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Original Article

Real-Time Quantitative PCR: Standardized Detection of Minimal Residual Disease in Pediatric Acute Lymphoblastic Leukemia

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Purpose: To develop a standardized real-time polymerase chain reaction (PCR) method of quantifying minimal residual disease (MRD) in patients with pre-B acute lymphoblastic leukemia (ALL).

Patients and Methods: In a series of 24 follow-up bone marrow (BM) samples in 11 patients (14 clonal markers), we performed real-time PCR assays using one consensus and one clone-specific primer for each marker. The markers analyzed included immunoglobulin heavy chain (IgH), T-cell receptor (TCR) and TEL-AML1 rearrangements.

Results: We achieved a detection limit of $3.3 \times 10^{-5} \pm 1.2 \times 10^{-5}$ and an accurate quantitation (r = −0.99) limit of $2.0 \times 10^{-4} \pm 8.8 \times 10^{-5}$ blasts. Both inter- and intra-assay reproducibility were exceptional. Additionally, we found comparable results to those of a "gold standard" limiting-dilution PCR assay (r = 0.62).

Conclusions: The IgH, TCR and TEL-AML1 markers can be used as targets by real-time PCR under the same cycling profile, allowing quantitation of MRD in more 95% of patients with pre-B ALL. This standardized, real-time PCR technique should simplify monitoring MRD in clinical trials.

Key Words: Real-time quantitative PCR—Minimal residual disease—Pediatric acute lymphoblastic leukemia.

Of children diagnosed with acute lymphoblastic leukemia (ALL), 95% to 98% achieve complete remission (1). The disease relapses in 20% to 30% of patients, whose event-free survival after relapse reaches only 30% to 60% (2). Therapeutic intensification regimens increase the incidence of treatment-related toxicity and long-term adverse effects (3). Discrimination of those at a high risk of relapse justifies earlier treatment intensification.

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Complete remission in patients with ALL is defined morphologically as less than 5% lymphoblasts in the BM. Leukemic cells below the 5% detection limit is termed "minimal residual disease'' (MRD). Molecular analysis of clone- or leukemia-specific genes by polymerase chain reaction (PCR), which can be performed in most patients, enables the detection of 1 leukemia cell in 100,000 to 500,000 normal cells (4–6). Commonly used clonal molecular markers include immunoglobulin heavy chain (IgH) and T-cell receptor (TCR) gene rearrangements, and the TEL-AML1 fusion transcript, detected in 80%, 60%, and 22% of patients, respectively (1,7).

Molecular MRD analysis can stratify children with ALL because certain levels of residual leukemia are highly predictive of outcome (8–10). But, methodologic differences among various laboratories can confound clinical interpretation. Therefore, the current focus is to design a simple, reliable, and standardized methodology. Real-time quantitative PCR can be ideal where a fluorescence detector monitors the reaction as it proceeds. Two strategies are available and both can use either the TaqMan (ABI, PE, Foster City, CA, U.S.A.) or LightCycler (Roche Diagnostics Corporation, Indianapolis, IN, U.S.A.) systems.

One strategy, which uses a fluorogenically labeled probe nested inside oligonucleotide primers, has been successful for numerous chromosomal translocations (11–13). In the case of IgH and TCR gene rearrangements, disadvantages include costly, labeled oligonucleotides; optimized cycling profiles for each patient; or both.

The second strategy takes advantage of an increased fluorescence that occurs as SYBR Green I dye binds newly synthesized DNA. The LightCycler is equipped with software that measures the presence of the PCR product and absence of nonspecific amplification (14). Although this method has been used to quantitate MRD in patients with ALL targeting the IgH and TCR clonal markers (15), two rounds of PCR were performed, possibly increasing the risk of contamination.

