Clinical Significance of bcr-abl Gene Rearrangement Detected by Polymerase Chain Reaction After Allogeneic Bone Marrow Transplantation in Chronic Myelogenous Leukemia

By Robert Delage, Robert J. Soiffer, Keith Dear, and Jerome Ritz

In chronic myelogenous leukemia (CML), amplification of a segment of bcr-abl messenger RNA (mRNA) by polymerase chain reaction (PCR) can be used to detect minimal residual disease after bone marrow transplantation (BMT). Previous studies have shown that this sensitive technique can often detect small numbers of leukemia cells in patients who are otherwise in complete remission. Nevertheless, the clinical significance of PCR positivity remains unclear because the majority of patients with PCR-detectable bcr-abl mRNA can remain disease-free for prolonged periods after allogeneic BMT. In the present studies, we applied PCR to detect bcr-abl-positive cells in 100 serial blood or BM samples from 24 patients with CML who underwent CD6 T-cell-depleted allogeneic BMT. After BMT, bcr-abl mRNA could be detected in 20 patients (83.3%) during complete cytogenetic or clinical remission. Patients in whom PCR positivity was sustained over time had a higher probability of CML relapse than patients in whom PCR was intermittently negative (P = .0095, log rank test). PCR detection of bcr-abl transcript between 2 and 10 weeks post-BMT also was associated with a high probability of subsequent relapse (P = .023, log rank test). In eight selected patients, we used a titration assay of the PCR-amplified product to estimate the number of residual tumor cells in each clinical sample post-BMT. PCR results in four patients showed a continuing increase in the number of tumor cells from early posttransplant until either cytogenetic or clinical relapse could be detected by conventional methods 1 to 2 years later. In contrast, PCR detected either no leukemia cells or relatively low and stable numbers of residual tumor cells throughout the follow-up period in four patients who remained in clinical remission. These results show that detection of the bcr-abl transcript by PCR after allogeneic BMT in patients with CML has important prognostic value. Estimation of the number of tumor cells in serial analyses can also be used to detect proliferation of the residual leukemic population. Sensitive detection of minimal residual disease can be used to assess the effectiveness of the transplant preparative regimen and to direct and evaluate further therapy post-BMT, before the development of overt relapse.

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PATIENTS AND METHODS

Patient population. Between September 1986 and October 1990, 24 patients (14 male, 10 female) with CML underwent allogeneic BMT and could be evaluated for detection of bcr-abl transcript by PCR analysis post-BMT (Table 1). Twenty patients were transplanted while in stable phase, three in accelerated phase, and one in blastic phase. The median age at BMT was 34 years (range, 20 to 49). All patients underwent allogeneic BMT using selective in vitro T-cell depletion with anti-T12 (CD6) monoclonal antibody (MoAb) and rabbit complement for prevention of graft-versus-host disease (GVHD). All patients and donors were matched at HLA-A, B, C, and D loci and were nonreactive in mixed lymphocyte culture. The transplant conditioning regimen consisted of cyclophosphamide (60 mg/kg/d × 2) followed by total body irradiation (TBI; initially at 5 cGy/min and since 1985 at 10 cGy/min) for a total dose of 1,200 to 1,400 cGy administered in six to seven equal fractions. All patients not previously splenectomized also received splenectomy. A total body irradiation dose of 1,000 to 1,200 cGy was given in 10 equal fractions over 4 days. All patients were transplanted while in stable phase, three in accelerated phase, and one in blastic phase. The median age at BMT was 34 years (range, 20 to 49). All patients underwent allogeneic BMT and could be evaluated for detection of bcr-abl translocation at any time after BMT. Clinical or hematologic relapse was defined as the detection of the 9:22 translocation at any time after BMT. Clinical or hematologic relapse was defined as the presence of an elevated white blood cell (WBC) count in conjunction with hypercellular BM and evidence of cytogenetic relapse.

