Polymerase Chain Reaction Detection of the $BCR-ABL$ Fusion Transcript After Allogeneic Marrow Transplantation for Chronic Myeloid Leukemia: Results and Implications in 346 Patients


We studied 346 patients after bone marrow transplantation (BMT) for chronic myeloid leukemia (CML) for the presence of the $bcr-abl$ transcript detected by the polymerase chain reaction (PCR) to understand the frequency and implication of a positive test. A total of 634 samples of BM and/or peripheral blood were obtained for PCR analysis between 3 and 192 months after BMT. A positive PCR test at 3 months post-BMT was not statistically significantly associated with an increased risk of relapse compared with PCR-negative patients. However, a positive PCR assay at 6 months and beyond was highly associated with subsequent relapse. The Kaplan-Meier estimate of relapse for patients testing PCR-positive at 6 to 12 months was 42% versus 3% for PCR-negative patients ($P < .0001$). The Kaplan-Meier estimate of survival at 4 years for the PCR-positive patients was 74% compared with 83% for the PCR-negative group ($P = .002$). Multivariable analysis indicated that a PCR-positive result at 6 to 12 months post-BMT, the type of BMT donor (allogeneic matched donor vs mismatched or unrelated), and the presence of acute GvHD were independent risk factors for subsequent relapse. The relative risk (RR) for relapse for patients PCR-positive at 6 to 12 months post-BMT was 26.1 (95% confidence interval, 8.9 to 76.1, $P < .0001$). The outcome of long-term patients (>36 months post-BMT) who tested PCR-positive was much better, as 15 of 59 (25%) tested positive for $bcr-abl$, but only one patient relapsed. There was a 91% concordance between PCR tests of simultaneously obtained BM and peripheral blood. These analyses show that the PCR assay of the $bcr-abl$ fusion transcript 6 to 12 months post-BMT is an independent predictor of subsequent relapse which provides an opportunity for early therapeutic intervention.

© 1995 by The American Society of Hematology.
ables associated with relapse in multivariable models. We find that a positive PCR assay at 6 to 12 months post-BMT is an independent predictor of relapse after separately controlling for phase of disease, type of donor, and acute and chronic GVHD. We also note that PCR positivity is less common in patients greater than 12 months from transplant and is less strongly associated with relapse. Like allogeneic, related transplants, unrelated donor (URD) transplants are commonly PCR-positive early after transplant but, in contrast, URD patients who are PCR-positive infrequently relapse.

MATERIALS AND METHODS

Patients. All patients receiving transplants for CML at the Fred Hutchinson Cancer Research Center between 1976 and 1993 were eligible for study. The transplant protocols were approved by the Institutional Review Board. Patients were identified after BMT before their discharge home, or for patients already home, through contact with our Long-Term Follow-Up (LTFU) program. Requests were made to receive both BM and peripheral blood (PB) at 6-month intervals. The LTFU staff were unaware of the results of PCR assays. Many patients elected to have samples obtained at their yearly LTFU check at our institution. These BM and PB samples were used to perform cytogenetic evaluations and the PCR assay for the presence of bcr-abl mRNA.

Transplant regimen. Patients receiving transplants in chronic phase before 1990 received a conditioning regimen of cyclophosphamide (CY) 60 mg/kg for 2 days (120 mg/kg total) and total body irradiation (TBI) at a dose of 200 cGy for 6 days (1,200 cGy total). Since 1990 chronic phase patients were randomized to the above regimen versus busulfan (BU) 16 mg/kg and CY 120 mg/kg. Accelerated- and blast-phase patients received a preparative regimen of CY 120 mg/kg and TBI of 225 cGy for 7 days (1,575 cGy total). Unrelated donor transplant recipients received a preparative regimen of CY 120 mg/kg and either 1,200 cGy of TBI if in chronic phase or 1,320 cGy if in accelerated or blast phase. All patients received GVHD prophylaxis with methotrexate and cyclosporine as previously described.8

Sample preparation. RNA isolation from leukocytes from fresh BM and PB samples was performed by the acid guanidinium thiocyanate-phenol-chloroform method as previously described.18 Aliquots frozen at -70°F were used for the detection of bcr-abl and the control b2 microglobulin mRNA (below).

