Curr Hematol Malig Rep (2012) 7:170–177
DOI 10.1007/s11899-012-0115-4

Should Minimal Residual Disease Monitoring in Acute Lymphoblastic Leukemia be Standard of Care?

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Published online: 29 February 2012

Abstract In acute lymphoblastic leukemia (ALL), the advent of methods to measure disease not detectable by morphology, ie, minimal residual disease (MRD), has set a new standard to define remission. The clinical importance of MRD has been demonstrated by numerous studies using either flow cytometry or polymerase chain reaction and involving thousands of patients. Results are in remarkable agreement on the association between MRD persistence and risk of subsequent relapse, regardless of the MRD detection method used. More recent data indicate that MRD can also be informative in specific subgroups of ALL patients, such as infants or those with T-lineage ALL. Hence, MRD is now being used in clinical trials to inform treatment decisions and guide patients' clinical management. This article reviews MRD methodologies and clinical applications with emphasis on recently reported technical advances and prognostic associations, and the practical issues related to the implementation of MRD monitoring in the clinic.

Keywords Acute lymphoblastic leukemia · Minimal residual disease · Flow cytometry · Polymerase chain reaction · Remission · Prognosis

Introduction

Soon after hematologists recognized that the aim of acute lymphoblastic leukemia (ALL) treatment should be cure rather than palliation, it was noticed that patients who required longer treatment to achieve remission had a lower likelihood to maintain remission [1], a concept that has been confirmed by numerous studies [2]. However, most relapses still occur among "good responders." Because the definition of remission depends on the capacity to detect leukemic lymphoblasts by their morphology, which is notoriously limited, it is reasonable to assume that a substantial proportion of "good responders" still have considerable levels of residual leukemia ("minimal residual disease"; MRD).

A PubMed search using the terms "leukemia" and "minimal residual disease" leads to the first paper that clearly demonstrated the presence of leukemic cells in the bone marrow of patients with ALL in morphologic remission [3]. In that study, reported three decades ago, Ken Bradstock, George Janossy, and colleagues capitalized on their previous observation that T-lineage ALL lymphoblasts expressed terminal deoxynucleotidyl transferase (TdT) and T cell antigens (a phenotype absent in the bone marrow of healthy donors); they used rabbit antisera against these markers and immunofluorescence microscopy to search for residual leukemia in patients with T-lineage ALL and detected cells with the immunophenotype of T-lineage ALL, ranging from 0.5% to 5% of bone marrow mononucleated cells, in 6 of the 18 patients studied. When it became possible to track leukemic cells in B-lineage ALL by using flow cytometry or polymerase chain reaction (PCR), similar observations were made [4–10], thus establishing the fact that post-treatment samples in morphologic remission could contain a variable amount of leukemic cells ranging from less than 1 in 10,000–100,000 to 5% or more. The wide range of MRD levels measured among patients in remission suggested the potential for correlations with relapse. As discussed here, a large body of evidence involving thousands of patients indicates that MRD monitoring in patients with ALL should now be standard of care.

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MRD Assays: Recent Technical Advances

Currently available methods to monitor MRD in patients with ALL include flow cytometric detection of leukemia-associated immunophenotypes, PCR amplification of fusion transcripts, and PCR amplification of immunoglobulin (IG) and T-cell receptor genes (TCR). The technical aspects of these tests, as well as their specific strengths and limitations have been reviewed elsewhere [2, 11]. Leukemia-associated immunophenotypes can be identified in virtually all patients at diagnosis; flow cytometry targeting these immunophenotypes affords a sensitivity of MRD detection of 0.01%. Oncogenic fusion transcripts, such as *BCR-ABL1, MLL-AFF1*, *TCF3-PBX1,* and *ETV6-RUNX1,* can be identified in approximately 40% of cases of childhood ALL, while clonal rearrangements of IG and TCR genes occur in approximately 90% of patients; by using PCR targeting either type of genetic signature, a sensitivity of 0.001% can be attained. Results of flow cytometry are usually in agreement with those of PCR amplification of IG/TCR genes if the level of MRD is at or above the 0.01% (1 leukemic cell in 10,000 normal bone marrow mononucleated cells) [12–14].

