Detection of Minimal Residual Disease in T-Cell Acute Lymphoblastic Leukemia Using Polymerase Chain Reaction Predicts Impending Relapse

By Geoffrey A.M. Neale, Javier Menarguez, Geoffrey R. Kitchingman, Thomas J. Fitzgerald, Maria Koehler, Joseph Mirro, Jr, and Rakesh M. Goorha

After achieving remission, approximately one-third of patients with T-cell acute lymphoblastic leukemia (T-ALL) lapse due to the resurgence of residual leukemic cells that cannot be detected in remission by morphologic methods. Thus, the early detection of residual disease is highly desirable to monitor the efficacy of therapy, or to institute an alternative mode of therapy. Toward this aim, we have examined the applicability of polymerase chain reaction (PCR) amplification in the detection of minimal residual disease (MRD) in bone marrow samples from patients with T-ALL in morphologic remission. Two different approaches were taken to identify leukemic clone-specific sequences that could be used as targets for PCR amplification. The first technique used T-cell receptor-β (TCR-β) gene rearrangements that were sequenced directly after PCR amplification of leukemic DNA. This method was successful in generating clone-specific probes for 76% of T-ALL patients screened. An alternative method was used to clone and sequence a TCR-β chain gene from leukemic cells to generate a specific probe. The PCR assays that we used were specific for each patient’s leukemic clone, and were capable of routinely detecting one leukemic cell in 10³ normal cells. Using these sensitive PCR-based assays, we found no evidence for persistence of the leukemic clone in any of the bone marrow samples from four T-ALL patients who are in long-term (3.9+ to 8.1+ years) remission. In contrast, we detected residual disease in clinical remission samples from two patients who subsequently relapsed. In one patient, where we had appropriate samples, we observed a dramatic expansion of the leukemic clone 3 months before clinical relapse. These results suggest that PCR-based assays for detection of MRD in T-ALL patients have great potential in predicting impending relapse, and in determining the efficacy of the anti-leukemic therapy. These methods may also allow the identification of long-term survivors.

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To test the potential clinical utility of this technique, we have performed a retrospective study on six T-ALL patients for whom we had diagnosis and follow-up DNA samples. For five of the six patients studied, we amplified a TCR-δ gene rearrangement in the leukemic clone and sequenced it directly. From the sequence of each gene rearrangement at diagnosis, we prepared a clone-specific oligonucleotide probe. This probe was then used for screening PCR-amplified remission sample DNAs. One patient did not have a TCR-δ gene rearrangement. To obtain a clone-specific probe for this patient, we cloned and sequenced a TCR-β gene rearrangement. The results of this study indicate that detection of the leukemic clone in remission samples by PCR-amplification is more sensitive than morphologic analysis, and appears to be a good indicator for predicting relapse in T-ALL.

MATERIALS AND METHODS

DNA samples were prepared from Ficoll/Hypaque-purified bone marrow cells (density, <1.077 g/mL) by the method of Wigler et al.2 This investigation was approved by the institution's Committee on Human Experimentation and informed consent was obtained from all patients and their parents as appropriate. Diagnosis of T-ALL was made by French-American-British (FAB) criteria and by immunophenotyping with CD7, CD2, CD3, CD11, and CD8 markers, and by the presence of TCR gene rearrangements. Marrows were blindly reviewed by a pediatric hematologist/oncologist and a hematopoietic pathologist.

