Prognostic Significance of Philadelphia Chromosome-Positive Cells Detected by the Polymerase Chain Reaction After Allogeneic Bone Marrow Transplantation for Chronic Myelogenous Leukemia

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Although rare cells expressing the bcr/abl fusion transcript can be detected by the polymerase chain reaction (PCR) in patient blood or marrow after allogeneic bone marrow transplant (BMT) for Philadelphia chromosome (Ph')-positive chronic myelogenous leukemia (CML), the prognostic significance of this finding is unknown. This paper reports clinical, cytogenetic, and molecular data derived from 64 CML patients following allogeneic BMT. Nested primer PCR was performed on patient blood and bone marrow samples to detect the presence of residual bcr/abl (+) cells in CML patients considered to be in clinical remission at the time of study. bcr/abl transcripts were detected in 37 of 64 patients for at least one timepoint post-BMT. Thirteen of these 37 bcr/abl (+) patients have subsequently relapsed, as defined by clinical and/or persistent cytogenetic findings, in contrast to 0 relapses among the 27 bcr/abl (-) patients (P = .0025).

ALLOGENEIC BONE marrow transplantation (BMT) is the only known curative treatment for patients with Philadelphia chromosome-positive (Ph') chronic myelogenous leukemia (CML). Disease recurrence remains a major obstacle, however, with the overall 4-year probability of relapse ranging from 20% to 60%, depending on the stage of disease at the time of transplant. Relapse from clinically undetectable residual malignant cells is thought to be the most likely source of recurrence. Recently, we and others have adapted the polymerase chain reaction (PCR) to detect the chimeric bcr/abl RNA transcript unique to Ph' CML cells. This assay is significantly more sensitive than cytogenetics or Southern blotting. The application of this approach to detect minimal residual bcr/abl (+) cells after allogeneic BMT might identify subsets of patients who are more likely to relapse after transplant and thus might benefit from additional therapy or alternative conditioning regimens. However, initial reports of bcr/abl PCR analysis after BMT have yielded conflicting results. To date, no consistent correlation with clinical outcome has been identified. We now report clinical, cytogenetic, and molecular data on 64 Ph' CML patients after allogeneic BMT. Our data indicate that CML patients with bcr/abl transcripts detected by PCR following allogeneic BMT are at a significantly increased risk of subsequent relapse, while those patients persistently bcr/abl (-) are less likely to relapse. The pattern of bcr/abl detection after transplant may aid in the development of trials designed to reduce the risk of relapse, or allow for early intervention in patients who fail to clear the malignant clone.
visualization on ethidium bromide stained agarose gels after electrophoresis.\textsuperscript{1} In reconstitution experiments, one CML cell per million normal mononuclear cells could be detected by this approach.\textsuperscript{3} The presence of intact RNA and adequate cDNA preparation was evaluated by a single round of PCR using \textit{abl} sequence-specific PCR primers as previously reported.\textsuperscript{2}

\textbf{Controls for PCR carryover.} To avoid sample contamination, RNA extraction, cDNA synthesis, and PCR preparation were performed in an area physically isolated from the gel electrophoresis analysis. Mock RNA preparations were also run in parallel as negative controls. Sample RNA were scored as \textit{bcr/abl} (+) only if the corresponding set of controls was negative. In addition, when adequate RNA was available, nested primer PCR was repeated on a separate RNA aliquot from the \textit{bcr/abl} (+) samples to confirm the presence of the \textit{bcr/abl} transcript. If a separate assay gave discordant results, the data point was discarded. For 53 of 82 repeat PCR analyses two new \textit{bcr} oligonucleotide primers were used (\textit{bcr}-3 [AGCAAGGCTACGAGGAGGCTAAGAAAG bp (–)365 – (–)336] and \textit{bcr}-4 [TCGGAAAGGGTACTGCATT GCTGCTGTTATT G bp (–)333 – (–)304]).\textsuperscript{20} These primers designed 5' to the earlier reported \textit{bcr} primers were used in conjunction with the previously described \textit{abl}-1 and \textit{abl}-2 primers for nested primer PCR. Although PCR using the new primers resulted in sensitivity and specificity similar to the previous primer sets (data not shown), carryover from earlier PCRs would not amplify and thus could be eliminated as a source of contamination. Analysis for the last 10 patients studied used the new primers exclusively.

