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Progress of Minimal Residual Disease Studies in Childhood Acute Leukemia

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Abstract

Submorphologic (ie, minimal) residual disease (MRD) can be monitored in virtually all children and adolescents with acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL) using methods such as flow cytometric detection of leukemic immunophenotypes or polymerase chain reaction amplification of fusion transcripts, gene mutations, and clonal rearrangements of antigen-receptor genes. Numerous studies have demonstrated the clinical importance of measuring MRD, spurring the design of clinical trials in which MRD is used for risk assignment and treatment selection. Emerging results from these trials suggest that the adverse prognostic impact of low levels of MRD during the early phases of therapy can be diminished by treatment intensification. This article discusses the methods used for detecting MRD in childhood AML and ALL, the data obtained in studies correlating MRD with treatment outcome, the results of the initial trials using MRD, and the practical aspects related to the design of MRD-based clinical studies.

Keywords

Acute myeloid leukemia; Acute lymphoblastic leukemia; Flow cytometry; Polymerase chain reaction

Introduction

For at least four decades, hematologists have known that the kinetics of early response to therapy in patients with acute leukemia predicts the risk of relapse [1]. However, many patients who appear to respond adequately to the initial courses of chemotherapy, as judged by the absence of morphologically identifiable leukemic cells in bone marrow smears, subsequently relapse. For example, among 1,657 patients with acute myeloid leukemia (AML) treated in the MRD AML 10 trial, 1,110 (67%) achieved a complete remission after one course of chemotherapy, but 46% of these subsequently relapsed [2]. Likewise, 373 (93%) of the total cohort of 400 evaluable children with acute lymphoblastic leukemia (ALL) treated in St. Jude Total Therapy Studies XI and XII had no detectable lymphoblasts in the bone marrow by day 25 of remission induction therapy, but nearly one third

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subsequently relapsed [3]. These results suggested that better ways to measure the kinetics of treatment response were required to sharpen its predictive power.

The fundamental methods for detecting submorphologic or “minimal” residual disease (MRD) were first developed in the 1980s and have been steadily refined since then [4]. MRD can currently be monitored by flow cytometry or polymerase chain reaction (PCR). Flow cytometry is applied to detect combinations of cell markers that are expressed in leukemic cells but not in normal bone marrow cells. PCR is applied to detect leukemic rearrangements of immunoglobulin (IG) or T-cell receptor (TCR) genes, fusion transcripts, gene mutations, or overexpressed mRNA. As shown in Table 1 and discussed elsewhere in more detail [4, 5], each method has its own strengths and limitations.

Acute Myeloid Leukemia

Methods

In patients with AML, MRD can be monitored by flow cytometry and by PCR amplification of fusion transcripts, gene mutations, and overexpressed genes. Using combinations of four markers and flow cytometry, leukemia-associated immunophenotypes can be identified in about 95% of patients at diagnosis. A sensitivity of 0.01% can be achieved in around 50% of patients; in those remaining, cell marker expression on leukemic cells is rather heterogeneous, limiting the sensitivity of the assay to 0.1% [6, 7]. By increasing the number of markers that can be simultaneously detected (a possibility afforded by new-generation flow cytometers equipped with more than two lasers), it is likely that a sensitivity of 0.01% could be achieved in virtually all patients.

Fusion transcripts arising from nonrandom genetic abnormalities, such as *RUNX1-RUNX1T1* (or *AML1-ETO*), *CBF β /MYH11*, and *MLL* gene rearrangements, can be used as targets for PCR-based studies of MRD [8]. These are present in approximately 40% of patients and afford a sensitivity of MRD detection of about 0.01%. *FLT3* internal tandem duplications and mutations, as well as *RAS* mutations, could serve as targets but are reportedly unstable and prone to cause false-negative results [9, 10]; their clinical usefulness is unclear. Nucleophosmin (*NPM1*) mutations could also be used as PCR targets, but these are found in less than 10% of children with AML [11], as are immunoglobulin (IG) and T-cell receptor (TCR) gene rearrangements [8]. Finally, the *WT1* gene is overexpressed in AML and measuring its levels by PCR is an option for MRD studies [12], but the exact proportion of childhood AML cases with sufficiently high overexpression and the sensitivity of the test remain to be conclusively determined [13, 14].