PCR. PCR was performed essentially according to the method of Saiki et al.3 One-hundred microliter reaction mixtures contained 1 μg of genomic DNA, 0.5 μmol/L of each of 5' and 3' oligonucleotide primer, 0.2 mmol/L each dATP', dCTP, dGTP, and dTTP, 10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.01% (wt/vol) gelatin, and 0.1% Triton X-100 (Promega, Madison, WI). Synthetic oligonucleotides used in PCR reactions were made on an Applied Biosystems model 380B (Foster City, CA) automated synthesizer (using phosphoramidite chemistry) and are listed in Table 1. The mixture was heated at 95°C for 10 minutes to denature the DNA and then chilled quickly on ice to prevent renatting of double-stranded DNA. Taq DNA polymerase (2.5 U) (Promega) was added before overlaying the samples with 100 μL of mineral oil. Except where stated otherwise, samples were subjected to 40 cycles of PCR amplification in a Genetic Thermal Cycler (Precision Scientific, Chicago, IL). PCR reactions containing TCR-δ primers or N-ras primers were cycled between 94°C, 55°C, and 72°C for 1, 2, and 3 minutes, respectively. Reactions containing TCR-β primers were cycled between 94°C and 72°C, for 1 and 3 minutes, respectively. After amplification, 20% of the product was analyzed by 3% or 4% agarose (NuSieve GTG; FMC Corporation, Rockland, ME) gel electrophoresis and ethidium bromide staining.

Direct sequencing of PCR products. To sequence the PCR products directly, the remainder of the samples were electrophoresed through 4% NuSieve agarose gels, ethroleuolized, and ethanol precipitated. Twenty percent of the purified product was sequenced using the Sequenase 2.0 protocol (US Biochemical Corporation, Cleveland, OH) with the 3' amplification primer. In some instances we modified the sequencing protocol by using 100 ng of [γ-32P]ATP-labeled primer in the sequencing reactions as described.23

Southern blot analysis of PCR products. Twenty percent of the PCR reaction was electrophoresed through a 1.5% agarose gel, transferred to a Nytran membrane (Schleicher and Schuell, Keene, NH) as described by Southern,29 and covalently attached to the membrane by UV irradiation. Membranes were prehybridized at room temperature in 6X SSC-1% sodium dodecyl sulfate (SDS) for 1 hour, and then hybridized in the same buffer containing 105 cpm/mL of a [γ-32P]ATP-labeled oligonucleotide probe for a further 1 hour at room temperature. The membranes were washed four times at room temperature in 6X SSC-1% SDS for 10 minutes and then for 20 minutes in the same buffer at 45°C.

Cloning and sequencing of the TCR-δ gene rearrangement. A genomic library was constructed by ligation of BamHI-digested patient diagnosis DNA into similarly digested EMBL-3 arms (Stratagene, La Jolla, CA) using standard techniques.29 After screening the library with a TCR-δ constant region probe (pB400),29 two positive A clones with inserts of 12 kb and 17 kb were isolated. A 4.1-kb BamHI/PstI fragment that hybridized to the TCR-β probe was subcloned from the 12-kb λ clone into pBS (−) (Stratagene). Restriction mapping showed that this fragment contained the second constant region of the TCR-β locus, and thus sequencing was initiated from within the Jδ region using oligonucleotide primers synthesized from published sequence data.30 Sequence data were obtained sequentially using oligonucleotide primers synthesized from the preceding sequence and were performed in both directions using the Sequenase 2.0 protocol (US Biochemical Corporation).

RESULTS

PCR amplification of TCR-δ gene rearrangements. The strategy that we used to amplify leukemic-specific TCR-δ gene rearrangements was based on that of Hansen-Hagge et al.,17 and is outlined in Fig 1. Genomic DNA isolated from T-ALL patient diagnosis bone marrow was amplified using various combinations of the TCR-δ V- and J-region primers listed in Table 1. The amplification primers were synthesized using published sequence data,4,9 and their positions within the TCR-δ locus are indicated in Fig 1. After recombination of the locus, these primers amplify an approximately 350-bp genomic DNA fragment containing the V(D)-J junction of the TCR-δ gene rearrangement.