\textbf{Cytogenetics.} Bone marrow cells from 31 patients were analyzed at the Mayo Clinic Cytogenetics Laboratory (Rochester, MN) for the presence of the Ph\textsuperscript{+} chromosome. Analyses used direct preparation of aspirates with slides stained by quinacrine mustard with fluorescence (QFQ banding).\textsuperscript{21} For each analysis, at least 20 metaphases were evaluated by light microscopy. The median number of separate cytogenetic evaluations after BMT for these 31 patients was four (range 1 to 7).

\textbf{Statistical analysis and definition of relapse.} Actuarial relapse-free survival and cumulative relapse probabilities were calculated using the Kaplan-Meier method.\textsuperscript{22} The Cox-Mantel statistic and Chi-squared analysis were used to compare \textit{bcr/abl} status with clinical parameters.\textsuperscript{23,24} Relapse was defined by the occurrence of clinical hematologic features (abnormal blood counts and/or marrow consistent with CML), or progressive cytogenetic changes (the appearance of Ph\textsuperscript{+} metaphases in increasing percentages on consecutive marrow examinations).

\section*{RESULTS}

\textbf{Detection of \textit{bcr/abl} by PCR after allogeneic BMT identifies patients at significant risk of relapse.} Samples from all 64 patients were adequate for evaluation based on the presence of intact \textit{abl} mRNA by PCR or the detection of \textit{bcr/abl} after two rounds of PCR. All 40 patients on whom pretransplant samples were available had detectable \textit{bcr/abl} transcripts before BMT. After BMT, a total of 221 RNA samples were analyzed, ranging from one to eight per patient, with a median number of three samples per patient. The median follow-up time post-BMT for the entire group was 365 days with a range of 14 days to over 5 years. In all, PCR-detectable \textit{bcr/abl} transcripts were present in 37 of 64 patients. The \textit{bcr} exon III/\textit{abl} exon 2 fusion was detected in 22 patients, the \textit{bcr} exon II/\textit{abl} exon 2 product in 12 patients, and both in three patients. Twenty-six of 40 patients with available pretransplant samples had a (+) assay after BMT. In two patients both bands were seen pretransplant, while only the larger transcript (\textit{bcr} exon III/\textit{abl} exon 2) remained post-BMT. In the remaining 24 patients, pre- and post-BMT transcripts matched in size. Repeat nested primer PCR analysis was performed on 82 of the 85 (+) RNA samples as described in Materials and Methods. Sixty-eight of the 82 (+) assays remained (+). The 14 discordant data points were discarded from the statistical analysis presented below. Inclusion of these additional points did not significantly alter the results. Thirteen of the 37 \textit{bcr/abl} (+) patients (5CP and 8AP) have relapsed, as documented by clinical parameters and cytogenetics in 11 or by a progressive increase in the percentage of Ph\textsuperscript{+} chromosomes detected by cytogenetics in two. The median time from first (+) \textit{bcr/abl} signal to relapse was 150 days (range 90 to 832). The larger \textit{bcr/abl} transcript was detected in eight of the relapsing patients while the smaller transcript was detected in four and both transcripts in one. Ten of the 37 patients with a (+) \textit{bcr/abl} assay had a detectable signal with one round of PCR. Seven of these patients have relapsed with a median time from (+) signal to relapse of 120 days (range 30 to 270). None of the 27 \textit{bcr/abl} (−) patients have relapsed with a median follow-up time of 372 days (range 14 to 1,289). The difference in the incidence of disease relapse between patients with 0 versus 1 or more (+) assays was highly significant ($P = .0025$, Fig 1).

\textit{Distinct \textit{bcr/abl} detection patterns identify patients at high, intermediate, and low risk of relapse after BMT.} The 54 patients studied at two or more timepoints post-BMT could be classified into three groups (Table 1). Group I consisted of eight patients with a persistent \textit{bcr/abl} (+) assay. Median follow-up for all group I patients was 268 days (range 63 to 1,140). In this group, five patients have relapsed, one died in clinical remission at day 60, and two are alive and clinically free of disease at days 63 and 1,140. The latter patient was first evaluated at day 660 after BMT.

