to define relapse, the need and timing for confirmatory testing if a positive result is obtained, and how to approach patients with persistent molecular MRD at low levels. Early relapse detection may allow early therapeutic intervention to prevent overt relapse. However, the clinical benefit from early intervention based on MRD results needs to be further investigated.

MRD as a surrogate endpoint for new drug testing

The strong prognostic impact of MRD in hematologic malignancies has sparked the interest in using MRD as a surrogate efficacy biomarker to expedite the drug testing and approval process (9, 10, 89). The use of MRD as a surrogate endpoint could also decrease the clinical trial cost, as it would shorten the required time to conduct a large clinical trial. Important factors for establishing surrogacy include biological plausibility, results from studies demonstrating the prognostic value of the surrogate endpoint, and evidence from clinical trials showing that treatment effects on the surrogate endpoint correlate to treatment effects on the clinical outcome. Some data from clinical trials have demonstrated a therapeutic effect on both MRD responses and survival (90, 91), which supports the application of MRD as an adequate surrogate efficacy biomarker. The U.S. Food and Drug Administration (FDA) has issued a guidance document on the regulatory considerations for the use of MRD in clinical trials (https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM623333.pdf). European medicines agency (EMA) *also publishes guideline on the use of MRD as a clinical endpoint in multiple myeloma* studies (https://www.ema.europa.eu/en/documents/scientific-guideline/draft-guideline-use-minimal-residual-disease-clinical-endpoint-multiple-myeloma-studies_en.pdf). Even with these guidelines, there remain challenges regarding how MRD can be utilized as a clinically meaningful endpoint. Technologies for detecting MRD are rapidly evolving, and the sensitivity of MRD testing keeps improving. With changes in assay techniques, the MRD thresholds and the goal of the treatment might become “moving targets”. And the optimal timing for MRD assessment remains unclear and needs to be further investigated.

CONCLUSION

MRD testing is the best way to assess therapeutic response in-depth for patients with acute leukemias, and MRD status has been demonstrated as the strongest independent prognostic factor to predict relapse and survival outcomes. During the past three decades, technological advances have significantly improved MRD detection techniques, making MRD assays more and more sensitive and accurate. Currently, MRD assessment has already become a part of standard care in the management of acute leukemia patients, and the MRD results have been used to

guide risk-adapted therapies as routine care or in clinical trials. However, many questions remain regarding the best detection method, optimal timing and frequency of the tests, optimal assay-specific thresholds, and how to incorporate MRD information accurately into risk-adapted therapies. More studies are needed to fully answer these questions, and there should be ongoing efforts to standardize and harmonize the MRD testing methods and ensure that accurate results can be safely translated into clinical applications.

Conflict of Interest: The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this manuscript.

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