We previously reported a limiting-dilution PCR assay (6), where stringent clone-specific primers for IgH and TCR gene rearrangements reproducibly allowed the quantitation of MRD as low as 2×10^{-6}. Herein, we applied the limiting-

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dilution PCR primers to real-time PCR technology, which could allow a standardized MRD quantitation assay in more than 95% of children with pre-B ALL, and in patients with other B-cell malignancies. We report here the intra- and inter-assay reproducibility and accuracy of this technology, and the comparative results of 24 remission samples in patients with pre-B ALL by limiting-dilution and real-time PCR.

MATERIALS AND METHODS

Patients and Samples

Bone marrow mononuclear cell DNA samples from 11 children with ALL were included in this study. Diagnostic IgH and TCRδ gene rearrangements were previously analyzed (6). Informed consents were properly obtained from these patients, who were enrolled in an institutional review board-approved MRD study at New York Medical College. Remission BM samples were previously monitored for MRD by limiting-dilution quantitative PCR (6). Peripheral blood mononuclear cell DNA samples from healthy volunteers were used for negative controls.

Characterization of TEL-AML1 Intron Fusion Sequences

Two patients positive for TEL-AML1 (patients 10 and 11) were identified by reverse transcription PCR using oligonucleotide primers 5′ TEL and 3′ AML1 (16). Intron fusion breakpoints were amplified using a panel of primers designed at the microcluster breakpoint regions (17), followed by automated sequencing (6).

Primer Design

The oligonucleotide primers used to quantify clonal IgH and TCRδ gene rearrangements and the rules of primer design have been reported (6). Sequences can be obtained by E-mail (sharon_mayer@nymc.edu). For IgH, a universal V primer was combined with a clone-specific D-N-J junction primer. Vδ2-Dδ3 and Dδ2-Dδ3 rearrangements were amplified with a universal Dδ3 primer and a clone-specific primer spanning the delta junction. For TEL-AML1, the upstream primer spanned the junctional sequence and the second primer was designed 100 to 150 base pair (bp) downstream of the breakpoint, in AML1. To maximize specificity under highly stringent conditions, the 3′ end of each clone-specific primer extended no more than 3 bp into a germline segment.

Normalization of DNA

The DNA concentrations were determined by spectrophotometry, then by normalization using the LightCycler control kit (Roche Molecular Biochemicals). Specifically, the standard curve was composed of 500, 200, 50, and 10 cell equivalents (CE) per reaction (1 μg DNA = 130,000 cells) and was tested in duplicate. Patient samples (200 CE) were tested in triplicate. Each reaction (20 μL) contained 0.5 μM β-globin primer mix, 4 mM $MgCl_2$, and 2 μL of DNA Master SYBR Green I. Purified water was used as a negative control. Reaction conditions consisted of denaturation at 95°C for 30 seconds, and 40 cycles at 95°C, 55°C, and 72°C, for 0, 5, and 10 seconds, respectively. Standard and melting curves were obtained at the end of the reaction. The normalization value was calculated by dividing the average result of the triplicates by 200.

Intra-assay variability from capillary positional effects was assessed by testing 21 replicates of the 500 cell equivalents DNA standard using the β-globin assay. Inter-assay variability was assessed by testing a normal peripheral blood (NPB) sample on seven different days and two patient samples in on two different days. To assess the variability introduced by multiple investigators, one research scientist performed six of the reactions and the second scientist in the same laboratory performed the remaining five assays. Variability was calculated by ANOVA.

Clone-Specific Real-Time PCR

After normalization, diagnostic DNA samples were diluted to 5×10^{-2} (1000 CE), 2.5×10^{-2}, 2.5×10^{-3}, 2.5×10^{-4}, 5×10^{-5}, and 2.5×10^{-5} (0.5 CE) per reaction, and tested in triplicate. The diluent was NPB DNA (20,000 CE per reaction). The reactions (20 μl) included 2.0 or 2.5 mM $MgCl_2$, 0.5 μM each primer, and 2 μL LightCycler Fast-Start SYBR Green. Negative controls were NPB DNA and water. Cycling conditions consisted of denaturation at 95°C for 90 seconds, then 70 cycles at 95°C, 68°C, and 72°C, for 0, 5, and 10 seconds, respectively. After the last cycle, standard and melting curves were obtained. Amplification efficiencies of different types of target genes were compared by ANOVA of the regression coefficients. The lowest dilution that yielded a signal in at least one of the replicates, in the absence of nonspecific amplification, was defined as the sensitivity threshold. The quantitative threshold was defined as the lowest dilution in which all three replicates were positive. Differences in the detection and quantitation thresholds among the types of clonal markers were assessed by ANOVA.