Frequency of TCR-δ gene rearrangements in T-ALL. To examine the applicability of the PCR amplification technique to screen patients for MRD, we randomly screened 17 T-ALL diagnosis DNA samples for TCR-δ gene rearrangements. Thirteen of the 17 (76%) samples showed

<table>
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The relative positions and orientations of the TCR-δ PCR primers are shown in Fig 1. The TCR-δ V-region primers match the sequence at the 5'-end of the second V-region exon, while the J-region primers match the sequence at the 3'-end of their respective regions. The relative positions of patient 14 PCR primers are shown in Fig 2. N-ras primers amplify the 197-bp second exon of the human N-ras gene.
Fig 1. Genomic organization of the TCR-δ locus, and PCR strategy for detection of MRD. (A) Somatic (unrearranged) configuration of the TCR-δ locus with limited V-regions (3), J-regions (3), and D-regions (3). (Not drawn to scale.) (B) Rearranged TCR-δ gene with arrows indicating the position and direction of amplification primers. Open boxes show open reading frames, while the shaded box represents the highly diverse V-D-J junction that is the target for PCR amplification. Comparison of our sequence data with published sequences (Fig 2) confirmed that the PCR products were TCR-S gene rearrangements as judged by an appropriate-sized band on an ethidium bromide-stained gel (data not shown). After gel purification, the PCR products were sequenced directly to establish their identity. Comparison of these nucleotide sequences was suitable for use in identifying the leukemic clone.

TCR gene rearrangements as judged by an appropriate sized band on an ethidium bromide-stained gel (data not shown). After gel purification, the PCR products were sequenced directly to establish their identity. Comparison of our sequence data with published sequences (Fig 2) confirmed that the PCR products were TCR-δ gene rearrangements. We observed the following frequencies of TCR-δ gene rearrangements: 7 V₁-J₁, 2 V₂-J₁, 2 V₂-J₂, 1 V₂-J₃, and 1 V₁-J₂. The V-(D)-J junction sequences amplified from different patient samples were unique and showed little homology despite the limited number of TCR-δ V-, J-, and D-regions. Overall, our results indicated that approximately 75% of T-ALLs can be screened for MRD using TCR-δ gene rearrangements, and that the V-(D)-J junction sequences were suitable for use in identifying the leukemic clone.

Preparation of leukemic clone-specific probes. We selected patients 1 through 5 for examination of MRD because multiple follow-up DNA samples were available for analysis from each of these patients. A clone-specific oligonucleotide probe for each patient (underlined in Fig 2) was synthesized using the nucleotide sequence data spanning the V-(D)-J junction of the TCR-δ gene rearrangement. In pilot Southern blot experiments to test the specificity of these oligonucleotide probes, we observed hybridization of each oligonucleotide with its respective patient PCR-amplified DNA, but not with amplified DNA from seven normal peripheral blood lymphocyte (PBL) samples (Fig 3B for patient 5; data not shown for patients 1 through 4). From these observations we concluded that the probes chosen for each patient were suitable for use in the analysis of MRD in remission samples from each of these patients.

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Fig 2. Nucleotide sequences of TCR-δ V-D-J junctions amplified from diagnosis bone marrow DNA samples from patients with T-ALL. Patients are grouped by their observed TCR-δ gene rearrangement. V-, D-, and J-regions were identified by comparison with germline sequences. The other nucleotides in this region were designated N-regions. The leukemic-specific oligonucleotide probes used in this study are shown by underlined sequences.
we made serial dilutions of the patient diagnosis DNA (in normal PBL DNA where indicated) and tested them simultaneously with each remission sample. Each set of patient DNA samples was tested in independent experiments, and representative autoradiographs of Southern blots are presented. For each experiment, 1 μg of DNA isolated from KB cells was also tested with the amplification primers. This sample served as a negative control because KB epithelial cells do not contain rearranged TCR genes. In addition, all DNAs used for MRD analysis were tested with a set of control (N-ras) primers (Table 1) to ensure that each sample was amplified equally by PCR, and therefore comparable with any other sample from the same patient.

We estimated the sensitivity of our assay from the serial dilutions made from the diagnosis DNA. By theoretical calculation, 1 μg of genomic DNA corresponds to approximately 10^6 diploid genomes, that is, about 10^8 cells. Therefore, a hybridization signal at 10^-5 dilution of diagnosis corresponds to the detection of a single leukemic cell in the PCR assay. In our experiments we were able to detect the leukemic clone routinely at dilutions of 10^-4 of diagnosis, or about 10 leukemic cells per sample. The sensitivity of detection was not affected by the presence of competing PBL DNA.