Oligonucleotides. Oligonucleotides were synthesized with an Applied Biosystem PCR Mate synthesizer from previously published sequences. Primers G (5'-GGT ATT ATA GCC TAA GAC CCG GAG 3') and F (5'-GGC CAA AAA ATC ATA CAG TGC 3') were both antisense oligonucleotides spanning, respectively, nucleotides 182 to 203 and 121 to 142 3' to the bcr-abl junction. Primers α (5'-GGC AAG AGT AGC TCC CTG ATC 3') and A (5'-GGA GCT GCA GAT CAC CAA 3') were both sense oligonucleotides spanning, respectively, nucleotides -262 to -241 and -152 to -130 5' to the bcr-abl junction. Oligonucleotide E (5'-GAC CCC TTC AGC GCC CAG TAG CA 3') is adjacent to the junction on the abl side and was used as a probe to analyze the PCR-amplified products.

Reverse transcription and PCR amplification. Total RNA isolated from 10⁶ cells was used for reverse transcription. cDNA was first synthesized using MLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) and antisense oligonucleotide G. The reaction was performed at 42°C for 60 minutes and then at 95°C for 5 minutes. The newly synthesized cDNA was transferred into the PCR reaction mixture where the first amplification round was performed with primers G and α. If no amplification product was seen on an ethidium bromide-stained agarose gel, a second round of amplification was performed using 1 μL of the negative reaction mixture. This second round of amplification was performed in the same conditions using nested primers F and A. Both amplification rounds had 30 cycles, consisting of heat denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and polymerase extension at 72°C for 2 minutes. A 10-μL aliquot of the PCR reaction mixture was loaded onto a 3% NuSeive/l% agarose gel and electrophoresis was performed in Tris/borate/EDTA (TBE) buffer for 4 cm migration. Gels were stained with ethidium bromide solution (0.5 μg/mL) for 30 minutes and photographed. A 123-bp ladder was used as the molecular weight marker. The first amplification disclosed a product of 465 bp if exon 3 of the bcr gene was present in the chimeric mRNA or 390 bp if bcr exon 3 was absent. Likewise, the nested primers in the second round of PCR amplified DNA segments of 292 and 217 bp, depending on the presence or absence of bcr exon 3. In control experiments, gels were transferred to nylon membranes (Zetabind; Bio-Rad, Melville, NY) and the presence of bcr-abl-derived product was confirmed by hybridization with 32P-labeled oligonucleotide E.

PCR controls. To avoid possible contamination, isolation of mRNA, mixing of reagents, and electrophoresis of PCR products were each performed in separate areas. Mixing of reverse transcription and PCR reagents was performed in a restricted UV-irradiated area under a laminar flow hood. Different sets of automatic pipettes were used for mRNA preparation and analysis of the amplified products. Contamination was monitored by including two negative controls in each assay. The first control consisted of a

Table 1. Clinical Characteristics of the 24 CML Patients

<table>
<thead>
<tr>
<th>UPN</th>
<th>Age/Sex</th>
<th>Phase at BMT</th>
<th>Cytogenetic Status*</th>
<th>Clinical Status†</th>
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<td>SP</td>
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<td>CR 62</td>
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<td>SP</td>
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</table>

Abbreviations: SP, stable phase; AP, accelerated phase; BP, blast phase; CR, complete remission; R, relapse.

*Time of the last cytogenetic analysis if in complete remission or time of the cytogenetic relapse in months post-BMT.

†Actual follow-up post-BMT if in complete remission or hematologic relapse date in months post-BMT.

‡Died in complete remission.
sterile water control that included all reagents without mRNA and the second included mRNA isolated from normal T cells in culture. Results from experiments in which bcr-abl-positive signals were detected in the negative controls were considered false positives and were not used for analysis. To assess the quality of the mRNA in PCR-negative samples, we amplified the normal counterpart of the bcr gene that is expressed in tumor cells as well as normal PB lymphocytes (PBL). Thus, samples negative after the second PCR amplification were tested by reverse transcription and PCR amplification of a segment between exon 2 and 3 of the bcr gene. If no amplification of the normal bcr gene product was seen in this assay, the mRNA in the sample was judged unsuitable for reverse transcription and the result of the bcr-abl amplification was considered a false negative.

Sensitivity and specificity of double amplification PCR. Serial 10-fold dilutions of CML-derived K562 cells were mixed with 10⁷ normal cultured T cells. In each sample, total mRNA was isolated, reverse transcribed, and a first round of amplification was performed with G and α primers. A specific PCR amplified product of 465 bp was detectable in samples from 10⁶ or more K562 cells in 10⁶ normal T cells. A second round of PCR using nested primers F and A was performed on negative samples after the first amplification and disclosed the specific bcr-abl-amplified sequence of 292 bp with a sensitivity of one K562 cell per 10⁶ normal T cells. Cell lines of different lineages negative for the bcr-abl translocation and PB mononuclear cells (PBMC) from normal donors were tested and were negative for the targeted specific products after both the first and second rounds of PCR amplification (data not shown).