Group II consisted of 23 patients with both \textit{bcr/abl} (+) and (−) timepoints. The median follow-up time posttransplantation was 480 days (range 50 to 1,460). The patterns of \textit{bcr/abl} detection and disease outcome for group II patients

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig1.png}
\caption{Kaplan-Meier plot of the actuarial probability of relapse for all 64 patients comparing those with no (+) assays to those with one or more (+) assays. The difference in the probability of relapse is statistically significant ($P = .0025$).}
\end{figure}
are shown graphically in Fig 2. Among the 23 patients in this group, five patients (nos. 18 through 22, Fig 2) have relapsed. Ten patients (nos. 1 through 10, Fig 2) had one or two initial (+) assays followed by one or more subsequent negative assays. Eleven patients (nos. 11 through 21, Fig 2) had one or more initial (−) assays within the first 180 days post-BMT followed by at least one (+) assay at a later timepoint. Two patients (nos. 22 and 23, Fig 2) had an initial (+) assay followed by (−) and then (+) assays.

Group III consisted of 23 patients with persistent bcr/abl negative assays after BMT. None of these patients have relapsed, with a median follow-up time of 372 days (range 14 to 1,289). Analysis of bcr/abl in patients from all three groups are shown in Fig 3.

To further determine if the number of (+) or (−) bcr/abl PCR assays was associated with disease relapse, relapse rates were compared among patients with 0, 1, or ≥2 (+) assays (Fig 4). The actuarial risk of relapse increased with the number of (+) assays (0% vs 41% vs 75%, respectively; \( P = .0082 \) ) (Fig 4). An increased risk of relapse with increasing number of positive bcr/abl signals was also observed when patients were stratified into CP vs AP/BC (data not shown).

Early detection (<day 30) of the bcr/abl transcript after BMT is not associated with an increased risk of leukemia relapse. The distribution of (+) bcr/abl signals relative to time after BMT and disease outcome is shown in Table 2. Fifteen of 32 patients evaluated within the first month after BMT were bcr/abl (+). The frequency of a bcr/abl (+) signal at this early time period was similar for both the relapse (3/6 or 50%) and remission (12/26 or 46%) groups (\( P > .7 \)). Seven patients (nos. 1 through 6 and 8, Fig 2) who were initially bcr/abl (+) in this time period became bcr/abl (−) at later timepoints. In contrast to the finding in the first 30 days, patients who relapsed were more likely to be bcr/abl (+) between days 30 and 365 when compared with patients who remained in remission (Table 2).

Detection of bcr/abl transcripts after BMT is not associated with T-cell depletion, donor source, or GVHD. There was no significant association between marrow manipulation or donor source with overall detection of bcr/abl transcripts. A PCR bcr/abl (+) assay was present in 28 of 44 T-cell-depleted and in 9 of the 20 unmanipulated transplants (\( P > .25 \)). Eight of 17 patients who received unrelated but closely HLA-matched transplants had detectable bcr/abl signals compared with 29 of 47 related donors (\( P > .4 \)). A detectable bcr/abl signal was not statistically associated with the presence or absence of acute (grades III or IV) or chronic (moderate or severe) GVHD. In 13 patients, simultaneous blood and marrow samples were available for study. Ten of these samples had similar PCR assay results, while three had a (−) blood assay with a (+) marrow.

The bcr/abl PCR assay is more sensitive than cytogenetics. Concurrent cytogenetic and PCR bcr/abl analysis was available for 30 patients. No patient had a positive cytogenetic analysis with a negative bcr/abl PCR assay. Thirteen patients had exclusively normal donor karyotype analysis by cytogenetics and a negative bcr/abl assay. None of these patients have relapsed, with a median follow-up of 420 days (range 60 to 1,460). Twelve patients had detectable bcr/abl transcripts with normal cytogenetics. Three of these patients have relapsed. One of these patients developed concurrent clinical and cytogenetic relapse, while in the other two detectable Ph' chromosomes appeared before clinical relapse. Five patients had both a bcr/abl (+) assay and detection by cytogenetics of a Ph' chromosome when first evaluated. Three of these five patients progressed to clinical relapse.