Reverse transcription (RT) PCR assay for bcr-abl. We used a two-step, “nested” RT/PCR to amplify the chimeric bcr-abl mRNA. The nested reaction yields either a 305-bp or a 234-bp PCR product depending on the expression of bcr exon 3. One microgram of total RNA was added to a combination 50 μL RT/PCR mix of 25 pmol of oligonucleotides CMLND and CMLNC, 50 mmol/L KCl, 10 mmol/L TRIS-HCL (pH 9.0), 0.1% Triton X-100 (Sigma, St Louis, MO), 200 μmol/L dioxynucleotides (dNTPs), 1.5 mmol/L MgCl2, 1.25 U Taq polymerase (Amplitaq; Perkin Elmer Cetus, Norwalk, CT), 10 U RNAsin, 7.5 U AMV RT (Boehringer Mannheim, Indianapolis, IN), and DEPC H2O. This reaction mixture was incubated for 30 minutes at 42°C, brought to 95°C for 5 minutes, and then amplified for 40 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute followed by a final elongation step at 72°C for 7 minutes. Amplification was performed at 15 μL of the final PCR product was electrophoresed through ethidium-bromide-stained 2% agarose gel and photographed.

For all PCR reactions a 10−6 dilution of K562 RNA into the Ph-negative control cell line HL60 RNA served as a positive control. Negative controls included both a no-RNA PCR mix ("blank") and HL60 RNA. A separate amplification of b2 microglobulin was used as a control of RNA integrity and was performed as previously described.20

Precautions to eliminate PCR carryover contamination included separate rooms for pre-PCR and amplification procedures, filter-containing disposable pipette tips, and no-nucleic acid PCR reactions as negative control in all PCR amplification reactions.4 Using these precautions we had an approximately 2% incidence of false positive assays, defined as a positive PCR result in the no-RNA or HL60 RNA reactions. In these instances all samples run in the amplification "batch" (usually 10 samples) was repeated.

The nucleotide sequences of the PCR primers used in bcr-abl amplification were: CMLNA 5' GGAGCTGACATGTGGC- CCAACTCG-3'; CMLNB 5'-ATCTCCACTGCGCCAACAATC- ATACA-3'; CMLNC 5'-GAAGTTTTTCAGAAGCTTCTCC-3'; CMLND 5'-TGATTATAGCTAAGACCCGGGA-3'.

Definition of positive and negative PCR assays. The PCR assays were performed without knowledge of the patient's cytogenetic results or hematologic remission status. A positive PCR test required a correct-size bcr-abl PCR product as well as negative "blank" and HL60 amplifications. A negative assay required the absence of a bcr-abl PCR product as well as no amplification of the "blank" and HL60-negative controls, successful amplification of the 10−6 diluted K562-positive control, and a successful b2 microglobulin PCR amplification. If a positive or negative control did not amplify as expected, the entire panel of patient samples done in that amplification "batch" was discarded and repeated.

Definitions of outcomes and statistical methods. In this study we defined relapse by both hematologic and cytogenetic criteria. Cytogenetic relapse was defined as ≥5 metaphases positive for the Ph chromosome at any point in time or the presence of any Ph chromosomes on two successive cytogenetic evaluations at least 6 months apart. Patients were classified as being PCR-positive or -negative at the time interval of sampling. Relapse and survival rates for patients appropriately classified at the stated time intervals were estimated by the method of Kaplan and Meier.33 For the endpoint of relapse patients who died without relapse were censored at the time of death. The hazard rates of relapse and survival for patients classified as PCR-positive and PCR-negative at specific time intervals were compared using the log-rank test.40 Multivariable analyses were performed by fitting Cox regression models and considering the data to be left truncated. This allowed the variable entry times onto study to be accommodated.33 PCR status was considered as a time-dependent covariate in these models.

If a patient had PCR results that were positive concurrently with a sample which showed hematologic or cytogenetic relapse, then such a patient was classified according to the PCR result at the last test before relapse. For example, a patient with a negative PCR assay at 6 months who at 12 months was PCR-positive for bcr-abl with cytogenetics positive for the Ph chromosome is regarded in this analysis as a patient who relapsed with a negative PCR assay at 6 months.

Patients were excluded from statistical analyses if (1) they had evidence of hematologic or cytogenetic relapse simultaneously to,
Age: Median 36 (range 2-60)

2634 RADICH ET AL

or before, their first PCR assay; or (2) they received a second transplant before their first PCR assay.

RESULTS

Patient characteristics. The clinical characteristics of the 346 patients included in these analyses are shown in Table 1. Six hundred thirty-four samples of PB and/or BM were successfully analyzed by PCR; in 15 other samples RNA did not amplify from the PB and BM. One hundred sixty-two patients had one PCR test performed, and 184 had two or more tests performed. Eighty-two patients had their first PCR sample at 3 months post-BMT, 146 patients at 6 to 12 months, 83 patients at 12 months to 3 years, and 35 patients at greater than 3 years post-BMT. The total number of patients tested in each interval after BMT, categorized by type of donor and phase of disease, is shown in Table 1.

