
Guest Commentary

Minimal Residual Disease Detection in Acute Lymphoblastic Leukemia: Real Improvement With the Real-Time Quantitative PCR Method?

Risk-directed therapy is important to avoid overtreatment or undertreatment of childhood acute lymphoblastic leukemia (ALL), a disease that is now curable in approximately 80% of patients. Adverse prognostic factors include increased initial leukocyte count ($>50,000/\mu\text{L}$, mainly in B-lineage ALL), patient age less than 1 year (especially in association with *MLL* rearrangement), t(9;22) chromosomal translocation, and hypodiploidy (<45 chromosomes per cell) (1–9). Growth of leukemic cells in vitro and their resistance to drug treatment in vitro also predict a poor clinical response (10–12). Favorable prognostic factors include hyperdiploidy (>50 chromosomes per cell, especially in association with the trisomies 4, 10, and 17) and the *TEL-AML1* gene fusion (1,2,13). However, none of these factors has high precision in predicting clinical outcome.

Response to remission induction therapy is a powerful and independent prognostic factor. Early studies were based on morphologic examination of bone marrow or blood after 1 to 2 weeks of remission induction (14). These measures are powerful discriminators of outcome but are inherently subjective and cannot discriminate between different levels of leukemic cells below 1%. A recent study showed that some patients with no morphologically identifiable leukemic cells had high levels of residual disease (15), with the leukemic cells being morphologically indistinguishable from normal lymphocytes.

Great efforts have been made during the past 10 to 15 years to develop sensitive and objective methods for the detection of minimal residual disease (MRD), defined as disease below the limit of detection by morphologic examination. It was reasoned that such methods would provide a more precise evaluation of the leukemic cell response in vivo and could forecast the re-emergence of leukemic cells prior to overt hematologic relapse. The two most common methods used for MRD assessment are the detection of cells expressing abnormal immunophenotypes by flow cytometry, and of leukemia-associated molecular targets by the polymerase chain reaction (PCR) (16–18). Assessment of MRD by flow cytometry can routinely detect as few as

0.01% leukemic cells in the bone marrow or peripheral blood, and this method is applicable to more than 90% of ALL cases (19). Targets suitable for PCR studies are leukemic-specific fusion transcripts, such as the *BCR-ABL*, *E2A-PBX1*, and *TEL-AML1* gene fusions, present in 35% to 40% of cases (1,2), and clone-specific immunoglobulin and T-cell receptor gene rearrangements, found in 85% to 90% of cases. PCR-based assays usually detect one leukemic cell in 10^4 to 10^6 normal cells (18).

Although it is not yet proven that planning treatment according to MRD results will improve outcome or decrease toxicity, numerous studies have demonstrated that MRD measured with these highly sensitive assays is a powerful and independent prognostic indicator (20–23). It was found that rapid cyto-reduction of leukemic cells during or following induction therapy, as indicated by undetectable or less than 0.01% leukemic cells, was associated with an excellent clinical outcome (3-year relapse rate $<5\%$), while patients with MRD of more than 0.01% had a five- to ten-fold higher risk of relapse (3-year relapse rate 23%–75%). The level of MRD was the single most powerful prognostic factor of patient outcome, independent of other risk factors such as age, leukocyte count, cytogenetic abnormalities, or prednisone response. Other studies demonstrated that MRD evaluation was the best predictor of clinical outcome in children with relapsed ALL (24) and in patients undergoing bone marrow transplantation (25–27). Thus, MRD evaluation during treatment is currently the most accurate method for identifying patients at a high risk of treatment failure, although scaling up such assays to analyze large numbers of patients in multi-institutional trials remains a challenge.

A major conclusion from MRD studies is that quantitative rather than qualitative evaluation of bone marrow samples provides the best prediction of clinical outcome. However, the traditional methods of quantification by PCR (e.g., comparative hybridization, competitive PCR, and limiting dilution analysis) are either too cumbersome or time-consuming for routine application in MRD studies (17,18). A recent advance in PCR methodology, namely real-time quantitative PCR (RQ-PCR), appears to have solved some of the complications associated with PCR quantification. By using a fluorescent reporter in the PCR and measuring the real-time accumulation of fluorescence during the reaction, the amount of target amplicon synthesized can be precisely determined at any time point because the increase in fluorescence is proportional to the amount of product synthesized. A set of standards, made from serial dilutions of diagnostic DNA, is used for quantification purposes. By monitoring the accumulation of fluorescence, data from the standards and unknown samples are obtained from the exponential phase of the reaction, and the initial target amount is extrapolated from the standard curve (28–36).