When we tested patients 1 through 4 by PCR analysis for MRD we were unable to detect the original leukemic clone in any of the patient remission samples (Fig 6). The date of diagnosis and the length of time in clinical remission for the patients are, respectively: patient 1: 12-82, 8.1+ years; patient 2: 4-84, 6.7+ years; patient 3: 5-86, 4.6+ years; and patient 4: 1-87, 3.9+ years. Despite being able to detect the leukemic clone at levels of one leukemic cell among 10^4, we

![Fig 3. Detection of MRD after relapse by PCR amplification and Southern blot analysis of remission bone marrow DNA from patient 5. (A) Diagnosis DNA (Diag) and 10-fold serial dilutions of diagnosis DNA are shown in the first six lanes. The bone marrow sample at 9 months post-diagnosis was taken at relapse (Rel), while the sample at 10 months was taken during clinical remission (Rem). The negative control KB DNA is shown in the last lane. (B) Specificity of hybridization of patient 5 leukemic-specific oligonucleotide probe to PCR-amplified DNA samples. Lanes 1 through 7, normal PBL DNA samples; lane 8, patient 5 diagnosis DNA; lane 9, KB DNA; lane 10, no DNA.](image-url)

**A.**

*Pt. 5*

Diag 10-1 10-2 10-3 10-4 10-5 9 mo (Rel) 10 mo (Rem) KB

**B.**

1 2 3 4 5 6 7 8 9 10

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**Fig 4. Sequence of the cloned TCR-β gene rearrangement from patient 14. The nonamer and remaining heptamer sequences up-stream of D, and the J2, region from the TCR-β locus are singly underlined and were identified from germine sequences. The nucleotide sequences used to make the 5'- and 3'-PCR primers are underlined with half arrows indicating the direction of amplification. The leukemic-specific oligonucleotide probe created from the gene rearrangement is doubly overlined.**
did not find evidence for persistence of the original disease in any of the remission samples from these four patients. Therefore, the PCR analysis data for these patients is in complete agreement with the clinical diagnoses of the bone marrows.

Patient 5 was diagnosed with T-ALL on 8-86, achieved clinical remission, but relapsed 9 months later. Unfortunately for this study, no remission DNA samples before the first relapse were available for PCR analysis of MRD. However, analysis of the bone marrow sample taken at the time of relapse (5-87) clearly shows the recurrence of the original leukemic clone (Fig 3A, 9 months). After relapse, patient 5 underwent further therapy and achieved a second clinical remission 3 weeks later. This second remission with less than 5% blasts was based on examination by two hematologists and two pathologists. When this remission sample was tested for MRD by PCR analysis we found evidence for persistence of the leukemic clone at a level of about 1% (Fig 3A, 10 months). These findings prompted a blind marrow review of the samples from this patient. The blind review of this sample indicated clinical remission was achieved but the marrow contained about 1% blasts. Thus, there was general agreement between the two methods as far as detecting blast cells, but the PCR assay confirmed that the blasts were leukemic.

Patient 14 was diagnosed with T-ALL on 8-86 and remained in clinical remission until relapsing approximately 2 years later on 6-88. After relapsing, the patient received additional therapy and achieved a second complete remission that lasted until the second relapse on 9-88. When we analyzed available DNA samples by PCR amplification we detected the leukemic clone in every bone marrow sample taken from this patient, including those taken during the initial remission period (Fig 5A). For purposes of estimating the amount of the leukemic clone in these samples, dilutions of diagnosis DNA were made in 1
μg of normal PBL DNA. Significantly, the leukemic clone was readily detected at 8 months postinduction (about 0.1%) and had increased dramatically to about 1% just 3 months before clinical relapse. Blind review of these bone marrow samples confirmed complete remission and that these samples were devoid of any morphologically identifiable leukemic blasts. The marrow reviews of these early remission samples are consistent with the PCR-based estimate of the number of leukemic cells because the sensitivity of the morphologic analysis is limited to 1% to 5%.

