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The significance of *bcr-abl* molecular detection in chronic myeloid leukemia patients "late," 18 months or more after transplantation

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The *bcr-abl* chimeric messenger RNA is frequently detected in chronic myeloid leukemia (CML) patients after bone marrow transplantation. It was previously reported that the relapse risk of *bcr-abl* detection 6 to 12 months after transplantation was greater than 40%. This risk decreased as the time between transplantation and detection increased. To further define the relapse risk associated with *bcr-abl* molecular detection in "late" CML survivors, 379 consecutive CML patients alive at 18 months after transplantation or later were studied. Ninety of 379 patients (24%) had at least one positive *bcr-abl* test 18 months after transplantation or later; 13 of 90 *bcr-abl*–positive patients (14%) and 3 of 289 *bcr-abl*–negative patients (1.0%) relapsed. The median time from *bcr-abl* detection to relapse was 916 days (range, 251-2654 days). The hazard ratio of relapse associated with *bcr-abl* detection was 19.2 ($P < .0001$). The stage of disease, chronic graft-versus-host disease, and the donor type did not alter the association between *bcr-abl* and relapse. Quantification of *bcr-abl* was performed on 344 samples from 85 *bcr-abl*–positive patients by means of a real-time quantitative reverse transcriptase–polymerase chain reaction assay. The median *bcr-abl* change of patients who relapsed was significantly greater than those that remained in remission ($P = .002$). The median *bcr-abl* level at relapse was 40 443 *bcr-abl* copies per μg RNA (range, 960-299 552). Of 73 *bcr-abl*–positive patients who failed to relapse, 69% had only one positive test at a median of 24 copies *bcr-abl* per μg RNA. The detection of *bcr-abl* is common following transplantation. The prognostic significance of a qualitative *bcr-abl* can be refined by quantitative assays and thus may target patients who would benefit from early intervention. (Blood. 2001;98:1701-1707)

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Introduction

The genetic hallmark of chronic myeloid leukemia (CML) is the Philadelphia chromosome (Ph), which occurs in at least 90% of cases.[1,2] The reciprocal translocation between chromosomes 9 and 22 places the 5′ regulatory domains of the *BCR* gene from chromosome 22 in juxtaposition with the 3′ tyrosine kinase domains of ABL from chromosome 9. This unique gene fusion leads to the expression of chimeric *bcr-abl* messenger RNA (mRNA) and the production of functional BCR-ABL fusion protein.[3] The chimeric *bcr-abl* mRNA is an attractive target for monitoring minimal residual disease (MRD) by reverse transcriptase–polymerase chain reaction (RT-PCR) assays. Several studies have examined the significance of *bcr-abl* molecular detection after bone marrow transplantation (BMT). The detection of *bcr-abl* molecular disease is strongly associated with relapse, though not absolutely predictive.[4-8] The significance of *bcr-abl* positivity in CML is influenced by the time at which the patient is tested after transplantation, the type of transplant, and the frequency of testing.[6,8-10] We previously reported that a single positive assay for *bcr-abl* after transplantation was associated with an elevated risk of relapse.[8] The detection of *bcr-abl* at 6 to 12 months after BMT was associated with a 42% risk of relapse at a median of 200 days from the first PCR-positive result, as opposed to a 3% risk of relapse in PCR-negative patients. This risk was greater for recipients of matched related compared with unrelated donor transplants. The relative risk of relapse associated with *bcr-abl* detection was approximately 30, even after clinical variables associated with relapse, such as phase of disease and graft-versus-host disease (GVHD) status, were controlled for. Because of this strong association, we initiated an intervention trial treating those patients with *bcr-abl* detected 6 to 12 months after BMT.

However, the significance of *bcr-abl* detection is less evident when the detection occurs a longer period of time after transplantation. Many CML patients persist with *bcr-abl* for years after marrow transplantation. We found that 25% of patients tested more than 36 months after BMT were *bcr-abl* positive, and only 10% of these relapsed. These data were supported by Costello et al,[11] who found that 66 of 117 patients (56%) were *bcr-abl* positive more than 36 months after BMT, but only 8% relapsed. In addition, Van Rhee et al[12] reported 19 patients in complete remission for more than 10 years after BMT, 2 of whom were still positive for *bcr-abl*. A semiquantitative assay demonstrated that *bcr-abl* transcript levels were very low in these patients. In summary, these data suggest that molecular relapse in CML patients years after BMT may not necessarily be a harbinger of relapse, and these patients with MRD may thus not be appropriate candidates for therapeutic intervention.