Results of Correlative Studies with Treatment Outcome

There is extensive published evidence in support of the clinical significance of MRD measurements by flow cytometry in childhood AML. This approach was used by investigators of the Children's Oncology Group to study 252 children with AML with responsive disease at first remission [15]. The 41 patients (16%) with detectable MRD were 4.8 times more likely to relapse than those with negative MRD. As a caveat, the low stringency in the definition of responsive disease (<30% blast cells by morphologic

examination in a bone marrow aspirate) in this study could have allowed the inclusion of patients with morphologically detectable blasts. Moreover, the sensitivity of the assay used was rather limited (0.5% at the most). Nevertheless, this was the first large prospective study that supported the clinical potential of MRD monitoring in childhood AML. At the same time, our laboratory studied MRD by flow cytometry in a small cohort of patients enrolled in the St. Jude Children's Research Hospital AML97 protocol [6]. The sensitivity of the assay used was at least 0.1%, and 17 (38.6%) of the 44 patients studied after induction I had MRD-positive findings. After excluding the 3 patients who had morphologically detectable leukemic cells, the mean ($\pm$ SE) 2-year overall survival estimate for MRD-positive (0.1%) patients was $30.0\% \pm 17.7\%$ versus $72.1\% \pm 11.5\%$ for MRD-negative patients ($P=0.044$). MRD after induction was the only significant prognostic factor in this series. A study performed in a cohort of 150 patients enrolled in the AML-BFM 98 study also demonstrated that MRD detected by flow cytometry after the first block of chemotherapy was significantly predictive of outcome, even in analyses restricted to patients without detectable blasts by morphology [16]. When the results of flow cytometry were included in a multivariate analysis model controlling for a risk classification schema based on FAB subtype, cytogenetics and blasts on day 15 by morphology, MRD was a slightly better predictor of failure-free survival. Only after adding the additional covariate of blast by morphology on day 28 did the schema have a better predictive value than MRD alone. It should be noted that these investigators studied MRD with markers of variable sensitivity: in some cases, the method allow the detection of leukemic cells only if these were present at a level of 1% or higher. Finally, investigators at the Fred Hutchinson Cancer Research Center studied 63 children and adolescents with AML prior to allogeneic hematopoietic stem cell transplantation (HSCT) in morphologic remission and identified 10 with MRD by flow cytometry [17]. The actuarial overall survival for these 10 patients was 15% versus 67% for those without MRD, and they had a relapse rate after transplantation of 65%, versus 17% for those without MRD.

Although PCR amplification of fusion transcripts is routinely performed at many centers [18], and its clinical significance is well established by studies in adult patients, there is a paucity of reports on the predictive value of this method in childhood AML [19, 20]. One recent study investigated the prognostic value of *WT1* expression levels measured in the peripheral blood of 36 children with AML 2 weeks before HSCT [13]. Of the 11 patients with *WT1* levels higher than those measured in reference samples from healthy individuals, 7 patients relapsed after transplantation, whereas none of the 25 patients with normal *WT1* expression levels had relapsed at the time of the report.

Application of MRD to Guide Therapy

In the multicentric AML02 study, we used MRD measurements for risk assignment [7[•]]. Briefly, patients with 0.1% or more MRD after induction I received subsequent chemotherapy with intensified timing, whereas those with at least 1% MRD received gemtuzumab ozogamicin in addition to cytarabine, daunorubicin, and etoposide; patients with persistent MRD of 0.1% or more were candidates for allogeneic HSCT.