The number of patients testing positive for bcr-abl mRNA, and their Kaplan-Meier estimate of relapse at 3.5 years is 32%, whereas the Kaplan-Meier estimate of relapse of those patients PCR-negative at 3 months is 24% (P = 0.27). The percentage of patients PCR-positive at 6 to 12 months was 26%. The Kaplan-Meier relapse estimates for patients testing positive for bcr-abl at 6 to 12 months is shown in Fig 2. For the 38 patients testing PCR-positive, the Kaplan-Meier estimate of relapse at 4.5 years is 42%, compared with 3% for the 112 PCR-negative patients (P <

![Fig 1. The percentage of patients testing PCR-positive and the Kaplan-Meier estimate of relapse associated with a PCR-positive and PCR-negative test, defined by time from BMT. The x axis represents the time interval from BMT, whereas the y axis is percentage. The Kaplan-Meier estimate of relapse for PCR-positive patients tested in the designated time interval from BMT. The estimate of relapse associated with a negative PCR-test in the time interval. The P values compare the risk of relapse for patients PCR-positive to PCR-negative.](image-url-

![Fig 2. The Kaplan-Meier estimates of relapse for patients testing PCR-positive (N = 38) at 6 to 12 months compared with patients who were PCR-negative (N = 112) for bcr-abl mRNA. Tick marks represent patients alive and still at risk of relapse.](image-url-
The median time from PCR-positivity to relapse was 7 months (range, 1.5 to 12 months). A separate Kaplan-Meier estimation was performed for just the allogeneic-related transplants. Ninety-eight patients were tested, and 20 were PCR-positive. The Kaplan-Meier estimate of relapse associated with a PCR-positive test at 6 to 12 months post-BMT was 62%, compared with 3% for PCR-negative patients ($P < .0001$).

Thirty of 151 patients (20%) tested from 12 to 36 months post-BMT were PCR-positive. Five of the PCR-positive and one of the PCR-negative patients have relapsed (Kaplan-Meier estimates of relapse 25% v 1%, $P < .0001$). The median time from PCR-positivity to relapse was 7 months (range, 0.5 to 20 months). The median follow-up of the patients who tested PCR-positive but have not relapsed by June 1, 1994 is 28 months from the first PCR-positive assay.

Fifteen of 58 patients (25%) tested greater than 36 months post-BMT tested PCR-positive, and one relapsed. No PCR-negative patient relapsed. The median follow-up of these patients from their first PCR-positive test to present time is 22 months. Nine patients had their first positive PCR assay detected greater than 36 months post-BMT, and none have relapsed (Fig 3). The latest finding of a new positive PCR assay in either multivariable model. The phase of disease at BMT (chronic phase v accelerated or blast phase), the presence or absence of acute (grade $\geq$ II) and chronic GVHD after BMT, and the use of a TBI-containing preparative regimen. Because of the relatively small number of relapses, it was feasible to include only two covariates in any given model (PCR status plus one clinical variable). PCR-positivity alone was associated with a 26-fold risk of relapse. Match status and acute GVHD were independently significantly associated with relapse. Patients who received an allogeneic matched transplant had a relative risk (RR) of relapse of 5.1 compared with URD or mismatched transplant recipients, and patients with acute GVHD grades 0 or 1 had an RR of relapse of 0.4 compared to patients with grades $\geq$ II. However, these variables did not influence the strength of the risk associated with a PCR-positive assay in either multivariable model. The phase of disease and the chronic GVHD status did not contribute to the risk of relapse independent of a PCR-positive assay.

### Table 3. Multivariate Analysis of Risk of Relapse for Patients Tested at 6 or 12 Months Post-BMT

<table>
<thead>
<tr>
<th>Variable</th>
<th>$P$ Value</th>
<th>RR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR-positive</td>
<td>$&lt;.0001$</td>
<td>26.1</td>
<td>8.9-76.1</td>
</tr>
<tr>
<td>PCR-positive and match*</td>
<td>$&lt;.0001$</td>
<td>29.3</td>
<td>10.0-86.4</td>
</tr>
<tr>
<td>PCR-positive and phase†</td>
<td>$&lt;.0001$</td>
<td>24.8</td>
<td>8.4-73.0</td>
</tr>
<tr>
<td>PCR-positive and cGVHD‡</td>
<td>$&lt;.0001$</td>
<td>24.9</td>
<td>8.5-73.0</td>
</tr>
<tr>
<td>PCR-positive and aGVHD§</td>
<td>$&lt;.0001$</td>
<td>30.1</td>
<td>10.4-91.5</td>
</tr>
<tr>
<td>PCR-positive and TBI</td>
<td>$&lt;.0001$</td>
<td>26.1</td>
<td>9.0-78.2</td>
</tr>
</tbody>
</table>

