Competitive Polymerase Chain Reaction to Estimate the Number of BCR-ABL Transcripts in Chronic Myeloid Leukemia Patients After Bone Marrow Transplantation

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We have developed a competitive polymerase chain reaction (PCR) titration assay that estimates the number of BCR-ABL transcripts in chronic myeloid leukemia patients to monitor minimal residual disease after bone marrow transplantation (BMT). The assay gave reproducible results and allowed differences in BCR-ABL message levels of half an order of magnitude to be distinguished. Of 91 patients studied by nonquantitative PCR, 28 who had a positive PCR result on at least one occasion posttransplant were analyzed by competitive PCR. Seventeen patients had no evidence in their marrow of cytogenetic relapse during the period of observation; BCR-ABL transcript numbers in these cases ranged from approximately 10 to 800/μg RNA. Ten of the 11 patients who relapsed cytogenetically were studied when Philadelphia-positive metaphases were first detected in their marrow; transcript numbers ranged from 1.60 to 7 × 10^9/μg RNA. Patients in hematologic remission had between 9 × 10^4 and 10^8 BCR-ABL transcripts/μg RNA. Patients who progressed from cytogenetic remission to cytogenetic relapse and then to hematologic relapse had increasing numbers of BCR-ABL transcripts in their blood. Three patients had clear evidence of rising numbers of BCR-ABL transcripts before routine detection of cytogenetic relapse. Conversely, patients without cytogenetic relapse generally had low or falling numbers of transcripts. We conclude that serial monitoring of residual disease post-BMT by estimating the number of BCR-ABL transcripts provides more information than conventional cytogenetics or nonquantitative PCR and may identify patients in need of therapeutic intervention before the onset of overt relapse.

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MATERIALS AND METHODS

Patients. CML patients received transplants of unmanipulated marrow from either HLA-identical sibling donors (SIBs) or matched unrelated donors (UDs). Patients in the latter group underwent in vivo T-cell depletion by the use of Campath 1G and all patients received cyclosporin A (CSA) plus methotrexate (MTX) posttransplant. A total of 91 such patients undergoing BMT at the Hammersmith were studied prospectively by PCR analysis from May 1991 to April 1993. Results are presented in this report on all 28 patients who had PCR-positive assays, and at least two sequential PCR results, during the 11-month period before the date of analysis (April 6, 1993). During this period, the remaining 63 patients either had only negative PCR assays, or had only a single or no PCR assay; all 63 remained in complete clinical and cytogenetic remission. Many of these patients and their qualitative PCR results have been described previously.8 The conditioning regimen in all groups included cyclophosphamide at 120 mg/kg and fractionated total body irradiation to a total dose of 10, 12, or 13.2 Gy at a dose rate of 15 cGy/min.11 Peripheral blood cells were studied at various intervals posttransplant; we have previously shown a good correspondence between PCR results obtained from blood and marrow.2 Cytogenetic analysis of marrow cells was performed routinely on all patients at 3, 6, 9, and 12 months posttransplant and subsequently.

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PCR. Preparation of RNA from peripheral blood samples by ultracentrifugation, cDNA synthesis, and the two-step nested PCR procedure used for detection of BCR-ABL transcripts have been described previously.\(^4\) The amount of RNA extracted was determined by the optical density at 260 nm. Usually 10 to 40 μg of RNA was recovered from 10 to 20 mL of peripheral blood leukocytes. Rigorous precautions were taken to avoid contamination of PCRs. Samples were processed in laminar flow hoods, plugged aerosol-resistant pipette tips were used at all stages, two negative controls were included in each run (one introduced at the RNA extraction step and one at cDNA synthesis), and all samples were processed in duplicate on separate days. Reaction products were electrophoresed on 1.5% agarose gels in a separate room using dedicated pipette. At least two dilutions were prepared in 1 mmol/L Tris, pH 8.0, 0.1 mmol/L EDTA, 50 μg/mL Escherichia coli tRNA. Dilutions were made in 10-fold dilution from each dilution. The equivalence point at which the competitor and sample band would be of equal intensity was estimated independently by two investigators. This approach was found to be preferable to performing further competitive PCRs to determine an exact equivalence point because of the limited amount of cDNA that could be obtained from samples. Any discordance was resolved by performing the analysis for a third time and scoring the majority result. We found that only approximately 5% of duplicate results were discordant. Due to the size difference between the competitor and BCR-ABL, the number of molecules of competitor at the equivalence point was multiplied by 1.2 for b3a2 patients and 1.5 for b2a2 patients (the ratio in size of competitor and b3a2 or b2a2 PCR products, respectively) to derive the number of BCR-ABL molecules in the sample. This number was converted to the number of molecules per microgram of sample RNA.