Quantitative analysis of bcr-abl mRNA. A titration assay of the bcr-abl-amplified product was evaluated to determine if a correlation could be made between the level of this PCR-amplified product and the number of leukemic cells in individual samples. A standard curve was first established using K562 cells. Total RNA was isolated from different samples containing 10⁶ K562 cells or 10⁷ cultured T cells admixed with decreasing numbers of K562 cells (from 10⁶ to 1) and reverse transcribed as described previously. The cDNA from samples containing 10⁶ to 10⁷ K562 cells (known to show a detectable PCR product after a first round of amplification) were serially diluted (10-fold dilutions) and the first PCR amplification was performed. As expected, analysis of ethidium bromide-stained gels showed decreasing intensity of the PCR-amplified product with each dilution. Samples without detectable bands after this first round of PCR were again diluted by serial 10-fold dilution and a second round of PCR with nested primers F and A was performed. All PCR reactions were run on a 2% agarose gel electrophoresis and stained with ethidium bromide for 30 minutes. The relative level of bcr-abl mRNA was established by counting the number of dilutions required for the last sample with a visually detectable PCR product. In samples in which dilutions were made after reverse transcription and after the first amplification, simple addition of the number of dilutions after each step determined the relative level of bcr-abl mRNA. Figure 1 shows the standard curve comparing the level of bcr-abl-amplified sequence with the known number of K562 cells. As seen in this analysis, an excellent correlation exists between the level of bcr-abl mRNA as determined by titration of the PCR assay and the added number of leukemic cells. This assay and standard curve were applied to the evaluation of standardized samples of PBMC from patients analyzed before BMT.

Statistical methods. The log rank test was used for testing difference in time to relapse between groups of patients defined by the result of PCR analysis. Two patients who died without evidence of relapse were considered censored for relapse at their times of death. The Mantel-Byar adaptation of the log rank test was used for testing the prognostic significance of continuing PCR positivity. In performing this test, one patient (UPN 1118), whose first negative PCR analysis was achieved at 29 months post-BMT, was considered to be PCR positive until that time. All other patients who became PCR negative showed a negative PCR analysis earlier than the earliest relapse time of any patient. This correction avoids a potential bias in the usual log rank test that would arise if patients who continued in remission for long periods would have a greater opportunity to show a negative PCR result.

RESULTS

Results of PCR analysis. Samples from 20 patients were analyzed before BMT, all of which were PCR positive after a single amplification. Exon 3 of the bcr gene was found in 8 of the 20 patients; in 11 patients, exon 2 of the bcr gene was adjacent to the junction of the chimeric transcript and in one patient both mRNA types could be detected. Interestingly, five patients expressed a different transcript on post-BMT analyses than the one originally identified. After BMT, 100 samples from 24 patients were examined. Overall, 65 of 100 analyses performed after BMT were found to contain a bcr-abl PCR product. Moreover, PCR positivity was found in at least one sample from 20 of the 24 patients examined during complete cytogenetic or clinical remission. Examples of serial PCR results after first and second amplification in two patients are shown in Fig 2. In both examples, all samples obtained early post-BMT were negative after the first amplification but positive after a second amplification. Later samples became PCR positive after the first amplification.

All results of PCR analysis after BMT are summarized in Figs 3 and 4. Based on these results, the 24 patients were divided into two groups. For the 13 patients comprising group 1 (Fig 3), all samples analyzed post-BMT were positive for the bcr-abl sequence (median, 3 analyses/patient; range, 1 to 8). Group 2 consists of 11 patients in which the specific bcr-abl sequence was undetectable in at least one analysis post-BMT (median, 4 analyses/patient; range, 1 to 12). As shown in Fig 4, 35 of 49 PCR analyses obtained in group 2 patients were negative for bcr-abl.
mRNA. In four patients, the PCR assay was negative in all samples (UPN 1039, 1250, 1356, and 1339). In seven patients in group 2, bcr-abl mRNA was detected in some but not all of the analyses post-BMT.