Of the 215 patients studied at diagnosis, 204 (94.9%) had leukemic cells expressing immunophenotypes that could allow MRD studies with a sensitivity of at least 0.1% [7•]. MRD studies were successful in 99% of samples received, demonstrating the feasibility of performing MRD monitoring for AML in a multicentric setting. MRD after induction I was at least 0.1% in 74 (36.6%) of the 202 patients studied; 50 had high levels (≥ 1%) and 24 had lower levels (0.1% to <1%). Despite treatment intensification triggered by MRD results, MRD positivity remained an unfavorable prognostic indicator. Thus, the 3-year cumulative incidences of relapse or induction failure were $38.6\pm5.8\%$ for MRD-positive patients and $16.9\pm3.4\%$ for MRD-negative patients after induction I ($P<0.0001$). However, the incidence of relapse was much higher among patients with MRD (≥ 1%) and the outcome for patients with low levels of MRD after induction I was identical to that of patients with negative MRD [7•]. These results suggest that treatment intensification with currently available therapies may benefit patients with lower levels of MRD after initial chemotherapy, but novel agents are likely to be required to significantly improve the outcome of patients with higher levels of MRD.

Acute Lymphoblastic Leukemia

Methods

In patients with ALL, MRD can be monitored by flow cytometry, PCR amplification of fusion transcripts, and PCR amplification of IG/TCR genes. Leukemia-associated immunophenotypes can be identified in over 95% of patients at diagnosis, and a sensitivity of MRD detection of 0.01% by flow cytometry can be routinely achieved in all these patients [21].

PCR amplification of mRNA transcripts derived from gene fusions can also be used to monitor MRD in ALL. The transcripts typically targeted include *BCR-ABL1*, *MLL-AFF1*, *TCF3-PBX1*, and *ETV6-RUNX1*, which can be identified in about 40% of cases of childhood ALL [18]. Clonal rearrangements of IG and TCR genes occur in about 90% of patients with ALL and afford detection of MRD with a routine sensitivity of up to 0.001% [22]; hence, these targets are widely used to monitor MRD. Because IG and TCR genes may undergo continuing rearrangements during leukemia development, it has been recommended to use at least two different rearrangements as a target in each patient, although this requirement may reduce the capacity to apply the method to only 70% of cases [23]. For both fusion transcripts and IG/TCR genes, quantification of MRD is most frequently performed by using “real-time” quantitative PCR (RQ-PCR) [18, 22]. Results of PCR amplification of IG/TCR genes are generally in good agreement with those of flow cytometry when MRD is at or above the 0.01% level [24–26].

Results of Correlative Studies with Treatment Outcome

Our laboratory used flow cytometry to prospectively measure MRD in the bone marrow of 195 children and adolescents with newly diagnosed ALL enrolled in the Total XIII study at St. Jude Children's Research Hospital [21, 27, 28]. The presence of at least 0.01% MRD on days 19, 46, or subsequent time points during treatment was strongly associated with a higher risk of relapse. A study of 108 patients enrolled in the ALL-BFM 95 protocol in

Austria used flow cytometry to measure MRD levels in bone marrow samples collected on days 33 and 78 of treatment and demonstrated a good concordance between the findings and subsequent relapse [29]. Investigators in the Children's Oncology Group measured MRD in bone marrow collected on day 29 of therapy from 2,086 patients with B-lineage ALL who were enrolled in the 9,900 series treatment protocols and found that 0.01% or more MRD by flow cytometry was significantly associated with a worse outcome [30]. More recently, the Italian cooperative group AIEOP studied bone marrow samples from 830 patients by flow cytometry on day 15 of treatment and identified three risk groups: standard (< 0.1% MRD, 42%), intermediate (0.1%–<10% MRD, 47%), and high (> 10% MRD, 11%). The 5-year cumulative incidence of relapse in these groups was 7.5%, 17.5%, and 47.2%, respectively [31].