**DISCUSSION**

This report defines the prognostic significance for patterns of RNA PCR detection of residual bcr/abl containing
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Fig 3. Patterns of bcr/abl expression detected after BMT. (A through D) Ethidium bromide-stained agarose gel electrophoresis analysis of PCR products from four patients. The bcr/abl analysis represents two rounds of PCR with nested primers, while the control abl PCR (for intact RNA and cDNA) is only after one round. pBR 322 plasmid digested with restriction enzyme MspI was used as a size marker. (A) Patient with persistent detection of the bcr/abl transcript. Despite the detection of bcr/abl-containing cells at days 108, 210, and 324, this patient remained in clinical and cytogenetic remission. By day 357, 8/20 marrow metaphases were Ph+ by cytogenetics. Clinical disease relapse occurred at day 480 post-BMT. (B) Complete elimination of detectable bcr/abl transcripts. The presence of intact RNA for each sample is demonstrated by the (+) abl control. The variable intensities of the abl signal seen may reflect RNA quality or RNA PCR efficiency. These factors should be less significant after two rounds of PCR as performed for all bcr/abl detection. This patient remains in clinical and cytogenetic continuous remission at day 570 after transplantation. (C) Intermittent bcr/abl-positive timepoints. A (+) bcr/abl assay was obtained from a bone marrow sample at day 30 but (−) assays from the blood at day 60 and marrow at days 120 and 180. Reappearance of a bcr/abl signal occurred in the blood at day 480 after transplant. This patient (no. 23, Fig 2) remained in clinical and cytogenetic remission until his death at day 618. (D) Transient detection of the bcr/abl transcript. Both sizes of the bcr/abl transcript were present pre-BMT and at day 90 after transplant with subsequent disappearance at all later timepoints. Simultaneous blood (B) and marrow (M) were analyzed at day 730. This patient (no. 9, Fig 2) remains in clinical and cytogenetic remission at day 730 after transplant.

cells after allogeneic BMT for CML. In all, bcr/abl transcripts were detected by PCR in 37 of 64 patients. Previous studies have reported widely varying frequencies of detection of the bcr/abl transcript after allogeneic BMT for CML. Consistent with our data, Hughes et al recently reported a high frequency of (+) bcr/abl PCR analysis in high-risk patients, including eight patients analyzed at more than one timepoint. Although not statistically significant, their analysis suggested an association with disease relapse and a (+) bcr/abl PCR assay, particularly between 6 and 24
months post-BMT. However, some studies have found that the majority of patients remain bcr/abl (+) for years after BMT regardless of the stage of disease or the techniques used for GVHD prophylaxis, while still others have reported a very low frequency of detection and occasional transient detection.\textsuperscript{3,4} The basis for these discordant results is unclear. While major differences may exist among the patient populations studied, these results could also reflect differences in assay sensitivity or, alternatively, the significant problem of PCR carryover.\textsuperscript{25} To minimize possible cross-contamination in our study, extensive technical precautions were implemented.\textsuperscript{25} In addition, repeat RNA PCR analysis verified a (+) signal in 68 of 82 (+) samples. The discordant results from 14 samples (in 10 patients) could reflect contamination of the original sample or, alternatively, the presence of a small number of Ph\textsuperscript{+} cells near the threshold for detection by the PCR assay. However, the overall conclusions from the statistical analysis were not altered by exclusion of these data.

In the present study the presence of a (+) bcr/abl assay after BMT was associated with a significantly increased risk of disease relapse (Figs 1 and 4). Among relapers, the median time from first (+) bcr/abl assay to relapse was 150 days (range 90 to 832). Evaluation of patients at multiple timepoints after BMT identified three distinct patterns of bcr/abl mRNA detection strongly associated with risk of relapse (Table 1). The highest incidence of relapse occurred in group I patients (persistently bcr/abl (+), 5/6 [actuarial relapse risk, 77%]), the lowest in group III patients (persistently bcr/abl (−), 0/23 [0%]), and intermediate in group II patients (both (+) and (−) assays, 5/23 [actuarial relapse risk, 20%]). Within this latter group, some patients were transiently bcr/abl (+) in the early posttransplant period, while others were initially (−) or even episodically (+) (Figs 2 and 3). The finding of (+) and (−) assays in the same patient over time may reflect the presence in the RNA sample of an absolute number of Ph\textsuperscript{+} cells near the threshold for detection by the PCR assay. Patients in this group may either not have had sufficient time to develop clinical relapse or to completely eliminate the remaining (+) cells. Only one patient was persistently (+) and alive beyond 100 days without relapse. This patient was first evaluated at day 660 after BMT with no data available from earlier timepoints. As shown in Fig 4, disease relapse was significantly more frequent as the number of bcr/abl (+) assays increased. Multiple (+) assays may reflect a higher number of persisting bcr/abl (+) tumor cells. Mixed (+) and (−) assays, as in group II, might then be indicative of a number of Ph\textsuperscript{+} cells close to the threshold of the RNA PCR assay. The relative number of Ph\textsuperscript{+} cells may be directly related to the differing risk of relapse in these groups. Although not a quantitative assay, the observation that 7 of 10 patients with a (+) assay after only one round of PCR have subsequently relapsed is consistent with this hypothesis.