The leukemic clone was barely detectable in the 13-month remission sample, and was substantially lower than expected in the 22-month first relapse sample. To further investigate these observations, we examined the kinetics of amplification of all DNA samples using the TCR-β primers (Fig 5A), and the control N-ras primers (Fig 5B). Twenty-microliter samples were taken for Southern blot analysis after 25, 30, 35, and 40 cycles of amplification. Amplification with the TCR-β primers showed a steady increase in the amount of product over the entire time course, while amplification with the N-ras primers was essentially complete after 30 cycles for all samples except for the 13-month remission and the 22-month relapse samples. The 22-month relapse sample showed very weak amplification, while the 13-month remission sample showed slower kinetics of amplification with the N-ras primers. The differences in amplification of these samples were reproducibly seen in several independent experiments and suggested that the hybridization intensities seen after amplification with the TCR-β primers were most likely affected by the reduced capacity of these DNA samples to be amplified.

To test this theory, and to confirm the detection of the leukemic clone in the 13-month remission sample, we extended the number of cycles of amplification with the TCR-β primers. Twenty-microliter samples were taken after 40, 45, 50, and 55 cycles of amplification and analyzed by slot blot (Fig 5C). Extended amplification of the 13-month remission sample with the TCR-β primers resulted in a steady increase in the amount of a detectable product that was completely absent in the control PBL DNA.

Thus, PCR analysis of the samples from this patient indicated that the disease persisted continuously in clinical remission at levels undetectable by morphologic analysis, and that it had increased during chemotherapy before relapse.

DISCUSSION

In this study we tested whether PCR-based methods for detection of MRD in T-ALL patients can predict relapse of the leukemia. The PCR assay can amplify target sequences by 10^5- to 10^6-fold, and thus provides the opportunity to detect leukemic cells with greater sensitivity than current clinical methods that are limited to 1% to 5%. We investigated the efficacy of sensitive PCR assay techniques in detecting persistence of leukemia in bone marrow samples from patients considered to be in clinical remission, and to see whether a correlation could be made between detection of the leukemic clone and subsequent relapse of the patient. The results of our study demonstrate that detection of leukemic cells in clinical remission samples is possible after amplification by PCR, and suggest that these techniques have great potential for identification of long-term survivors and, in other cases, early prediction of clinical relapse.
Our method for preparing clone-specific probes from TCR-δ gene rearrangements differs from that originally proposed by Hansen-Hagge et al. We chose to directly sequence the TCR-δ gene rearrangements that were detected in the diagnosis sample, and then used that sequence data to synthesize a clone-specific oligonucleotide probe. These oligonucleotide probes provide, in our opinion, greater specificity of detection of the leukemic clone, because they do not include sequences from minor populations of blasts that may be present in the diagnosis sample.

Our results regarding the frequency of usage of TCR-δ V- and J-regions are similar to those reported previously. Recombination between Vδ1 and Jδ1 occurs more frequently than any other TCR-δ gene rearrangement in T-ALL. The implications of these findings in T-ALL are unclear, but the observed frequency presumably reflects what occurs during normal T-cell ontogeny.

When we tested patient bone marrow DNAs for MRD with their respective leukemic-specific probes, the patients fell into two distinct groups. In one group of patients (1 through 4), we found no evidence of the persistence of leukemia in their remission samples. Thus, although the PCR assay cannot provide proof for the elimination of leukemic cells in these remission samples, it can be used as a diagnostic aid in confirming induction of remission (less than 1% to 5% blasts) in T-ALL patients.

In addition to verification of clinical remission, we believe that the PCR assay has great potential as an indicator for prediction of cure. From our study, albeit with a small number of patients, the absence of detectable disease correlates well with long-term survival in each patient. However, we wish to stress that the prediction of cure would be purely qualitative and based on the absence of detection of the leukemic clone by PCR. The results presented in this report suggest that absence of the leukemic clone as early as 16 months post-diagnosis (patient 2) may be significant in predicting long-term survival. However, additional studies are required to determine the behavior of the leukemic cell clone during remission and whether clone elimination during induction will predict patients who will have a long-term disease-free survival.