Unequivocal evidence for the prognostic importance of MRD is also provided by studies using PCR amplification of IG/TCR genes. In an early study performed at the Flinders Medical Centre in Australia, DNA was extracted from bone marrow slides obtained from 88 children with ALL when they attained remission; 38 patients had detectable PCR IG signals, and 26 of these patients subsequently relapsed, whereas relapse occurred in only 6 of the 50 patients with negative PCR findings [32]. In 178 patients enrolled in a European Organization for Research and Treatment of Cancer (EORTC) protocol, levels of MRD among bone marrow mononuclear cells quantified by PCR amplification of IG/TCR genes during the first 6 months of therapy were significant predictors of outcome [33]. In 240 patients treated on the International BFM Study Group protocols, combined information from MRD measurements performed on days 33 and 78 allowed the definition of three risk groups, each with a significantly different prognosis [34]. The initial observations of this group of investigators were consolidated by a more recent report indicating that among 3,184 patients with B-lineage ALL, those considered to have standard risk by MRD criteria (42%) had a 5-year event-free survival of 92.3%, in contrast to 50.1% for the 6% of patients who were high-risk and 77.6% for the 52% of patients with intermediate-risk ALL [35]. In 284 patients with B-lineage ALL studied by the Dana-Farber Cancer Institute ALL Consortium, the 5-year risk of relapse was 44% in the 108 patients with detectable MRD at the end of remission induction therapy and 5% in the remaining 176 patients [36]. Finally, a recent study from our laboratory in 455 patients with B-lineage ALL confirmed the adverse prognostic impact of MRD as detected by PCR amplification of IG and TCR genes at the end of remission induction therapy and demonstrated that patients with low levels of MRD (ie, 0.001% to <0.01%, $n=63$) had a significantly higher risk of relapse than those with findings below 0.001% ($n=319$): patients with this low level of MRD had a $12.7\% \pm 5.1\%$ (SE) cumulative risk of relapse at 5 years, compared with $5.0\% \pm 1.5\%$ for those with lower or undetectable MRD ($P=0.0467$) [37].

There is strong evidence that MRD measurements by flow cytometry or PCR amplification of IG and TCR genes can also predict clinical outcome in patients with specific subtypes of newly diagnosed ALL as defined by presenting features [28, 38–40], in those with first-relapse ALL who achieve a second remission [41–44], and in patients with “isolated” extramedullary relapse [45]. Detection of MRD before allogeneic HSCT is associated with an increased risk of relapse after transplantation [46].

Application of MRD to Guide Therapy

The feasibility of flow cytometric MRD studies in a large multicentric study was demonstrated by investigators of the Children's Oncology Group who studied 2,143 patients with B-lineage ALL enrolled on 9,900 series treatment protocols [30]. Samples were shipped to the reference laboratory at the Johns Hopkins Medical Institutions. Day 29 samples were submitted from 2,086 patients (97.3%) to be studied for MRD: in only 4% of cases, sample cellularity was too low or the immunophenotype of the leukemic cells (determined at diagnosis) was not sufficiently distinct to allow a sensitivity of detection of 0.01%. Overall, a test with the sensitivity of at least 0.01% was performed in 92% of patients [30]. The feasibility of PCR amplification of IG/TCR genes in the context of a large multicenter study was clearly shown by investigators of the AIEOP-BFM ALL 2,000 trial, who studied bone marrow samples on days 33 and 78 of therapy [23]. Samples were shipped to six reference laboratories and adequate data for MRD-based stratification were obtained for 2,594 (78%) of the 3,341 patients.