Reproducibility of clone-specific real-time PCR on different days and by different investigators was assessed. Normalized diagnostic DNA from patient 4 was diluted to 25 and 5 CE in a total of 20,000 CE per reaction. One research scientist (blinded) tested the samples three separate days. A second research scientist (not blinded) in the same laboratory tested the samples on the fourth day. Variability was assessed by ANOVA.

Quantitation of MRD in Patient Samples

Minimal residual disease in 24 normalized remission BM samples was quantitated by real-time PCR. Each sample was tested in triplicate (60,000 total CE) simultaneously with the appropriate standard curve. Error was calculated as the standard deviation from the triplicate reaction. Each sample was previously assessed for MRD by limiting-dilution quantitative PCR (6), the results from which were blinded. Results were compared by Pearson correlation. Concordance was assessed using McNemar's test.

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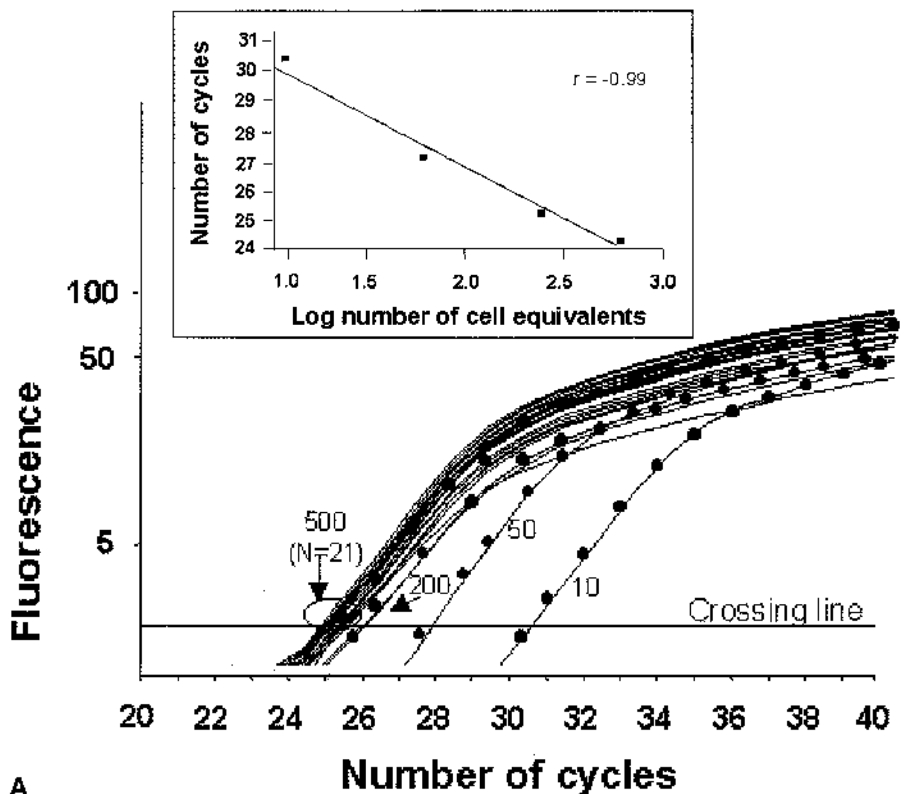
All statistical analyses were performed using SAS, version 6.12 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

We show that real-time PCR with the LightCycler instrument is suitable for standardized detection of MRD in patients with ALL. Normalization of DNA is precise, both in terms of sample position within the instrument, and inter-assay reproducibility. Clone-specific real-time PCR that uses one universal and one clone-specific primer requires only one cycling profile for IgH, Vδ2-Dδ3, Dδ2-Dδ3, and TEL-AML1 rearrangements. Therefore, "MRD kits," containing one of two $MgCl_2$ concentrations can be used, where only primers and DNA need to be added. No significant difference was seen in sensitivity between the type of clone tested. A high degree of inter-assay reproducibility of quantifying MRD was found in remission samples.