Although classification as group III [bcr/abl (−)] was associated with an excellent prognosis in this study, median follow-up time is still relatively short (372 days). Late relapses of CML greater than 4 years after BMT have been reported as having a slow evolution to clinical relapse among patients known to be Ph\textsuperscript{+} chromosome (+) after BMT,\textsuperscript{26} particularly among T-cell–depleted transplant recipients.\textsuperscript{27} Eight patients (nos. 11, 13, 15 through 18, 22, and 23, Fig 2) with bcr/abl (−) assays within the first year post-BMT have become bcr/abl (+) after 1 year. Two of these patients (nos. 18 and 22, Fig 2) have relapsed. Interestingly, this finding parallels the reports of late first detection (>1 year) of Ph\textsuperscript{+} marrow metaphases in patients after BMT.\textsuperscript{27} The initial RNA PCR (−) assays may again reflect numbers of Ph\textsuperscript{+} cells below the threshold for detection, or alternatively the existence of Philadelphia chromosome (−) precursor leukemic cells. However, this latter hypothesis is not supported by recent gene transfer experiments demonstrating that expression of the bcr/aml oncogene and the p210 protein in mouse marrow is sufficient to produce a myeloproliferative disorder closely resembling human chronic-phase CML.\textsuperscript{28} A late perturbation in immunoregulatory mechanisms responsible for early leukemia cell suppression may account for delayed expansion of the leukemic clone from a previously low residual or dormant tumor burden.\textsuperscript{29,30} The finding in some patients of a (+) bcr/abl assay from bone marrow cells with a simultaneous (−) assay in peripheral blood (patients 10 and 16, Fig 2) might also reflect early sequestration of tumor cells.

Thirty patients in our study had concurrent cytogenetic and PCR analysis. The PCR assay was significantly more sensitive than cytogenetics, with 12 patients demonstrating detectable bcr/abl transcripts by PCR despite normal cytogenetics. No patient had a positive cytogenetic analysis with a negative bcr/abl PCR assay. In two patients, initial (+) bcr/abl analysis followed by subsequent (+) cytogenetics predicted clinical relapse. Taken together, these data sug-

Table 2. Distribution of (+) bcr/abl Signals Relative to Time After BMT and Disease Outcome

<table>
<thead>
<tr>
<th>Time of Study (d)</th>
<th>Relapse*</th>
<th>Remission*</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;30</td>
<td>6 (60)</td>
<td>26 (46)</td>
<td>&gt; .70</td>
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<tr>
<td>31-120</td>
<td>9 (89)</td>
<td>31 (65)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>121-365</td>
<td>8 (87)</td>
<td>33 (12)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>&gt;365</td>
<td>2 (100)</td>
<td>23 (39)</td>
<td>&gt; .30</td>
</tr>
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*Refers to the total number of patients studied within the indicated time period for each group. The numbers in parentheses represent the percent of patients with a (+) bcr/abl assay.
gest a gradual increase in tumor-cell burden in those patients destined to relapse after allogeneic BMT. Leukemia is first detectable by PCR, followed by cytogenetic relapse, and finally clinical disease.

We conclude that the bcr/abl PCR assay is a useful prognostic tool in the management of CML patients post-BMT. The persistent detection of the bcr/abl transcript by PCR after allogeneic BMT for CML is associated with a significantly increased risk of relapse. In contrast, persistently bcr/abl (−) patients have a very low risk of relapse. As a significant number of patients may be transiently (+) in the early post-BMT period (<30 days), it is important that any assessment for prognosis should include multiple evaluations. The presence of multiple (+) assays between 1 month and 1 year post-BMT is associated with a significant risk of early disease relapse. Cells containing bcr/abl may also persist for prolonged periods in some patients who remain in clinical remission. The long-term outlook in these patients remains to be determined.

NOTE ADDED IN PROOF

Since submission of this manuscript, two additional bcr/abl (+) patients (nos. 11 and 17, Fig 2) have progressed to clinical relapse. Both patients were first bcr/abl (+) at >365 days post-BMT. The crossover of these patients into the relapse group now makes the association of a (+) bcr/abl assay beyond day 365 with disease relapse statistically significant (P < .06, Table 2).

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REFERENCES

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