Although flow cytometry is widely applicable and has proven to be well-suited for clinical studies of MRD, immunophenotypic differences between ALL cells and normal lymphoid cells can be hard to perceive without extensive expertise. This task might be helped by the availability of additional markers which should make the distinctive features of ALL more evident and therefore facilitate their recognition. To this end, we compared genome-wide gene expression of lymphoblasts from 270 patients with newly diagnosed childhood ALL to that of sorted normal $CD19^{+}CD10^{+}$ B cell progenitors from four healthy donors [15•]. We then selected 30 genes differentially expressed by ≥ threefold in at least 25% of cases of ALL (or 40% of genetic subtypes of ALL) and tested their expression by flow cytometry in 200 B-lineage ALL and 61 non-leukemic bone marrow samples. We included recovering bone marrow samples, which pose a particular challenge in MRD studies because of their high proportion of normal immature lymphoid cells. Of the 30 markers, 22 (CD44, BCL2, HSPB1, CD73, CD24, CD123, CD72, CD86, CD200, CD79b, CD164, CD304, CD97, CD102, CD99, CD300a, CD130, PBX1, CTNNA1, ITGB7, CD69, CD49f) were found to be differentially expressed in up to 81% of ALL cases. When the new markers were applied to study MRD in clinical samples, they yielded results that correlated well with those obtained by standard flow cytometric methods and PCR-based analyses [15•]. Moreover, sequential studies during treatment and diagnosis-relapse comparisons documented their stability. The addition of the new markers to established panels allowed the identification of unique leukemia profiles in all patients and afforded the detection of 1 leukemic cell in 100,000 normal bone marrow cells [15•]. Initial successful attempts to automate, at least in part, the process for interpretation of flow cytometric MRD data are now being reported. Pedreira et al. [16] applied a probabilistic approach based on pattern classification tools and the Bayes theorem to distinguish leukemic and normal peripheral blood B cells. Fiser et al. [17] developed a method based on hierarchical clustering analysis and Mahalanobis distance measure which, when applied to ALL follow-up samples, yielded results that correlated well with standard procedures. These new approaches, if incorporated into easily accessible software, should be useful for learning and quality control; ultimately, they may alleviate the interpretation process or automate it entirely.

One limitation of current studies of MRD based on PCR amplification of IG/TCR genes is that specific PCR primers need to be developed for each patient and, thus, PCR assay conditions need to be individually optimized. The process is laborious, expensive, and time consuming. A new approach is to use consensus primers which can be used universally to amplify all rearranged IG or TCR segments at diagnosis and subject them to high-throughput parallel sequencing [18]. The frequency of the different rearrangements is then measured, identifying all clonal rearrangements above normal background; the process is repeated in follow-up samples to identify the prevalent sequences determined at diagnosis. Faham et al. [19] studied diagnostic and follow-up samples from 10 ALL patients. The sequencing-based method that they developed identified all five samples that were MRD-positive according to established flow cytometry and PCR methods, with highly concordant estimates of MRD levels, demonstrating the reliability of the new approach. Notably, among the remaining five samples, scored as MRD-negative by both flow cytometry and conventional PCR, the sequencing method detected residual unequivocal leukemic sequences at a very low level (about 1 in a million) in one of the samples, suggesting potential for extraordinary sensitivity.

Clinical Significance of MRD

Measurement of Early Treatment Response

Correlative studies from different groups have demonstrated that MRD testing during and at the end of remission induction therapy, and in the early phases of post-remission treatment provide strong prognostic information in childhood ALL [20–27, 28•, 29•, 30]. Importantly, the prognostic strength of MRD typically exceeds that of presenting clinical and biological features. Thus, MRD measurements on days 33 and 78 yielded a risk-assignment schema that was superior to one based on leukocyte count, age, early response to prednisone, and genetic subtype in a study of 3184 B-lineage ALL patients enrolled in the AIEOP-BFM ALL 2000 protocol [29•]. Corroborating the independent predictive nature of