Since the significance of "late" *bcr-abl* detection is unclear, we have followed such patients without intervention. To further

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understand the significance of molecular relapse, we examined the prevalence of *bcr-abl* positivity in CML patients surviving without cytogenetic or hematologic relapse at least 18 months after transplantation. Furthermore, since the predictive value of *bcr-abl* detection in CML may be strengthened by quantification of the *bcr-abl* expression,[13-15] we performed quantification of *bcr-abl* burden by a real-time fluorogenic assay to study the kinetics of *bcr-abl* emergence in patients who relapsed compared with those with molecular disease but no other evidence of disease.

Patients, materials, and methods

Patients

All patients receiving transplants for CML at the Fred Hutchinson Cancer Research Center between 1976 and 1996 were eligible for study. Some patients were described in an earlier study.[8] All transplantation protocols were approved by the Institutional Review Board. Patients were identified and followed through contact with the Long-Term Follow-Up program. Requests were made for submission of both bone marrow and peripheral blood at 6-month intervals. These bone marrow and peripheral blood samples were used for cytogenetic evaluations, and the PCR assay was used to detect the presence of *bcr-abl* mRNA.

Transplant regimen

Patients receiving transplants while in chronic phase prior to 1990 received a conditioning regimen of 60 mg/kg cyclophosphamide (CY) for 2 days (120 mg/kg total) and total body irradiation (TBI) at a dose of 200 cGy for 6 days (1200 cGy total).[16] Since 1990, chronic phase patients received either the above regimen or 16 mg/kg busulfan and 120 mg/kg CY.[17] Accelerated and blast phase patients received a preparative regimen of 120 mg/kg CY and 225 cGy TBI for 7 days (1575 cGy total).[16] Unrelated donor transplant recipients received a preparative regimen of 120 mg/kg CY and either 1200 cGy TBI if in chronic phase or 1320 cGy if in accelerated or blast phase. All patients received GVHD prophylaxis with methotrexate and cyclosporine as previously described.[18]

Sample preparation

RNA isolation from leukocytes from fresh bone marrow and peripheral blood samples was performed by Trizol extraction per manufacturer's protocol (Gibco BRL, Rockville, MD). Aliquots from the RNA were used for the detection of *bcr-abl* and the control β2 microglobulin mRNA (β2μ).

RT-PCR for *bcr-abl*

A 2-step, nested RT-PCR was used to amplify the chimeric *bcr-abl* mRNA, as previously described.[8] The nucleotide sequences of the PCR primers used in *bcr-abl* amplification are listed in Table 1. For all PCR reactions a 10^{-5} and 10^{-6} dilution of an Amplichek BCR/ABL–positive CML control RNA (BioRad, Hercules, CA) into the Amplichek BCR/ABL–negative RNA served as the positive controls. Negative controls included a no-RNA PCR mix ("blank") and the Amplichek negative control RNA. A separate amplification of β2μ was used as a control of RNA integrity and was performed as previously described.[8] Precautions to eliminate PCR carryover contamination included separate rooms for pre-PCR and amplification procedures, aerosol-resistant pipette tips, and no–nucleic acid PCR reactions as negative controls in all PCR amplification reactions.[19]

Quantitative RT-PCR

The 5′ exonuclease-based fluorogenic real-time RT-PCR of *bcr-abl* and β2μ was performed in single tubes in an ABI Prism 7700 sequence detector (PE Applied Biosystems, Foster City, CA). Quantification was performed on samples that were qualitatively positive. The initial qualitative assay allowed for the determination of the b2a2 or b3a2 *bcr-abl* breakpoints. In the quantitative PCR (Q-PCR) assay, different *bcr* primers were used, depending on the *bcr* breakpoint, in order to generate PCR products in the optimal size range. Thus, the Q-PCR assay yielded an 85–base pair (bp) product for the b2a2 *bcr-abl* breakpoint and an 84-bp product for the b3a2 breakpoint. The amplification product for the β2μ gene was 70 bp.