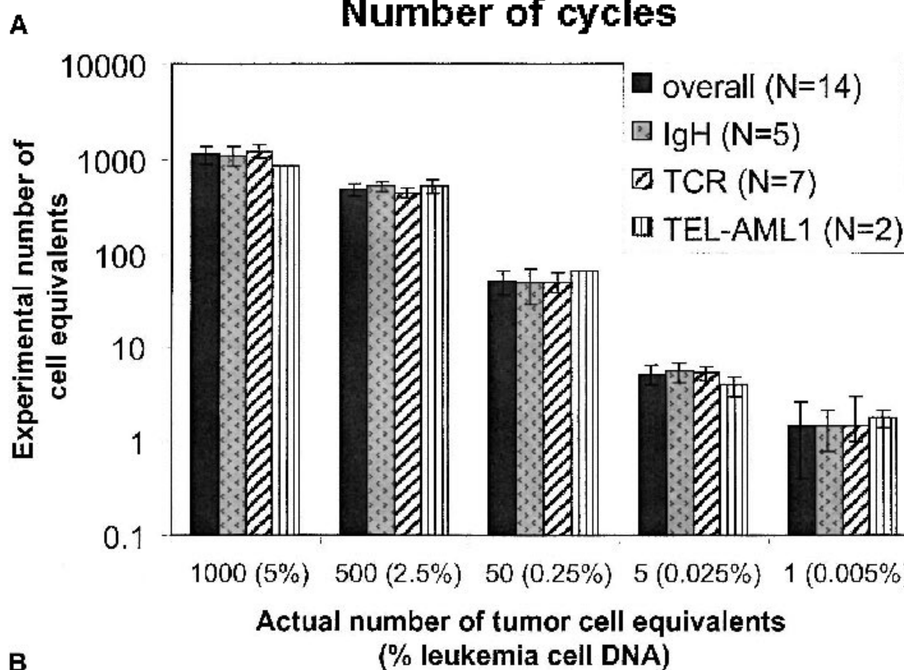


FIG. 1. Intra-assay reproducibility of normalizing DNA samples. Intra-assay reproducibility is demonstrated by quantifying 21 replicates of a standard human DNA sample from the kit containing 500 cell equivalents (CE) per reaction, in addition to a standard curve for the β-globin gene containing 500, 200, 50, and 10 CE. **(A)** Amplification plot showing that all 21 replicates' fluorescence surpassed the crossing line within 1 cycle, and corresponding standard curve, where the number of starting copies is plotted against the number of cycles at which the fluorescence surpassed the crossing line. **(B)** Mean β-globin normalized experimental values with standard deviations are plotted against the expected values based on the dilutions added to the reactions.

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TABLE 1. *Inter-assay reproducibility of normalizing patient samples*

Sample	Technician	Day	CE*	Average value	SD	Coefficient of variation (%)	Source	P‡
NPB†	A	1	156.5	137.2	22.8	16.6	Between days	0.531
	A	2	128.6				Between samples	0.403
	A	3	132.5				Between technicians	0.667
	A	4	130.0					
	A	5	126.2					
	B	6	156.8					
	B	7	130.0					
Patient 3	B	6	123.5	132.8	14.5	10.9		
	B	7	142.0					
Patient 11	B	6	147.8	148.2	16.9	11.4		
	B	7	148.4					
Overall				138.4	12.3	8.89		

CE, cell equivalents; SD, standard deviation.
*Average result of triplicate samples.
†NPB, peripheral blood mononuclear cells from a healthy volunteer.
‡P using analysis of variance.