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In this issue, Mayer et al. report a set of standardized conditions for detection of MRD in B-lineage ALL using a single-step real-time fluorescence assay. The authors describe the development of quantitative PCR assays based on the detection of leukemia clone-specific *IgH*, *TCR-delta*, and *TEL-AML1* gene rearrangements. In reports from other investigators, the detection of leukemic cells using RQ-PCR required either the inclusion of a separate fluorescent reporter probe in the PCR reaction, or a two-step PCR strategy using nested primers (28–36). However, in their study, Mayer et al. used an alternative strategy, taking advantage of the fact that the SYBR green dye undergoes a shift in fluorescence when bound to DNA. Thus, as the leukemia-specific target is synthesized during the PCR, more SYBR green dye is bound to DNA, producing a proportional increase in fluorescence. As described earlier, the authors calculated the percentage of leukemic cells by comparing the fluorescent signals produced in remission samples to those generated by known quantities of the leukemic cells from the diagnostic samples. Using their method, the authors have demonstrated the ability to quantify MRD using an assay that should be less costly than comparable RQ-PCR methods because it involves only a single round of amplification and does not require synthesis of a relatively expensive probe. The authors also show that minor adjustment of a single reaction component, namely the $MgCl_2$ concentration, is sufficient to detect a diverse array of clonal markers (*IgH*, *TCR*, and *TEL-AML1* rearrangements) under otherwise identical assay conditions. The only components that need to be added to a standard commercial mix are primers and sample DNA. Thus, the technique described by Mayer et al. could be easily implemented in other laboratories with MRD experience.

A cautionary note is that SYBR green will bind any DNA product that is synthesized during the PCR, including nonspecific products that may arise from template mispriming or primer dimerization. Thus, the amplification of any nonspecific product will also produce an increase in fluorescence during the reaction. Without appropriate controls, this outcome could result in false-positive detection of the leukemic clone. Alternatively, a high background of nonspecific fluorescence can reduce the sensitivity of leukemic cell detection. Therefore, great care must be taken in designing amplification primers that minimize the chance of producing nonspecific fluorescence. Other investigators pointed out that the use of fluorescent TaqMan probes enhanced specificity because the reporter probe hybridizes to the target sequence between the amplification primers, and fluorescent signal is detected only when the leukemia-associated target is amplified (28–33,35,36). Thus, the use of TaqMan probes in RQ-PCR provides both the sensitivity and specificity of leukemic cell detection.

The potential limitations of SYBR green RQ-PCR can be overcome by the judicious use of primers, appropriate controls, and suitable post-PCR analyses. In their study of 11

children with ALL, Mayer et al. used stringent criteria previously established (37) to design leukemia clone-specific primers. They also included appropriate negative controls—water and DNA from polyclonal mononuclear cells from healthy donors. In addition, they performed post-PCR melting curve analyses that confirmed the absence of multiple products synthesized during the PCR. In 14 assays, the authors demonstrated an average sensitivity of 3.2×10^{-5} , a level that is well suited for MRD evaluation in clinical studies. The authors state that detection of very low levels of MRD may not be clinically relevant because they are compatible with long-term cure. However, the results of the study cited (38) may not apply universally, and further studies are needed to determine if the presence of a very low level of MRD correlates with late recurrence of disease.

The authors then showed that their assay method was precise and reproducible. MRD data produced on different days and/or by different operators were virtually identical. They also compared MRD data obtained from the one-step SYBR green RQ-PCR assay with data obtained by limiting dilution analysis. Comparison of positive versus negative calls showed that MRD data from both methods were concordant in 31 of 32 analyses. The single discordant result was positive by limiting dilution and negative by RQ-PCR, at a level just above the threshold of the real-time PCR assay. Comparison of MRD results that were positive by both methods showed a high degree of correlation. Thus, the authors conclude that their assay method is accurate, precise, and reproducible.

The MRD findings were then evaluated with respect to clinical outcome. In agreement with previous MRD studies, the authors found that a slow cytoreduction of leukemic cells during therapy was associated with a higher risk of relapse, while the rapid eradication of leukemic cells to levels beneath the assay limits was associated with long-term remission (up to 35 months).

Overall, Mayer et al. have shown that PCR detection of MRD can be simplified by establishing a set of standard conditions that can be applied to a wide variety of molecular targets. Although their study included only a limited number of patients, the findings point the way toward simplified PCR methods that may help widen their applicability for large-scale MRD assessment in clinical studies.

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