Relative risks (RR) of relapse were estimated by placing the PCR status (positive v negative) by itself into a multivariable model and then adding an individual covariate to the model. Thus an RR $> 1$ reflects a elevated risk of relapse associated with the presence of the covariates defined as: (*) allogeneic matched related transplant v mismatched or URD; (†) chronic phase v accelerated, blast, or second chronic phase; (‡) presence v absence of chronic GVHD; (§) acute GVHD grades $\geq$ II v grades 0-1; and (I) use of a TBI-containing preparative regimen v chemotherapy only.
Moreover, the PCR assay was the strongest predictor of relapse, compared with only transplant while in chronic phase. A step-up regression analysis was also performed in this group, with the variables of PCR and GVHD status. PCR status was the only variable that entered the model, with an RR of 18 ($P < .0001$). Neither acute nor chronic GVHD entered the model.

The correlation of blood and BM samples. PB and BM samples obtained on the same day post-BMT were evaluated on 514 samples. Concordance between the PB and BM PCR result occurred in 468 samples (91%). Discordance with BM PCR-positivity and peripheral blood PCR-negativity occurred in 6.5% of tests, whereas a negative BM PCR assay with a positive PB assay occurred in 2.5% of tests.

**DISCUSSION**

Although the presence of the bcr-abl chimeric mRNA has been studied in CML patients previously, the predictive impact of a positive PCR, especially in light of clinical variables associated with relapse, has been unclear. Does the PCR detection of bcr-abl predict relapse even when controlling for factors such as GVHD and phase of disease? The present study of 346 patients allowed us to evaluate the predictive power of the PCR in light of other clinical variables as well as examine the significance of the PCR assay at different time points after BMT. We found that a single PCR assay positive for the bcr-abl chimeric mRNA was associated with a high risk of subsequent relapse. The PCR assay discriminated best at 6 to 12 months post-BMT, when patients who were PCR-positive experienced a 42% risk of relapse, compared with only 3% for those PCR-negative. Moreover, the PCR assay was the strongest predictor of relapse and remained highly associated with relapse in multivariable models when adjusted for other clinical variables associated with relapse in CML. In the multivariable model of all patients PCR-positivity carried an RR = 30, whereas in the subgroup of those receiving an allogeneic, matched transplant while in chronic phase, the RR = 18. In patients sampled greater than 36 months after BMT, and in URD transplants, a PCR-positive test was not strongly associated with relapse. It should be emphasized that these data do not mean that clinical variables such as phase of disease are unimportant in describing relapse risk, but instead indicate that PCR-positivity is in itself a strong independent predictor of relapse.

Our finding of a 42% Kaplan-Meier estimate of relapse in patients with PCR-positivity for bcr-abl at 6 to 12 months post-BMT is in agreement with the study by Roth et al. Moreover, we found a similar influence of multiple PCR testing on predicting relapse, with a 78% estimate of relapse for patients with two or more positive PCR tests, a 22% risk for patients with only one positive test, and a 3% risk for patients with repetitive PCR-negative assays. However, we have chosen to emphasize the predictive power of a single test, thus avoiding potential biases associated with multiple testing, such as controlling for patients who either did not receive subsequent testing for a variety of reasons or who received testing at a different interval.