Normalization of DNA

Each DNA sample was normalized using an external β-globin reference, because internal standards reduced the sensitivity of detection in our laboratory and in others (18). Normalized were 11 diagnostic BM, 24 remission BM, and 2 NPB samples. The average value was 92.2 CE; the coefficient of variation was 61%. A lower than expected result can reflect the inaccuracy of the spectrophotometric method or the presence of PCR inhibitors and emphasizes the necessity for DNA normalization.

Reproducibility is a major concern when a research tool is implemented in assays that can influence therapeutic decisions. To assess intra-assay variability, 21 replicates of a human DNA standard was quantified. Figure 1A illustrates the fluorescence data and linear regression analysis. The signal surpassed the crossing line at progressively higher cycle numbers as the CE were reduced ($r = -0.99$). The values for the 21 samples fell within one cycle number, between cycles 24.7 and 25.5. The average number of CE for the 21 replicates was 495.6 ± 82.2 (median = 510.5, coefficient of variation = 17%), indicating a high degree of intra-assay reproducibility.

Inter-assay reproducibility of β-globin standardization was assessed by testing NPB on seven different days, and two patient diagnostic samples each on two different days. One research scientist performed six of the reactions and a second scientist in the same laboratory performed the remaining five assays. Observed values are shown in Table 1. The average value was 138.4 CE and the coefficient of variation averaged 8.9%. ANOVA revealed no significant differences between days, samples, investigators, or any in-

TABLE 2. *Conditions and characteristics of clone-specific standard curves*

Patient	Clone	Optimal $MgCl_2$ (mM)*	Quantitative threshold†	Threshold of detection‡	Linear regression (r)§
1	IgH	2.5	2.5×10^{-4}	5.0×10^{-5}	−1.00
1	TCR1	2.0	2.5×10^{-4}	2.5×10^{-5}	−0.99
1	TCR2	2.5	2.5×10^{-4}	5.0×10^{-5}	−0.97
2	TCR	2.0	2.5×10^{-4}	2.5×10^{-5}	−0.97
3	IgH	2.5	2.5×10^{-4}	5.0×10^{-5}	−0.99
4	TCR	2.0	2.5×10^{-4}	2.5×10^{-5}	−1.00
5	TCR	2.5	2.5×10^{-4}	5.0×10^{-5}	−1.00
6	IgH	2.5	2.5×10^{-4}	2.5×10^{-5}	−0.99
7	TCR	2.5	5.0×10^{-5}	2.5×10^{-5}	−0.99
8	TCR	2.5	2.5×10^{-4}	2.5×10^{-5}	−0.98
8	IgH	2.5	5.0×10^{-5}	2.5×10^{-5}	−0.99
9	IgH	2.0	2.5×10^{-4}	2.5×10^{-5}	−0.98
10	TEL-AML1	2.5	5.0×10^{-5}	2.5×10^{-5}	−0.99
11	TEL-AML1	2.5	5.0×10^{-5}	2.5×10^{-5}	−0.98

IgH, immunoglobin heavy chain; TCR, T-cell receptor; TEL-AML1,
*The concentration of $MgCl_2$ that yielded efficient amplification of the diagnostic DNA in the absence of amplification in the negative controls.
†The lowest concentration of diagnostic DNA that yielded a positive result in all three of the replicate reactions.
‡The lowest concentration of diagnostic DNA that yielded a positive result in at least one of the triplicate reactions.
§Regression analysis of the clone-specific real-time polymerase chain reaction standard curve.

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TABLE 3. *Inter-assay reproducibility of quantitating the concentration of leukemia cells in an unknown DNA sample*

Actual CE	Day	Technician	Experimental CE*	Average value	SD	Coefficient of variation (%)	Sample	Source	*P*†
25	1	A	25.7	29.6	5.38	18.2	25 copies	Between trials	0.659
	2	A	25.4					Between technicians	0.184
	3	A	30.4				5 copies	Between trials	0.342
	4	B	36.9					Between technicians	0.324
5	1	A	4.4	4.6	0.89	19.3			
	2	A	5.9						
	3	A	4.2						
	4	B	3.9						

CE, cell equivalents; SD, standard deviation.
*Average result of triplicate samples.
†*P* using analysis of variance.

teractions among these three main order effects, assuring that the assay results were reproducible.