In the Q-PCR assay, 100 nmol/L of a template-specific (either *bcr-abl* or β2μ) oligonucleotide probe was used. The probes were labeled at the 5′ end with 6-carboxy fluorescein and at the 3′ end with 6-carboxy-tetramethyl rhodamine. Labeled probes were synthesized by Synthegen (Houston, TX). RT-PCR was carried out in 50-μL reactions containing 1 × PCR buffer (PerkinElmer, Foster City, CA), 1.5 mmol/L $MgCl_2$, 100 μM each deoxynucleoside triphosphate, template-specific primers (see below), 7.5 U Prime Rnase Inhibitor (5 Prime–3 Prime, Boulder, CO), 50 U Superscript II RT (Gibco BRL), 1.25 U Amplitaq DNA Polymerase (PerkinElmer), and 1.25 U Platinum Taq Antibody (Gibco BRL). In addition, each reaction contained a 6-mer attached to a fluorescent dye, 6-carboxy-X-rhodamine to serve as a passive internal reference.

The sequences of the primers and probes used for the *bcr-abl* and β2μ Q-PCR assays are listed in Table 1. The specific primers and amounts for the Q-PCR reaction were as follows: for b2a2, ABLTM (37.5 pmol) and BCRTM2 (37.5 pmol); for b3a2, ABLTM (37.5 pmol) and BCRTM3 (25 pmol); and for β2μ, β2TMF (12.5 pmol) and β2TMR (12.5 pmol). In the Q-PCR assay, the initial RT step was carried out at 42°C for 30 minutes followed by a inactivation step at 95°C for 5 minutes. The complementary DNA was immediately subjected to 40 cycles of a 2-step PCR. Each cycle consisted of a 15-second denaturation step at 95°C and a 1-minute combined annealing/extension step at 60°C.

Table 1. Reverse transcriptase–polymerase chain reaction primers and probes

Qualitative RT-PCR	
First-round RT-PCR	
CMLNA	5′TGGAGCTGCAGATGCTGACCAACTCG3′
CMLNB	5′ATCTCCACTGGCCACAAAATCATACA3′
Second-round nested PCR	
CMLNH	5′GATCTCCTCTGACTATGAGCGTCCA3′
CMLNI	5′-TTCACCTTTAGTTATGCTTAGAGTG3′
Quantitative RT-PCR (Q-PCR)	
bcr-abl b2a2 primers	
BCRTM2	5′CAT TCC GCT GAC CAT CAA TAA3′
ABLTM	5′AAC GAG CGG CTT CAC TCA GA3′
bcr-abl b3a2 primers	
BCRTM3	5′CCA CTG GAT TTA AGC AGA GTT CA3′
ABLTM	5′AAC GAG CGG CTT CAC TCA GA3′
bcr-abl probe	
ABLTMPROBE	5′AGCGGCCAGTAGCATCTGACTTTGAGC3′
β2-microglobulin primers	
β2TMF	5′CAT TCG GGC CGA GAT GTC3′
β2TMR	5′CTC CAG GCC AGA AAG AGA GAG TAG3′
β2-microglobulin probe	
β2TMPROBE	5′CCG TGG CCT TAG CTG TGC TCG C3′

Collection and analysis of data

The fluorescent emission data resulting from the amplification of target RNA was stored in real time during the run by the Prism 7700 and was analyzed following the completion of PCR. For each sample, the change in the fluorescence signal intensity from background, ΔRn, was calculated for each RT-PCR cycle. The cycle threshold (C_t) was defined at the cycle at which the fluorescence signal from the RT-PCR reaction crosses a defined fluorescence threshold level. In order to construct a standard curve, the C_t can be adjusted (C_t') to optimize the relationship of the C_t and the initial starting amount. The curve of C_t' vs the log of the target amount has an optimal correlation coefficient (r^2) of 1.0. Since PCR should proceed as an exponential amplification, the theoretical slope of this line should be 3.32; ie, each log of target dilution should be separated by 3.32 cycles of the PCR.