We found that 15 of 58 patients who were studied greater than 36 months post-BMT had at least one PCR-positive test. Nine of these 15 had their first PCR test become positive at greater than 36 months after at least one negative PCR test (Fig 3). However, with a median follow-up since first PCR-positivity of nearly 2 years none of these long-term patients has relapsed. These data suggest that PCR-positivity in these long-term patients is associated with a lower risk of relapse, or predicts a considerably slower tempo of relapse, than PCR-positivity at 6 to 12 months post-BMT. This interesting trend is most clearly demonstrated in Fig 1, which shows that the relapse risk associated with a PCR-positive assay steadily decreases from 42% at 6 to 12 months to less than 10% for those patients testing positive at greater than 36 months after BMT. These data suggest that CML may be a heterogeneous disease which may be more or less aggressive. Early PCR-positivity (6 or 12 months post-BMT) may delineate a group of patients with more aggressive disease in which the tempo of PCR-positivity to relapse is relatively quick. In contrast, patients who become PCR-positive late after BMT may have more indolent disease, which may relapse at a slower pace after PCR-positivity, if at all. Thus, although patients PCR-positive at 6 or 12 months the median of first PCR-positive test to relapse was 7 months, for patients PCR positive at greater than 36 months post-BMT, the median follow-up from time of first PCR-positive test to last contact is 22 months. Previously the most extensive investigation of long-term CML patients was reported by Guerrasio et al who tested 27 patients at greater than 36 months post-BMT. Five of those 27 patients had at least one PCR-positive test. One had no subsequent test, one had a subsequent PCR-positive assay, and the remaining three reverted to PCR-negativity. None of the five patients relapsed. These data combined with our experience suggest that PCR-positivity in long-term patients may not carry so high a risk of relapse compared with those patients testing positive sooner after BMT. Further follow-up will be important to see if these long-term PCR-positive patients are truly unlikely to relapse, or merely relapse at a slower rate than those patients with early PCR-positive tests.

We were interested in separately evaluating patients receiving an URD transplant because these patients experience relapse rates less than half of that of allogeneic, related transplants for CML. Interestingly, these patients have a similar rate of PCR-positivity to the aggregate CML group at 3 months and 6 to 12 months. The difference in the URD experience is that the Kaplan-Meier estimate of relapse associated with a positive PCR assay is relatively low, only 18%. There are few reports with which to compare this experience. Cross et al report on 15 URD recipients, 10 of whom were PCR-positive at some time during the post-BMT course. They report only one cytogenetic relapse in this group. The low progression to relapse in PCR-positive patients parallels the low risk of relapse experienced in all URD transplants for CML, and may be related to
the graft-versus-leukemia effect. Thus, the common occurrence of PCR-positivity early after transplantation implies that the preparative regimen frequently does not completely eliminate the leukemic cells and that the subsequent lower relapse rates in the URD recipients revolves around the immunomodulatory effect associated with GVHD.

Although our findings contrast with several studies which suggest that PCR-positivity after BMT is not associated with a dramatic increase of relapse risk, most studies, including our own, agree that persistently negative PCR assays from 6 to 12 months after BMT place the patient in a low-risk category. In our study, a negative PCR test at 6 to 12 months post-BMT was associated with only a 3% Kaplan-Meier estimate of relapse. At longer intervals from transplant, the predictive power of a negative exam was even more dramatic, as no patient with a negative test after 12 months has relapsed, with a median follow-up of 19 months. Taken together, all studies agree that a negative PCR test is associated with a very low risk of relapse. However, the implications of an early positive test are less clear. This may be caused largely by the sundry differences between studies both in variables related to therapy (eg, the composition of the cohort in regard to phases of disease, unmanipulated T-cell–depleted marrow, preparative regimens, and immunosuppression regimen), and to PCR testing (eg, PCR test assay conditions and sensitivity, frequency and time points of testing, and rigor of cytogenetic testing).

Our study has practical implications for the study and treatment of CML patients post-BMT. First, there was a median of approximately 6 months from PCR-positivity to subsequent relapse, long enough to launch intervention trials. Therapeutic approaches include the reduction in the amount of immunosuppression given to prevent GVHD, the use of interferon, or the administration of donor buffy coat infusions. Secondly, the high concordance between results obtained from PB and BM shows that PB can be used for monitoring. Thirdly, the PCR test has less predictive value in long-term patients and URD recipients who may not benefit from routine testing for bcr-abl.

Recently studies using a semi-quantitative or competitive PCR assay for the bcr-abl fusion mRNA have suggested that a progressive increase in bcr-abl copy number can be used to identify those patients who will relapse from those who will not. Validation of these data may not improve on the predictive power of a negative PCR test but could improve the predictive power of a positive test. However, caution may be required to interpret quantitative results from center to center for reasons involving both center-specific treatment and patient population as well as methods of sampling and PCR testing.

In summary, the PCR assay appears to discriminate between patients at a very low risk of relapse and those at a higher risk of relapse. The predictive power of the PCR assay appears independent of other relapse risk factors. Our data indicate that the PCR test is of greatest predictive value when performed at 6 to 12 months after BMT in allogeneic-related donor recipients. PCR testing makes possible early intervention to avert relapse.

REFERENCES


Polymerase chain reaction detection of the BCR-ABL fusion transcript after allogeneic marrow transplantation for chronic myeloid leukemia: results and implications in 346 patients

JP Radich, G Gehly, T Gooley, E Bryant, RA Clift, S Collins, S Edmands, J Kirk, A Lee and P Kessler