MRD in childhood ALL are studies focusing on specific subgroups of patients. Thus, Van der Velden et al. [31•] studied MRD in 99 infants with ALL enrolled in the Interfant-99 protocol and found that all patients classified as "high-risk" because of MRD ≥ 0.01% at the end of induction and/or consolidation (26%) relapsed, while relapse occurred in only 13% of those with MRD < 0.01% at both time points (44%); the remaining patients had a relapse rate of 31%. Conter et al. [29•] showed that an MRD-based risk classification was predictive of outcome among subsets of patients defined by *TEL-AML1*, high hyperdiploidy, or *BCR-ABL1*. Finally, Schrappe et al. [32•] studied 464 patients with T-ALL and found that MRD < 0.01% at the end of induction was the most favorable prognostic factor, with patients converting to MRD negativity at the end of consolidation also having a favorable outcome, while patients with MRD ≥ 0.1% at this time point had a high relapse hazard.

Several studies have also demonstrated the clinical impact of MRD in adult patients. The German Multicenter Study Group for Adult ALL studied 196 patients younger than 65 years; eligibility criteria included leukocyte counts < 30×10^9/L for patients with B-lineage ALL or < 100×10^9/L for those with T-ALL, absence of *MLL-AFF1* or *BCR-ABL1* gene fusions, and achievement of complete remission after the first phase of induction therapy [33]. MRD was measured on days 11 and 24 and thereafter; patients who had MRD < 0.01% at both time points (10%) had a 3-year relapse rate of 0%; those with MRD ≥ 0.01% until week 16 (23%) had a relapse rate of 94%; and the relapse rate for the remaining patients was 47%. This group also monitored MRD prospectively in samples collected from 105 patients who had completed the first-year chemotherapy, and were MRD-negative prior to the study: MRD became positive in 28 patients followed by relapse in 17; only 5 of the 77 patients who remained MRD-negative relapsed [34]. MRD was studied at the end of consolidation in patients enrolled in the Northern Italy Leukemia Group-ALL 09/00 protocol: 5-year overall disease-free survival was 14% for the 54 patients with MRD ≥ 0.01% and 72% for 58 with MRD < 0.01% [35]. Investigators of the UKALL XII/ECOG2993 trial studied MRD in 161 patients with non T-lineage, *BCR-ABL1*-negative ALL and found that the relative risk of relapse was 8.95 (2.85–28.09)-fold higher in patients who were MRD-positive after phase 2 induction than in those who were MRD-negative [36•]. Finally, in a study of 116 patients with Philadelphia-chromosome negative ALL enrolled in the Polish Adult Leukemia Group ALL 4-2002 trial, MRD ≥ 0.1% after remission induction therapy was an independent predictor for relapse [37].

Pane et al. [38] evaluated MRD after induction and after consolidation in 42 adults with *BCR-ABL1* ALL who achieved remission after a high-dose daunorubicin induction schedule. Those who had a > 2 log reduction of residual disease after induction and > 3 log reduction after consolidation therapy (n=28) had a 27% disease-free survival while this was 0% for the remaining patients. By contrast, Yanada et al. [39] studied MRD in 100 adult patients with *BCR-ABL1* ALL treated with imatinib-containing chemotherapy and found that negative MRD at the end of induction therapy was not associated with longer relapse-free survival or a lower relapse rate. Of note, we found that MRD was detectable at the end of induction in 16 of 18 children with *BCR-ABL1* ALL treated without imatinib as compared to 1 out of 5 who received imatinib as part of the remission induction therapy (P=0.008; D Campana, CH Pui, S Jeha, unpublished observations).