Clinical significance of PCR analysis post-BMT. The clinical outcome of patients who always tested PCR positive (group 1) was compared with that of patients who had at least one negative PCR analysis post-BMT (group 2).

Figure 5 compares the probability of maintaining hematologic remission for these two groups of patients. Of 13 patients in group 1, eight have relapsed and five remain alive without clinical evidence of leukemia. Two patients who remain in hematologic remission have detectable Ph'-positive metaphases by cytogenetic evaluation. Of 11 patients in group 2, nine remain alive without clinical evidence of leukemia. Two patients in group 2 died within 6 months of transplantation without evidence of relapse. One patient in group 2 who remains in clinical remission was found to have a cytogenetic relapse 37 months post-BMT.

Comparison of the clinical outcome of group 1 and group 2.
patients clearly suggests that sustained PCR positivity on serial analyses is associated with a higher probability of relapse post-BMT ($P = .0095$, log rank test).

We also analyzed the predictive value of the first PCR analysis in 16 patients in whom this analysis had been performed between 2 and 10 weeks post-BMT. Ten of 16 patients had a positive PCR result during this period and six of these patients subsequently relapsed. Six patients were negative on their first analysis post-BMT and none have relapsed after BMT, although two patients died in remission of transplant-related complications at 3 and 6 months post-BMT. This difference in clinical outcome is statistically significant ($P = .023$, log rank test), indicating that detection of minimal residual disease by PCR early post-BMT is associated with a higher probability of subsequent relapse.

Quantitative assessment of MRD post-BMT. To evaluate changes in minimal residual tumor burden post-BMT, we developed a titration assay of bcr-abl–amplified sequences to estimate the number of residual leukemic cells in patient samples. This analysis was performed with serial samples of PBMC from eight patients. The level of bcr-abl determined in each clinical sample was converted to a relative number of residual leukemic cells using the standard curve established with K562 (Fig 1). During the follow-up period (6 months to 4 years), three of these patients have had a clinical relapse, one patient has had a cytogenetic relapse only, and four have remained in complete remission with detectable bcr-abl sequences in some analyses. Figure 6 summarizes the results of this serial quantitative analysis in the three patients who developed a clinical relapse (UPN 1095, 1102, and 1079) and in one patient with a cytogenetic relapse (UPN 1112). In each patient, bcr-abl mRNA could be detected in every sample. In UPN 1095, the relative number of residual tumor cells appeared to remain stable at a low level during the first 3 months post-BMT. However, subsequent samples obtained at 6 and 12 months post-BMT contained progressively increasing numbers of bcr-abl–positive cells. This patient relapsed 12 months post-BMT when the proportion of tumor cells reached the level estimated before BMT ($5 \times 10^3$ CML cells/10$^6$ PBMC). A similar progression was seen in UPN 1102, although there was a slower increase in the relative number of tumor cells and relapse did not occur until 2 years post-BMT. Estimation of the number of leukemic cells in UPN 1079 showed that they remained relatively low during the first 9 months post-BMT but began to increase 14 months post-BMT. Concurrent cytogenetic analysis of BM cells showed a single Ph$^+$-positive cell out of 35 metaphases. By 24 months post-BMT, the number of PCR-detectable leukemic cells had increased further and simultaneous cytogenetic analysis of BM cells showed that 8 of 31 metaphases were Ph$^+$-positive. This patient remained hematologically normal at this time and only showed evidence of clinical relapse 1
year later. Finally, UPN 1112 was found to have a relatively low level of bcr-abl-positive cells at 3 months post-BMT that subsequently increased at 9 and 11 months post-BMT. Concurrent cytogenetic analysis showed that 4 of 37 metaphases were Ph'-positive. However, the relative number of tumor cells detected by PCR did not show any further increase at 15 months post-BMT. This correlates with the clinical course in this patient who has remained in hematologic remission for more than 2 years after the first positive cytogenetic analysis.