The second group of patients comprises patients 5 and 14, for whom we found their respective leukemic clone in every sample tested. In patient 14, the leukemia persisted below the limits of detection of morphologic analysis during remission and increased dramatically 3 months before the first clinical relapse. Therefore, it appears that the sensitivity of the PCR assays provides detection of persistent disease in T-ALL and early warning of its re-emergence before clinical relapse. This PCR assay for MRD, if confirmed in a larger series, could select the patients most likely to relapse and indicate the most appropriate time for alternative therapy (such as bone marrow transplantation). However, one cautionary note should be made regarding quantitation of MRD by PCR. The relative amplification of each DNA sample can vary and, thus, have a great impact on the interpretation of results. For example, the leukemic clone was barely detected in the 13-month remission sample from patient 14, and was weakly detected in the 22-month relapse sample (Fig 5A). Use of control N-ras primers showed that the weak leukemic signals in these two samples are due to poor amplification of DNA (Fig 5B). The cause of the poor amplification is not known, but presumably is due to contaminants in the DNA. We strongly advise the use of control primers to monitor the relative ability of each DNA sample to be amplified, and recommend that samples exhibiting poor amplification characteristics should be treated with caution, if not discarded altogether from analysis of results.

When we undertook this retrospective study, we focused on patients for whom we had multiple bone marrow DNA samples. However, evaluation of MRD by PCR analysis can be applied prospectively to many areas of ALL, including the presence of central nervous system (CNS) disease, residual disease posttransplantation, recurrent disease, and measuring the effects of maintenance therapy. It is noteworthy in this regard that recent studies with chronic myelogenous leukemia (CML) have shown the applicability of PCR analysis in detection of MRD after bone marrow transplantation.

On the basis of the observed frequency of TCR-δ gene rearrangements in T-ALL, the PCR assay of Hansen-Hagge et al. is limited to screening 70% to 80% of these patients. Absence of TCR-δ gene rearrangements as determined by PCR detection can occur quite normally in cells through the deletion of the TCR-δ locus during TCR-α rearrangement, or by incomplete gene rearrangement. Thus, it seems that a combination of approaches will be necessary to ensure that all T-ALL patients can be screened for MRD.

Yamada et al. recently reported the detection of minimal residual disease in B-lineage ALL using PCR. Three main issues are raised when their findings are compared with ours. First, these investigators detected residual leukemic cells in every bone marrow sample obtained up to 18 months after diagnosis. These results differ from ours because we do not detect residual disease in samples taken as early as 16 months after diagnosis from patients who are in long-term remission. This difference in studies may reflect differences between B-ALL and T-ALL in the response of the leukemic clone to therapy. Second, similar to our findings, they observed a dramatic increase in the leukemic clone of one patient 3 months before relapse. Their observation and ours strengthen the hypothesis that PCR analysis of bone marrow samples is a valuable aid in predicting clinical relapse. Third, a cautionary note must be raised from the observations of Yamada et al.; they found no evidence of the leukemic clone in the bone marrow of one patient who relapsed in the CNS. It appears that PCR analysis of bone marrow samples may not be sufficient to predict the likelihood of relapse in the CNS.

We would recommend PCR assays for the study of MRD over immunophenotyping assays because the latter methods, although very sensitive, can generate false-negative results in 20% of patients who relapse. In this report, we have shown the applicability of using another TCR gene
rearrangement (β-chain gene) for detection of MRD in T-ALL. However, this method is more time consuming, and is therefore less applicable than the TCR-β gene method for routine screening of T-ALL patients. Alternative PCR-based strategies for detection of MRD in T-ALLs lacking TCR-β rearrangements include amplification of TCR-γ gene rearrangements, and amplification of TCR-β cDNA.

We are currently exploring these approaches in the detection of MRD in T-ALL samples.

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Detection of minimal residual disease in T-cell acute lymphoblastic leukemia using polymerase chain reaction predicts impending relapse

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