We monitored MRD by flow cytometry and PCR amplification of IG/TCR genes in patients with newly diagnosed ALL who were enrolled in the St. Jude Total XV trial. Of the 492 patients, 482 (98%) were monitored by flow cytometry and 403 (82%) by PCR, which was used only in patients with B-lineage ALL; the two methods in combination could be applied to study 491 (99.8%) of the 492 patients [47•]. The remaining patient's cells had no suitable immunophenotype or IG/TCR gene rearrangements, but had an *MLL-AF9* fusion transcript, which was used as a target for MRD studies by RQ-PCR. Any patient with at least 1% MRD in the bone marrow on day 19 of remission induction or 0.01% to <1% residual leukemia after completion of 6-week induction therapy, was considered to have standard-risk (ie, intermediate-risk) ALL and received more intensive remission induction and/or post-remission therapy. Patients with 1% or more MRD after completion of induction therapy were assigned to the high-risk group and were candidates for HSCT. This treatment strategy abrogated the unfavorable prognostic weight of MRD 0.01% to <1% at the end of induction, although 1% or higher MRD at this time point remained a significant predictor of relapse [47•].

In our current trial, Total XVI, we use MRD levels on day 15 and day 42 for treatment assignment. Patients with MRD of 1% or higher on day 15 receive intensified remission induction therapy; further intensification is reserved for patients with at least 5% leukemic cells. On the other hand, patients with MRD less than 0.01% on day 15 receive a slightly less intensive reinduction therapy and lower cumulative doses of anthracycline. Patients with standard-risk ALL who have MRD of 0.01% or higher on day 42 are reclassified as high-risk; patients with 1% or higher MRD are eligible for HSCT. Based on the observation that MRD levels in peripheral blood are similar to those in bone marrow in patients with T-lineage ALL [48, 49], it is our current practice to use blood instead of marrow to monitor MRD after day 42 in patients with T-lineage ALL. (Bone marrow is used for B-lineage ALL patients.)

We also use MRD measurements to direct treatment for patients with first-relapse ALL who achieve a second remission. In this setting, patients with persistent MRD are candidates for allogeneic HSCT. For all patients who undergo transplantation, the aim is to perform the

transplant when MRD is at the lowest levels and possibly undetectable. Monitoring of MRD after HSCT informs decisions about reducing immunosuppressive therapy, donor lymphocyte infusions, and preparation for a second transplant.

Conclusions

The definition of remission in patients with acute leukemia is shifting from the criteria defined by morphology to those set by MRD assays. The cutoff level to define MRD positivity in AML varies. We adopted the 0.1% threshold, which can be achieved in 95% of patients with current flow cytometry methods and which provides prognostic information when applied at the end of the first and second blocks of induction [6, 7]. Patients with 1% or higher MRD appear to have a markedly higher risk of relapse than those with lower levels [7]. Whether detection of MRD at levels below 0.1% will provide useful prognostic information in AML is unclear. The most commonly used cutoff level to define MRD positivity in ALL is 0.01%, as this represents the typical limit of detection for routine flow cytometric and molecular assays. The 0.01% threshold can be used to identify patients with an overall higher risks of relapse [21, 23, 27, 28, 30, 34], but the relapse risk increases for patients with higher levels of MRD, such as 0.1% or higher [23, 29, 33, 34, 36] or 1% or higher [28]. Among patients with MRD less than 0.01%, typically regarded as “MRD-negative,” those with levels of MRD at 0.001% or more have a higher risk of relapse than those with lower levels [37], corroborating the concept that the risk of relapse is directly proportional to the level of MRD at the end of remission induction. Measurements of MRD in children with ALL at earlier points (2 to 3 weeks after diagnosis) may also provide important information and allow early adjustment of treatment intensity [21, 31, 50, 51].

Application of MRD for risk stratification suggests that the prognostic impact of low levels of MRD after the initial phases of chemotherapy can be considerably suppressed or abrogated by subsequent treatment intensification [7, 47]. As a caveat, these trials did not specifically address the usefulness of MRD in a randomized fashion, so it can be argued that the overall improvement in outcome for patients with low levels of MRD was due primarily to other changes in the protocol rather than to the adoption of an MRD-directed strategy. Though this is true, it is difficult to justify addressing this question in a clinical trial, considering the overwhelming evidence of the prognostic strength of MRD. Indeed, one would be hard-pressed to find a paper reporting the results of a randomized study testing the value of using other established prognostic factors, such as karyotype or molecular genetics, to guide therapy.