Figure 7 summarizes the results of similar quantitative studies in four patients who have remained in hematologic and cytogenetic remission with PCR-detectable bcr-abl-positive cells. In UPN 1107, most of the PCR analyses performed over a 4-year period were negative except at 24, 40, and 44 months post-BMT. Although positive at these times, the relative number of leukemic cells determined by the titration assay was low, varying between 1 and 10 CML cells/10^6 PBMC, and did not increase over time. In fact, CML cells were undetectable on the last PCR analysis obtained 48 months post-BMT. Similar patterns were seen in UPN 1342 and 1071, although in the latter patient the level of CML cells increased dramatically (approximately 100-fold) in the most recent sample obtained 44 months post-BMT. In UPN 1162, PCR analysis was positive on all three occasions at 5, 16, and 28 months after transplantation. However, quantitation of bcr-abl-positive cells in this patient showed that the relative number of leukemic cells remained stable at a low level throughout this period.

DISCUSSION

Immediately after allogeneic BMT for CML, the Ph' chromosome usually cannot be detected by conventional cytogenetic methods. Its reappearance at a later time post-BMT is frequently associated with concurrent or incipient clinical relapse, although in some individuals detection of cytogenetic relapse post-BMT predates hematologic relapse by several years. There are also well documented cases of the disappearance of Ph'-positive cells several years after BMT. That Ph'-positive cells can persist post-BMT and yet not result in clinical disease is intriguing. Unfortunately, cytogenetic studies are not sufficiently sensitive to accurately detect small numbers of residual leukemia cells. In contrast, PCR techniques that amplify mRNA of the hybrid bcr-abl gene produced by the 9,22 translocation have allowed the specific and sensitive detection of very small numbers of leukemia cells. Indeed, in control experiments using PCR amplification, this method can detect the presence of one CML cell admixed with 10^6 normal cells. Given the marked increase in sensitivity of PCR, application of this technique provides a unique opportunity to study the biologic and clinical implications of the presence of small numbers of leukemia cells after BMT.

In the present study, we used a sensitive double-amplification PCR technique with nested primers to detect residual leukemic cells in 24 patients with CML after allogeneic BMT. The great majority of these patients (83.3%) were transplanted in the chronic phase of their disease. The BMT preparative regimen consisted of splenic radiation, high-dose cyclophosphamide, and TBI. Each patient received marrow from an HLA-matched, MLC-nonreactive sibling donor. Selective T-cell depletion of donor marrow with anti-T12 (CD6) MoAb and complement was used for prevention of GVHD. Patients did not receive additional prophylactic immune suppressive therapy or chemotherapy post-BMT.

Samples from 20 patients were available before BMT. All pre-BMT samples disclosed a specific bcr-abl band after a single round of PCR amplification, including two samples...
that were negative for the Ph' chromosome by cytogenetic analysis. Chimeric transcripts using junctional regions derived from bcr exon 2 as well as from bcr exon 3 were both easily detected with this method. After BMT, bcr-abl mRNA could be detected in either blood or BM samples by PCR in 20 patients (83.3%) at times when neither cytogenetic nor cytologic assessment was able to detect the presence of leukemic cells. This finding confirmed the striking sensitivity of this method and that persistence of small numbers of CML-derived cells is a common occurrence after allogeneic BMT.\textsuperscript{16,18,22} However, despite detection of minimal residual disease, 10 of 20 PCR-positive patients continue in remission for 6 to 46 months after BMT. Although all four patients who were consistently PCR-negative remained in remission, the detection of the bcr-abl transcript by itself does not appear to accurately predict clinical outcome.\textsuperscript{16,18,22,26}

To better define the role of PCR in CML post-BMT, we undertook further analysis to evaluate the clinical significance of serial PCR results. Based on the sensitivity of our PCR analysis, a negative result indicates a tumor burden less than 1 CML cell/10\textsuperscript{6} normal cells. We therefore reasoned that patients with any negative results on serial PCR analyses would be more likely to have lower numbers of residual tumor cells than patients with consistently positive PCR results, and that this may contribute to different clinical outcomes. To examine this hypothesis, we divided patients into two groups based on their serial PCR results: group 1 consisted of 13 patients in whom we could detect bcr-abl mRNA in all analyzed samples; group 2 included 11 patients with at least one negative PCR analysis after BMT. All eight patients who eventually relapsed and two of three patients who experienced a cytogenetic relapse were in group 1. In contrast, no hematologic relapse has thus far been seen in group 2; only one patient has had a cytogenetic relapse and all others have remained in complete remission (two patients died while in remission post-BMT). Although relapses may ultimately occur in group 2 patients with further follow-up, statistical analysis of our results clearly indicates that patients with at least one negative PCR analysis post-BMT have a significantly lower risk of relapse than patients who were consistently PCR positive (P = .0095).