Clone-Specific Real-Time PCR

A complication of MRD analysis of pre-B ALL in patients is the uniqueness of the clonal markers. Previously, either internal probes or a nested PCR was required for real-time PCR. Leukemia clone-specific real-time PCR reactions were performed on 14 clonal markers (2 TEL-AML1, 5 IgH, 5 Vδ2-Dδ3, and 2 Dδ2-Dδ3) in 11 patients. Remarkably, all reactions were successful using the same cycling profile. Of 14 clones, 10 amplified optimally at 2.5 mM $MgCl_2$ and 4 at 2.0 mM $MgCl_2$ (Table 2). Melting curve analysis revealed single peaks for each reaction, indicating specific PCR products. The water and NPB-negative controls yielded no amplification. In the future, we will investigate normal BM mononuclear cell DNA as a source for a negative control. No significant difference in linear regression analysis was found between types of clonal marker, IgH, and TCR (Table 2). Figure 1B shows the relationship between observed and expected copy numbers. The detection threshold was $3.2 \times 10^{-5} \pm 1.2 \times 10^{-5}$, with no significant difference between IgH and TCR rearrangements. The quantitation threshold equaled $1.9 \times 10^{-4} \pm 9.4 \times 10^{-5}$, with no significant difference between IgH and TCR (Table 2). Results of the TEL-AML1 leukemia-specific markers were excluded from the statistical analysis because of few patients harboring this translocation (n = 2). Overall sensitivity of detection was a half log lower than that of limiting dilution; however, very low levels of residual leukemia, which can be compatible with long-term cure (4), may not be clinically relevant. We expect that using these different clonal markers will not impede accurate comparisons in clinical studies.

Accuracy, day-to-day reproducibility, and researcher-to-researcher reproducibility of the clone-specific real-time PCR assay were assessed. Normalized diagnostic DNA dilutions from patient 4 were tested for MRD by two different research scientists in the same laboratory. As shown in Table 3, the average experimental values were 29.6 ± 5.38, and 4.6 ± 0.89 CE. No significant difference was seen between 1) trials on different days or between scientists, indicating a high degree of reproducibility, or 2) the actual

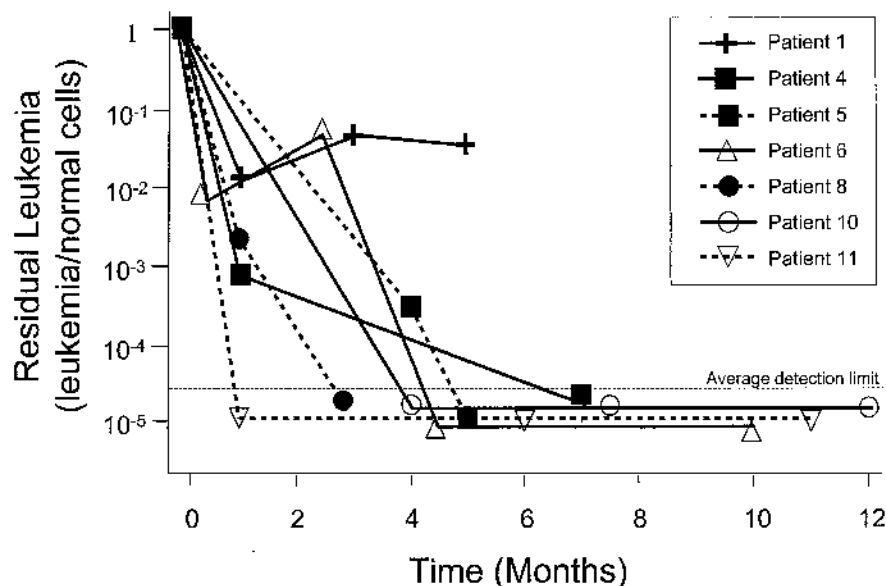

FIG. 2. Real-time polymerase chain reaction (PCR) minimal residual disease analysis of patient samples. Follow-up bone marrow samples from seven patients were analyzed for residual leukemia. Each data point represents a "fit" of the fluorescence from the follow-up sample to its respective standard curve. Points below the line labeled "average detection limit" were negative but plotted for visual clarity.