**Primer extension.** Oligo 185-1 (5' CCACAGTTATCCAAG-TGGGA 3') was designed to hybridize to the 18S RNA between positions 137 and 156.\(^1\) The oligo was end-labeled using polynucleotide kinase\(^1\) and γ-32P-ATP, and 20 ng was hybridized to 1 μg RNA in 1× cDNA buffer containing 10 mM dithiothreitol (DTT), 1 mM/L each of the four deoxynucleotides, 400 U Mo-loney murine leukemia virus (MMLV) reverse transcriptase, and 40 U RNasin. After adding an equal volume of formamide dyes, samples were heated to 80°C for 5 minutes and electrophoresed on 6% polyacrylamide sequencing gels.\(^\text{13}\) Gels were dried and exposed to autorigraphic film at −70°C for 16 hours.

**RESULTS**

**Competitive PCR titration assay.** We engineered a plasmid construct to introduce an extra DNA sequence between the primer sites used for PCR of BCR-ABL cDNA (Fig 1). Amplification of this clone, pBK5, produces a larger product than either b2a2 or b3a2 BCR-ABL under identical conditions and the three are readily distinguishable after agarose gel electrophoresis. We have used this clone as a competitor in a PCR titration assay.\(^\text{14}\) Serial dilutions of linearized pBK5 were added to fixed amounts of test cDNA and the mixture subject to standard two-step nested PCR. The competitor clone competed with the endogenous sample BCR-ABL for primers, enzymes, and nucleotides in the reaction. If the initial concentration of the competitor is much higher than that of sample fusion gene message, then only the competitor band is visible on the final gel. Conversely, if many fewer molecules of clone are added, then only the sample BCR-ABL band is visible. If the starting reaction contained equal numbers of molecules, then the gel showed both bands with the ratio of the fluorescence intensity between the two in proportion to their sizes. In this case, the competitor, b3a2, and b2a2 result in band sizes of 559 bp, 458 bp, and 383 bp, respectively. If, after titration, a number of competitor molecules is obtained that results in half order of magnitude on a logarithmic scale, ie, 10\(^7\), 3.2 × 10\(^7\), 10\(^8\), 3.2 × 10\(^8\), etc.
competitor and BCR-ABL in RNA derived from peripheral blood leukocytes of a patient in cytogenetic relapse after BMT and reverse transcribed into cDNA, and competitive PCR was performed with $3 \times 10^5$, $10^5$, and $3 \times 10^4$ molecules of linearized competitor plasmid. The figure above each track indicates the number of competitor molecules added. The amount of cDNA in each reaction was that generated from 1 µg RNA. The equivalence point in fluorescent intensity between the competitor and b2a2 bands is between $10^5$ and $3 \times 10^4$ competitor molecules added. A hybrid band is also visible that does not interfere with the equivalence point in fluorescent intensity between the competitor and sample bands of equal fluorescence intensity, as seen in Fig 2. Dilution of the CML blood into normal blood at factors of 10-, 100-, and 1,000-fold results in a reduction of detectable BCR-ABL transcripts by approximately 10-, 100-, and 1,000-fold, respectively. The normal blood used for dilution is PCR negative, as expected.