Monitoring MRD Post-Relapse and Prior to Transplant

MRD studies are also clinically informative in children with first-relapse ALL who achieve a second remission [40, 41]. Paganin et al. [42] studied 60 such patients and found that MRD after the first course of chemotherapy was associated with outcome: 3-year event-free survival (EFS) was 73% for patients with undetectable MRD, 45% for those with detectable MRD below 0.01%, and 19% for those with MRD ≥ 0.01%. Raetz et al. [43] examined the significance of MRD after each block of reinduction therapy in 77 children with relapsed ALL: MRD-negative patients after all three blocks of therapy (n=21) had a 12-month EFS of 86%±8% while this was 19%±10% for those with persistent MRD (n=16); patients who were MRD-positive only at the end of block 1 (n=30), or were MRD-positive at all three time points but with a >1 log reduction in MRD levels (n=10), had an EFS of 73%±8% and 70%±16%, respectively. Of note, Hagedorn et al. [44] studied 64 patients with apparently isolated extramedullary relapse and found MRD in bone marrow in 57 (≥ 0.01% in 46).

The prognostic significance of MRD prior to allogeneic hematopoietic stem cell transplant (HSCT) in children and adolescents with ALL is also well established [45, 46]. Bader et al. [47•] measured MRD in 91 children with relapsed ALL receiving HSCT in second or subsequent remissions and found that probability of EFS was 0.27 for the 45 patients with MRD ≥ 0.01% prior to HSCT compared with 0.60 for the 46 patients with MRD < 0.01%; multivariate Cox regression analysis showed that MRD was the only independent prognostic factor in this cohort. The Northern Italy Leukemia Group studied MRD in 43 adult patients with ALL undergoing HSCT and found that the relapse rate at 36 months post-transplant was 0% and 46% for patients who were MRD-negative (n=12) and MRD-positive (n=31), respectively [48]. Patel et al. [36•], however, found that MRD prior to allogeneic HSCT was not significantly predictive of a higher risk of relapse in their series: 23 recipients were MRD negative (< 0.01%) and 4 relapsed as compared to 2 of the 13 MRD-positive patients. Interestingly, MRD positivity prior to autologous transplant did predict relapse in this study: 3 of the

4 patients who had MRD-positive harvest samples relapsed at 3, 6, and 9 months after the transplant, while none of the 5 who received an MRD-negative harvest sample relapsed at a median follow-up of 8 years after transplant. The European Study Group for Adult ALL evaluated the prognostic significance of MRD in 123 patients before autologous transplant and found that the 5-year probability of leukemia-free survival was significantly higher for patients with MRD < 0.01% (57%) as compared to that of patients with MRD ≥ 0.01% (17%), a difference that was primarily due to the results of the 46 patients with T-lineage ALL (62% vs 8%) [49]. In the study by Yanada et al. [39] in *BCR-ABL1* ALL patients receiving imatinib, increasing MRD levels were followed by relapse in 12 of the 13 who did not receive allogeneic HSCT, as compared to 6 of 16 among patients who received transplant. Wassmann et al. [50] studied 27 patients with *BCR-ABL1* ALL who received imatinib upon detection of MRD after HSCT. BCR-ABL1 transcripts became undetectable in 14 of 27 patients, after a median of 1.5 months; these patients remained in remission for the duration of imatinib treatment. By contrast, failure to achieve MRD negativity shortly after starting imatinib predicted relapse, which occurred in 12 of 13 patients after a median of 3 months.

Practical Considerations for Implementing MRD Testing

Time Points and Cutoff Levels

MRD is typically measured at predetermined time points during therapy, with extra assays performed earlier than planned if recurrent disease is suspected. The treatment intervals for MRD measurement are usually dictated by long-established practices at each cancer center or cooperative group. For example, because the end of remission induction bone marrow sample at St Jude Children's Research Hospital was historically obtained 6 weeks from diagnosis, after 4 weeks of remission induction therapy and 2 weeks of recovery, MRD studies were performed at that time [22, 24]. The clinical significance of MRD varies depending on the time of treatment at which it is measured. Therefore, when MRD is implemented to guide therapy, it is usually measured at the same time points used in the preceding correlative studies.