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and experimental values, indicating a high level of accuracy.

Quantitation of MRD in Remission Samples

Twenty-four remission bone marrow samples from seven patients were monitored by limiting-dilution quantitative PCR and by real-time PCR LightCycler technology. The samples from patient 1 were tested for three clones, the samples from patient 8 for two clones. The remaining samples were tested for one clone, totaling 32 assays. Concordance was defined as positive or negative in both tests, or negative by real-time PCR, but positive by limiting-dilution below the detection threshold of real-time PCR. The correlation ($r = -0.62$) was moderate and McNemar's test revealed high concordance. In fact, 31 of 32 analyses were concordant; 15 were positive and 12 were negative by both assays, and 4 were positive by limiting-dilution PCR below the detection threshold of real-time PCR. The discordant sample was positive for limiting dilution at $4.2 \times 10^{-5} \pm 3.3 \times 10^{-6}$, but negative by real-time PCR (sensitivity 2.5×10^{-5}). Two samples, although concordant, were extreme outliers and did not conform to the elliptic region of the correlation. These results could not be explained by technical problems during MRD analysis but could be related to the fact that the real-time PCR was a retrospective analysis of stored DNA samples. The PCR products from the two positive samples of patient 2 were confirmed by automated sequencing (6) (data not shown). Assuming that the "gold-standard" limiting dilution assay is an accurate measure of MRD, the real-time PCR assay reported here is accurate.

Real-time PCR analysis results of the remission samples are shown in Figure 2, where only one clone per patient is shown for simplification. Patient 11, who responded well to therapy, remains in clinical remission 24 months after diagnosis. Patients 4, 6, 8, and 10 had a less rapid molecular response to chemotherapy, but eventually achieved a molecular remission. These children are in clinical remission 23, 22, 27, and 35 months after diagnosis, respectively. Patient 5 was a poor responder and relapsed 33 months after diagnosis. The level of MRD decreased slowly in patient 1 who is still in clinical remission 17 months after diagnosis. Comparison in MRD status between multiple clones was performed. Patient 8 was positive for both IgH (9.4×10^{-4}) and Vδ2-Dδ3 (1.9×10^{-3}) clones at 1 month, and negative for both clones at 3 months after diagnosis. Patient 1 was positive for IgH (7.6×10^{-3}), Vδ2-Dδ2 (3.3×10^{-2}), and Dδ2-Dδ3 (1.4×10^{-2}) at 1 month, positive for IgH (7.4×10^{-3}), Vδ2-Dδ2 (1.1×10^{-1}), and Dδ2-Dδ3 (5.3×10^{-2}) at 3 months, and positive for IgH (1.4×10^{-2}), Vδ2-Dδ2 (2.8×10^{-2}), and Dδ2-Dδ3 (3.9×10^{-2}) at 5 months after diagnosis. It is likely that both rearrangements are from the same leukemic clone in patient 8, whereas patient 1 may have two leukemic clones, one harboring the IgH and the other harboring the Vδ2-Dδ3 and Dδ2-Dδ3 rearrangements.

Inter-laboratory variability was not assessed in this study. But, because the SYBR Green kit requires the addition of only oligonucleotide primers, $MgCl_2$, and DNA sample, inter-laboratory variability can be minimized. A comprehensive study composed of two or more research laboratories is necessary to assess critical issues of standardization.

We conclude that clone-specific IgH and TCR gene rearrangements and leukemia-specific TEL-AML1 fusion breakpoint markers can be used as targets by real-time PCR under the same cycling conditions, allowing reliable and reproducible quantitation of MRD in more than 95% of patients with pre-B ALL, an essential component for translating MRD research into clinical practice.

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