Accuracy and reproducibility of the assay. A number of experiments were performed to establish the accuracy and reproducibility of the competitive PCR titration assay. First, serial dilutions of a sample of CML blood were made into normal blood and quantification performed to see if the number of BCR-ABL transcripts detected corresponded to the dilution factor. The equivalence point between the competitor and BCR-ABL in RNA derived from peripheral blood leukocytes of a patient in cytogenetic relapse after BMT was found to lie between $3 \times 10^5$ and $10^5$ molecules/µg of total RNA (Fig 2). Inspection of the intensity of the competitor and patient b2a2 PCR bands suggested a level of roughly $7 \times 10^4$ competitor molecules and therefore $1.1 \times 10^5$ BCR-ABL molecules. Dilution of the CML blood 10-fold into normal blood resulted in a reduction of detectable BCR-ABL transcripts, with an equivalence in fluorescent intensity at roughly $7 \times 10^3$ competitor molecules. Similarly, dilution of 100- and 1,000-fold results in equivalence points 100-fold and 1,000-fold less, respectively. This experiment establishes linearity of the assay over at least 4 orders of magnitude and shows that the competitor and BCR-ABL cDNA are amplified with equal efficiency.

To show the reproducibility of competitive PCR, cells from a patient with CML in chronic phase were collected and divided into eight parts. RNA was extracted independently from each fraction and 10 µg used for cDNA synthesis and quantitative PCR. The results are shown in Fig 3. In each of the eight samples A to G, the point at which the fluorescence intensity of the b3a2 and competitor bands would be equal can be seen to lie between $3 \times 10^5$ and $10^5$ molecules of competitor added. The equivalence points were estimated to be $5 \times 10^5$, $7 \times 10^5$, $5 \times 10^5$, $5 \times 10^4$, $4 \times 10^5$, $6 \times 10^5$, $5 \times 10^4$, and $6 \times 10^4$ (mean, $5.25 \times 10^5$; standard deviation, $8.9 \times 10^4$) molecules of competitor added, respectively. The standard deviation found here is only 17% of the mean and thus shows that results of the assay are reproducible. Estimation of BCR-ABL transcripts to within half an order of magnitude is therefore achievable because 95% of determinations fall within two standard deviations of the mean, assuming a normal distribution.

Because the competitor is DNA and is therefore added to the first PCR, there is no direct control for the reverse transcription step. To see if this step was a significant source of variation, we routinely performed quantitative PCR on the M5 dilute positive RNA controls that had been reverse transcribed over a number of months using different batches of reverse transcriptase and other reagents (Fig 4). Again, a high reproducibility between samples can be seen with fluorescence intensity equivalence points lying between 30 and 100 molecules of competitor added. This experiment shows that variation in the efficiency of cDNA synthesis, once conditions have been established, is low. Occasional instances of inefficient reverse transcription were found by failure of the M5 to produce a b3a2 band after nested PCR; in these cases, results from all samples in that batch were discarded. A potentially far greater source of variation is the quality of the sample RNA. We have used a primer extension assay to check for the integrity of the 18S RNA (Fig 5)
and, thus, by implication, all RNA molecules in the sample. Intact RNA was indicated by a discrete band at 156 bp, partially degraded RNA by a ladder of bands, and severely degraded RNA by absence of bands. All samples assayed in this way showed a discrete band.