In the current Total XVI protocol for patients with newly diagnosed childhood ALL at St. Jude Children's Research Hospital, MRD levels in bone marrow on days 15 and 42 are used for risk assignment. Patients with MRD ≥ 1% on day 15 receive intensified remission induction therapy; with further intensification for patients with MRD ≥ 5%. Patients with MRD <0.01% on day 15 receive less intensive reinduction therapy and lower cumulative doses of anthracycline. Patients with standard-risk ALL according to presenting features who have MRD ≥ 0.01% on day 42 are reclassified as high-risk; patients with MRD ≥ 1% at that point are eligible for HSCT in first remission. Post-remission studies of MRD in B-lineage ALL patients are performed only in those who are MRD-positive on day 42, while all patients with T-lineage ALL are periodically monitored post-remission using peripheral blood [51]; persistent or emerging MRD is an eligibility criteria for transplant. For any patient scheduled for transplant, additional courses of chemotherapy may be given to reduce MRD as much as possible. MRD at the end of remission re-induction is also used by the St. Jude investigators to guide treatment for patients with first-relapse ALL. Those with MRD ≥ 0.01% are eligible for allogeneic HSCT; achievement of MRD negativity (with other favorable clinical features) is an indication for proceeding with chemotherapy. Finally, monitoring MRD post-transplant is done monthly for the first 3 months, then at 6 and 12 months, and yearly thereafter. The COG AALL08B1 protocol for children with B-lineage ALL includes MRD measurements in peripheral blood on day 8 and in bone marrow on day 29 [27]. MRD ≥ 0.01% on day 29 indicates high-risk or very high-risk leukemia, depending on presenting features, and MRD ≥ 1% on day 8 indicates high-risk leukemia even if MRD on day 29 is < 0.01%. Bruggemann et al. [11] thoroughly reviewed the application of MRD in European trials.

Methodology

Routine monitoring of MRD with delivery of results in a timely fashion is feasible in the majority of patients. In the St. Jude Total XV trial for children with newly diagnosed ALL, we used flow cytometry and/or PCR amplification of IG/TCR genes to monitor MRD (PCR studies were performed only in patients with B-lineage ALL) [52••]. Of the 492 patients enrolled, 482 (98%) were monitored by flow cytometry, and 403 (82%) by PCR; the two methods combined could be applied to study 491 of 492 (99.8%) of the patients, with the single remaining patient being monitored by targeting the *MLL-MLLT3* fusion transcript [52••]. According to the requirements of the protocol, MRD results on day 19 were provided within 24 h, while those at later time points were released within 1 week of sample receipt. Most samples in this study were from a single institution. In large multicentric studies, compliance with sending samples for MRD testing, and quality of samples due to shipping delay, may not be as high. However, in the reported COG experience [27], day 29 samples were submitted from 97% of patients to be studied for MRD, and 92% of patients had successful studies.

Ideally, both flow cytometry and PCR amplification of IG/TCR genes should be available to monitor MRD. Thus, all patients can have access to the test and unexpected

results with one technique can be validated by the other. However, this is unrealistic for most centers. Then, which method should have priority? Because of the time required to develop a patient-specific PCR assay (often more than 2 weeks; please see above for newer PCR methods which may be more rapid), flow cytometry is preferable for studies at very early time points during therapy, while PCR might be best for studies at the end of therapy or post-HSCT because of its higher sensitivity. On the other hand, flow cytometry, if adapted to do so, can provide information beyond detection of MRD, such as a description of normal hematopoiesis, identification of drug-resistant cell subsets, and determination of the pathways targeted by tyrosine kinase inhibitors [53]. Hence, it may ultimately have a broader informative potential than PCR. Although it has been estimated that PCR is more expensive than flow cytometry, such calculations are complex and, in our experience, the two methods have comparable costs. Regardless, MRD testing provides true value for money and can help to achieve significant savings in health care costs.