In addition to demonstrating the importance of serial PCR analyses post-BMT, our data also indicate the value of such studies in the early posttransplant period. The results of the first PCR analysis performed between 2 and 10 weeks post-BMT were evaluated; 10 patients were positive on their first PCR analysis and six of these patients have relapsed. Six patients tested negative on the first PCR analysis and none have relapsed, although two patients died of complications early post-BMT. This finding suggests that a greater reduction in tumor burden was achieved in response to the ablative transplant regimen in some patients, and that this resulted in a negative PCR result on the first analysis early post-BMT.

Because our data suggest that patients with at least one negative PCR analysis on serial examinations have a lower probability of clinical relapse, it is interesting to note that five patients who always tested positive on serial PCR analyses have also remained in hematologic remission. Cytogenetic analysis has detected a small fraction of Ph'-positive cells in two of these patients, but three remain in cytogenetic as well as hematologic remission. These findings are consistent with previous studies that have shown that the presence of significant numbers of residual leukemic cells post-BMT is not followed by clinical relapse in all patients.\textsuperscript{18,22,25} The reasons that residual CML cells do not invariably result in clinical relapse have not been clearly defined but several hypotheses have been proposed: (1) alteration of the malignant clone by the ablative regimen resulting in decreased proliferative activity; (2) the persistence of long-lived bcr-abl-positive cells without tumorigenic potential that survived the transplant preparative regimen; and (3) the presence of GVL activity that is able to suppress the malignant clone.

One of the major drawbacks of PCR analysis for the detection of minimal residual disease has been that a positive result, by itself, yielded little information about the actual number of leukemia cells present or their proliferative potential. However, estimating the number of bcr-abl-positive cells, when used in serial analyses, would show whether residual cells derived from the leukemic clone are increasing, remaining stable, or decreasing. In CML, measuring the level of bcr-abl mRNA appears ideal for this purpose because, in addition to being a marker of the leukemic cells, the level of bcr-abl mRNA has also been shown to correlate with the proliferative state of the cell.\textsuperscript{32} To address this issue, we developed a method of estimating the level of bcr-abl mRNA based on titration of the two-step PCR assay. Analysis of serial PBMC samples was compared with a single standard with K562 cells, thus allowing us to detect changes in the relative number of bcr-abl-positive cells over time in individual patients post-BMT. Using this quantitative assay, the level of bcr-abl mRNA post-BMT was examined in four patients who had relapsed (three hematologic relapse and one cytogenetic relapse) and four patients with detectable bcr-abl product but who remained in complete remission. In three patients who eventually relapsed, we observed a progressive increase in the relative number of residual leukemic cells during the entire period of complete remission. At the time of relapse, the proportion of leukemic cells had increased to the level observed before BMT. In the patient who had a cytogenetic relapse but remained in hematologic remission, the estimated number of CML cells has remained stable at a level of approximately 10\textsuperscript{2} to 10\textsuperscript{4}/10\textsuperscript{6} PBMC throughout a 2-year period. In contrast, the number of detectable leukemic cells remained stable and generally at a much lower level in four patients who remained in complete remission with evidence of residual leukemic cells by PCR on some analyses. Thus, our method of quantitation of residual tumor cells clearly shows that the leukemic clones of some patients retain their proliferative capabilities after BMT. In contrast, bcr-abl-
positive cells in other patients appear to either have lost their proliferative advantage over normal hematopoiesis or their expansion is being effectively controlled.

As indicated by the results of this study, the ability to estimate the number of residual leukemia cells with PCR after BMT in patients with CML is likely to have significant clinical utility. First, this technique may be useful for comparing the cytoreductive efficacy of different transplant ablative regimens. Second, PCR can be used to assess the early progression of disease while patients remain in cytogenetic and hematologic remission. As a result, we may be able to gain a better understanding of the mechanisms and effectiveness of GVL activity after allogeneic BMT. In patients with PCR-detectable disease, further therapy or specific immunologic manipulations can be initiated early post-BMT, before the development of overt relapse.

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