Correlation between cytogenetics and BCR-ABL levels. Having established the accuracy and reproducibility of the competitive PCR assay, we estimated the number of detectable BCR-ABL transcripts in 4 untreated chronic-phase patients. All had levels of between 5 × 10^3 and 10^5 BCR-ABL transcripts/μg RNA. We proceeded to apply the assay to patients who were PCR positive after BMT. Results of patients in whom Ph-positive metaphases were found at some time after BMT are shown in Fig 6 and results of patients who remained in complete remission are shown in Fig 7. Patients who remained in complete remission generally had low levels of BCR-ABL transcripts and many had PCR positivity approaching the limit of detection at 10 or less than 10 molecules per microgram of RNA. In contrast, patients in whom Ph-positive metaphases were detected in the marrow had higher levels (Fig 8). For example, 10 patients who relapsed cytogenetically had contemporaneous PCR assays; all had greater than 1,600 BCR-ABL transcripts/μg RNA at the time that Ph-positive metaphases were first detected (range, 1,600 to 7 × 10^4 transcripts/μg RNA). Three patients had BCR-ABL levels within this range, but no Ph chromosomes were found in the first 30 contemporaneous marrow metaphases examined. Specifically, patients no. 1, 3, and 8 had 4,500, 6,000, and 10,000 BCR-ABL transcripts detected at 6, 8, and 24 months post-BMT, respectively. In view of these relatively high levels, the cytogenetics slides were reexamined. For patient no. 1, 0 of 52 metaphases were Ph positive; 2 of 50 were positive for patient no. 3 and 3 of 72 were positive for patient no. 8. Each of these patients was examined 3 to 4 months later; each had increased BCR-ABL levels and Ph-positive metaphases were detected in the first 30 evaluable metaphases examined. Cytogenetics slides were reevaluated in two further patients: patient no. 13 had 800 BCR-ABL transcripts/μg RNA at 3 months post-BMT and 0 of 31 Ph-positive metaphases; patient no. 7 had 500 BCR-ABL transcripts/μg RNA at 12 months post-BMT and 0 of 40 Ph-positive metaphases. In each case, there were no further evaluable metaphases. Therefore, the lowest BCR-ABL level at which Ph-positive metaphases were detected by routine cytogenetics (ie, 30 metaphases examined) was 1,600 transcripts/μg RNA and the highest level at which only cells of normal karyotype were found was 10,000 transcripts/μg RNA.

BCR-ABL levels and relapse. A total of 11 patients relapsed either cytogenetically or hematologically during this study. Of those who had serial semiquantitative PCR assays, all were found to have increasing numbers of detectable transcripts during progression from complete remission to cytogenetic relapse and finally to hematologic relapse. Patients no. 1, 3, 6, and 10 had not relapsed clinically, and only a small minority of metaphases in these individuals are Ph positive. It will be of interest to see whether these patients progress to full hematologic relapse. Of the 7 patients who did proceed to hematologic relapse, patients no. 4, 8, and 9 relapsed into chronic phase, no. 5 and 7 relapsed into accelerated phase, and no. 2 and 11 relapsed into blast crisis. Relapse into accelerated phase or blast crisis appears to be a rapid process. Patients no. 7 and 11 both showed dramatic increases in the level of BCR-ABL transcripts of 200- and 660-fold, respectively, over a period of 3 to 5 months, and patient no. 2 relapsed 2 months after a finding of only 200 BCR-ABL transcripts/μg RNA. However, relapse into chronic phase may proceed at a slower pace; transcript levels in patient no. 9 increased 10-fold and then 10-fold again over two 6-month periods, whereas relapse for patient no. 4 was rapid. A clear increase of more than 250-fold in the estimated number of BCR-ABL transcripts was observed in

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Fig 4. Reproducibility of quantitative PCR. Quantification was performed on eight dilute positive control cDNAs derived from a dilution of K562 cells in HL60 cells at a ratio of 1 in 10^6. Each cDNA was prepared using different batches of reverse transcriptase or buffer reagents over a period of several months. PCR was performed after the addition of 0, 10, 30, or 100 competitor molecules and the equivalence point in each case is between 30 and 100 molecules.

Fig 5. Primer extension of 18s RNA to check for integrity of sample RNA preparations. A discrete band at 156 bp indicates undegraded RNA. Track 1, no RNA; tracks 2 through 14, sample RNA.
Fig 6. PCR and cytogenetics results on 11 patients in whom Ph-positive metaphases were detected at some time point after BMT. PCR results (---) are shown as (0) PCR negative or (1) PCR positive. Quantitative PCR results, when performed, are shown above PCR positive results and are expressed as the number of BCR-ABL molecules detected per microgram of RNA. Cytogenetic results (---) are shown as (0) if all metaphases examined were Ph negative or (1) if Ph-positive metaphases were detected. The proportion of Ph-positive metaphases detected are shown in each case; eg. 2/30 indicates the presence of 2 Ph-positive metaphases of a total of 30 metaphases examined. Disease status at time of transplant and donor type are shown. Abbreviations: CP1, first chronic phase; CP2, second chronic phase; AP, accelerated phase. *No Ph-positive metaphases were detected in the first 30 metaphases examined by cytogenetics. Due to the high number of BCR-ABL transcripts found by competitive PCR, cytogenetics slides were reexamined, yielding 0 of 52 Ph-positive metaphases (patient no. 1), 2 of 50 Ph-positive metaphases (patient no. 3), and 3 of 72 Ph-positive metaphases (patient no. 8).