MRD tests are high complexity assays and require specific expertise. This necessity is widely accepted for PCR testing, which is typically performed in a few, highly specialized, laboratories. However, perhaps because flow cytometers are widely available and sample processing for MRD resembles that used for routine leukemia immunophenotyping, it is often not understood that flow cytometric studies of MRD also need specialized skills. Indeed, detection of rare events requires a degree of meticulousness that goes well beyond that of cell marker analysis of bulk cell populations. Moreover, to identify leukemic cells by flow cytometry, one needs to know exactly what are the boundaries between normal and abnormal expression of cell markers; this knowledge requires extensive analysis of normal and recovering samples to build a reference database. Hence, many centers prefer to submit samples to reference laboratories with proven expertise. For example, all MRD samples from children with ALL enrolled in COG trials are sent to one of two laboratories at Johns Hopkins University and University of Washington. Other groups have opted to share the workload among a larger number of laboratories [54–56]. This approach builds local expertise and fosters standardization but takes time, effort, and expense to reach satisfactory concordance; this approach is also slow in catching up with the latest methodological developments. An alternative approach, which can be implemented even if resources are limited, is to perform MRD studies by flow cytometry only during remission induction therapy (eg, on day 15–26). Because of the paucity of normal lymphoid progenitors at this stage of therapy, studies during this treatment interval are technically much simpler and less expensive [57], while still being clinically very informative [28•, 58]. With the aid of newer computerized analytical methods, well standardized reagents, shared reference databases, and web-based remote training and consultation, even laboratories with little experience in MRD (but solid cell processing protocols) should be able to deliver interpretable MRD results.

Conclusions

MRD testing in leukemia has redefined remission. It is unquestionable that MRD levels are clinically meaningful and that their informative value cannot be matched by any other currently available parameter. The evidence supporting the prognostic significance of MRD is particularly strong in ALL, with studies performed with different techniques, involving a wide range of age groups and genetic subtypes, and applied to different chemotherapy regimens; arguably, no other currently used prognostic parameter is backed up by such an overwhelming body of supporting data. Data from the St. Jude Total XV trial for children and adolescents with ALL suggests that the adverse prognostic impact of MRD during the early phases of treatment can be mitigated by subsequent treatment intensification [52••]. Hence, the prognostic significance of MRD needs to be reevaluated in the context of different therapeutic regimens. Nevertheless, in Total XV, high MRD (≥ 1%) at the end of induction remained an adverse predictor of outcome (the only independent prognostic factor, in addition to CNS3 status at diagnosis or traumatic lumbar puncture) despite treatment intensification.

In the MRD era, the relative importance of other prognostic ALL parameters needs to be reconsidered. The predictive power of clinical features such as age and leukocyte counts, at least in pediatric ALL, appears to be far too weak in comparison with MRD. The prevalence of MRD differs among different genetic subtypes of childhood ALL. During and at the end of induction therapy, it is significantly more prevalent in patients with early T-cell precursor (ETP)-ALL [59] or abnormalities of the *IKZF1* gene [60], and less prevalent in those with *ETV6-RUNX1*, hyperdiploid (> 50 chromosomes), and *TCF3-PBX1* ALL [2]. Associations between MRD and genetic features of leukemic lymphoblasts [61–63], and germline gene polymorphisms [64, 65], have also been described. However, none of these features can predict MRD with sufficient accuracy, and MRD has shown prognostic significance among ALL subtypes when sufficient number of patients were studied [29•, 31•, 32•]. Risk classification algorithms combining genetic presenting features and MRD are likely to offer the highest predictive accuracy, allowing both adjustments in the intensity of remission induction therapy before MRD is measured as well as MRD-directed therapy.

Morphological analysis of bone marrow smears is fraught with subjectivity and imprecision. With MRD testing available, the credibility of morphology to detect residual leukemic

blasts is increasingly put in question. Nevertheless, this practice is deep-seated in the clinical practice and may provide information complementary to MRD testing about overall bone marrow cellularity (particularly if performed on bone marrow biopsies), tri-lineage regeneration, or presence of myelodysplasia. Some hemopathologists insist in having relatively large amounts of bone marrow from the first aspirate for morphologic analysis but it should be clear that MRD testing performed on subsequent, more hemodiluted aspirates may seriously affect the reliability of the MRD assay [66].

Beyond quantifying leukemia cytoreduction, MRD studies have multiple other applications in the management of patients with ALL, such as determining remission status prior to and after transplantation, and detecting early relapse. In sum, they offer the opportunity to tailor intensity of therapy with unprecedented accuracy. The introduction of reliable MRD testing as a standard of care would provide the platform supporting true personalized medicine for patients with ALL.

Disclosure D. Campana: Consultant for Celgene.

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