Genetic abnormalities are clearly associated with MRD levels during the initial phases of therapy. In AML, we found that the odds of having detectable MRD after induction I were significantly lower for patients with core binding factor leukemia t(8, 21)/*AML1-ETO* or inv(16)/*CBF β -MHY11* and significantly greater for those with *FLT3-ITD*-positive leukemia [7]. In ALL, MRD during and at the end of induction therapy was much more prevalent in those with *BCR-ABL1* ALL and less prevalent in patients with *ETV6-RUNX1*, hyperdiploid (>50 chromosomes), and *TCF3-PBX1* ALL [52]. Recent studies have identified novel subtypes of ALL with a significantly higher prevalence of MRD. Thus, patients with B-lineage ALL and abnormalities of the *IKZF1* gene [53], and patients with early T-cell

precursor (ETP)-ALL had significantly higher levels of MRD during remission induction therapy [54]. Finally, MRD measurements also correlate with the gene expression profiles of leukemic lymphoblasts [55–57] and with germline gene polymorphisms [58].

MRD assays are complex and require expertise to be performed well. The relationship between cellular and biologic features identifiable at diagnosis and treatment response according to MRD raises the question as to whether MRD studies could eventually be replaced by risk classification schemas based on presenting features. Regardless of the extent and depth of studies performed on tumor and host cells at diagnosis, it is likely that MRD studies will continue to provide unique and clinically valuable information, as they reflect the composite effect of presenting features as well as therapeutic variables such as drug dosages, timing, pharmacokinetics, and compliance. Moreover, in addition to their capacity to predict outcome on the basis of early response to therapy, MRD methods can also be used to recognize leukemia relapse before it is morphologically overt, to determine the leukemia burden before transplantation, and to measure the efficacy of a treatment regimen in relation to that of its predecessor. Therefore, MRD studies may contribute in many ways to the improved clinical management of patients with leukemia.

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Table 1

Assays to monitor MRD in pediatric acute leukemia: their main features

Method	Target	Applicability/Sensitivity		Main strengths	Main potential pitfalls
		AML	ALL		
Flow cytometry	Leukemic immunophenotypes	95%/0.1%	95%/0.01%	Widely applicable; rapid; accurate quantitation of MRD.	Lack of expertise may lead to false-positive and false-negative results.
PCR	Fusion transcripts	40%/0.01%	40%/0.01%	Provides overview of hematopoiesis	Phenotypic shifts require the use of multiple sets of markers.
				Rapid; unequivocal link with leukemic/preleukemic clone	Uncertain quantitation of MRD because of unknown number of transcripts per cell.
					RNA degradation may produce false-negative results; cross-contamination may produce false-positive results
	IG/TCR rearrangements	<10%	90%/0.001%	High sensitivity; accurate quantitation of MRD	Laborious because reagents are tailor-made for each patient.
					Oligoclonality and clonal evolution may produce false-negative results.
					If more than one target is required, ~ 30% of patients are excluded.
	WT1	??	??	Potential as a universal MRD marker	Background from normal cells may produce false-positive results.
					Percentage of cases with sufficient overexpression is unclear.
	<i>NPM1</i> mutations; <i>N-RAS</i> mutations; <i>FLT3</i> mutations	~10%/0.01%	–	Rapid; unequivocal link with leukemic/preleukemic clone	Limited use in pediatric leukemia; instability; not validated in clinical studies

ALL acute lymphoblastic leukemia, *AML* acute myeloid leukemia, *IG* immunoglobulin, *MRD* *minimal residual disease*, *PCR* polymerase chain reaction, *TCR* T-cell receptor