Disease status at time of transplant and donor type are shown. Abbreviations: CP1, first chronic phase; CP2, second chronic phase; AP, accelerated phase. *No Ph-positive metaphases were detected in the first 30 metaphases examined by cytogenetics. Due to the high number of BCR-ABL transcripts found by competitive PCR, cytogenetics slides were reexamined, yielding 0 of 52 Ph-positive metaphases (patient no. 1), 2 of 50 Ph-positive metaphases (patient no. 3), and 3 of 72 Ph-positive metaphases (patient no. 8).

three cases (patients no. 3, 6, and 7) of the 11 before relapse. In the remaining 8 cases, insufficient contemporaneous PCR and cytogenetics results were obtained to unambiguously detect an increase in transcript levels before relapse.

DISCUSSION

We have developed a competitive PCR titration assay that allows us to estimate the number of BCR-ABL transcripts present in samples from CML patients. The method is accurate and reproducible; we have derived a standard deviation of the assay and shown that differences in the level of BCR-ABL transcripts of half an order of magnitude can be distinguished. The variation in the reverse transcription step is generally low and can be effectively controlled for by routine quantification of a control sample, which means that a DNA, rather than an RNA, competitor can be used. This greatly simplifies the procedure and also has the advantage that the competitor is much less likely to deteriorate over long periods of time. The quality and amount of sample RNA is controlled by a primer extension assay. Although estimation of the equivalence point, currently performed by inspection, could be improved by direct densitometry from the agarose gels or by use of radioactive/fluorescently labeled primers, we have aimed to keep the method as simple as possible and have shown that the variation thus obtained is acceptably low.
Patients in untreated chronic phase showed a level of between $5 \times 10^5$ and $10^6$ BCR-ABL transcripts per microgram of RNA. Assuming the peripheral blood of these patients to contain nearly 100% CML cells, and the average amount of RNA per cell to be 10 pg, then each cell would contain on average 5 to 10 copies of BCR-ABL mRNA. Several data points have been obtained in this study at about this level in patients after transplant, suggesting that single CML cells are being detected.

We and others have found that qualitative PCR assays are of limited predictive value for individual patients after BMT for CML.\(^{2,8-10,16}\) In particular, some patients are PCR positive for a number of months after transplant before becoming PCR negative. Although all patients are PCR positive before relapse, those who are persistently or intermittently PCR positive at or longer than 1 year after BMT have an increased probability, rather than a certainty, of relapse. We have shown here that competitive PCR effectively discriminates between those patients who have increasing numbers of BCR-ABL transcripts, and so may be in an early stage of relapse, and those who have decreasing or very low numbers of transcripts and therefore may be cured. For instance, patients no. 9 and 23 were both persistently PCR positive for 2 years after BMT. At 18 months after transplant, patient no.
Three of three evaluable patients showed a clear increase in the number of BCR-ABL transcripts before the routine detection of Ph-positive metaphases in the marrow. Other patients who had serial assays showed increasing numbers of detectable transcripts in the progression from complete remission to hematologic relapse. To effectively predict relapse, PCR results would have to be obtained more frequently. We suggest that patients should be monitored routinely at 3, 6, 9, 12, 18, 24, etc. months post-BMT. If a patient converts from PCR negative to PCR positive, or shows increasing numbers of transcripts on sequential analyses, then assays should be performed at 1- to 2-monthly intervals.

Studies by other groups using selected patients also suggest that estimation of the number of BCR-ABL transcripts by either a dilution assay or competitive PCR may be useful in determining which patients are likely to relapse. This study suggests that routine monitoring of CML patients after BMT by competitive PCR may effectively distinguish between those who will relapse and those who may be cured. It may soon be possible to consider therapeutic intervention, eg, donor buffy coat infusion\textsuperscript{19,20} or \textit{\alpha}-interferon, for such individuals before the development of overt relapse.

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