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Calculation of *bcr-abl* messages

To calculate the amount of unknown *bcr-abl* messages in a given sample, a standard curve of *bcr-abl* was created, with the use of the BCR/ABL–positive Amplichek cell line. RNA from the Amplichek CML cell line underwent serial log dilution into Amplichek negative cell line, ranging from 100 ng to 1 pg of Amplichek control RNA. In addition, a standard of fresh Amplichek positive RNA was diluted in water from 100 ng to 1 pg, and amplified with β2μ primers.

Unknown samples had equal volumes of RNA subjected to amplification with *bcr-abl* and β2μ primers. From the *bcr-abl* Q-PCR, the initial amount of *bcr-abl* RNA was back-calculated by means of the control CML standard curve. Amplification with the β2μ primers yielded an estimate of the amount of amplifiable RNA in picograms. From past experiments we have estimated that each picogram of Amplichek BCR/ABL–positive RNA has approximately 4 copies of *bcr-abl*. Thus, the formula for *bcr-abl* copy number can be estimated by: number of *bcr-abl* copies = *bcr-abl* BioRad RNA (pg)/β2μ RNA (pg) × 4 *bcr-abl* copies per RNA (pg). Any sample with a positive qualitative RT-PCR for *bcr-abl* but a nondetectable Q-PCR was arbitrarily given the value of one copy of *bcr-abl* RNA per microgram.

The reproducibility of the assay was investigated several ways. First, to evaluate the intra-experiment variation, 10 replicated dilution series of the Amplichek CML cell line were mixed into a background of Amplichek negative RNA, and Q-PCR was performed on all samples simultaneously. The coefficient of variation (CV) in the C_t value for all dilutions was then calculated for both the *bcr-abl* and β2μ assays. Next, CVs in the C_t were calculated for 10 standard curves (performed on different days) for each of the b3a2, b2a2, and β2μ assays. Finally, we used the standard curves where the threshold had been moved (C_t') to optimize the correlation coefficient (r^2) of the standard curve. This is a representation of the characteristics of the method used in actual calculation of patient data. We calculated the CV of the r^2 of the standard curve and the slope of the standard curve for 67 separate experiments.

Definition of positive and negative RT-PCR assays

The RT-PCR assays were performed without knowledge of the patient's cytogenetic results or hematologic remission status. A positive *bcr-abl* test required a correct size *bcr-abl* PCR product as well as negative blank and negative RNA control amplifications. A negative assay required the absence of a *bcr-abl* PCR product as well as no amplification of the blank and negative controls, successful amplification of the 10^{-5} diluted Amplichek positive control, and a successful β2μ RT-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 the assay repeated.

Definition of outcomes

Relapse was defined by hematologic and cytogenetic criteria. Cytogenetic relapse was defined as 5 or more metaphases positive for the Ph at any point in time or the presence of any Ph on 2 successive cytogenetic evaluations at least 6 months apart. Patients were classified as being *bcr-abl* positive or *bcr-abl* negative at the time of sampling. A patient who had positive *bcr-abl* results concurrently with a sample that showed hematologic or cytogenetic relapse was classified according to the *bcr-abl* status at the last test prior to relapse. Patients were excluded from statistical analyses if they had evidence of hematologic or cytogenetic relapse simultaneously to, or before, their first *bcr-abl* assay.

Statistical methods

The hazard of relapse according to *bcr-abl* status was estimated by considering *bcr-abl* status as a time-dependent covariate in a Cox regression model,[20] and the hazard ratio was estimated by taking the ratio of the hazards. The data were also considered as being left-truncated so that a patient did not contribute to the regression analysis until the first PCR test more than 18 months after transplantation occurred. At this time, the time-dependent covariate for *bcr-abl* status took on the value "1" if the *bcr-abl* test was positive and "0" otherwise. The value of the time-dependent covariate remained the same until the next *bcr-abl* test, at which time its value was determined as above. Any *bcr-abl* samples taken within 14 days of the date of relapse were not included in the regression models, as these were considered to be simultaneous with relapse. Multivariable regression models were fit by including various factors known to be associated with relapse, such as phase of disease, type of transplant, and presence or absence of chronic GVHD. Patients who died without relapse were censored at the time of death for purposes of estimating the hazard of relapse in regression models. The probability of relapse was estimated with a cumulative incidence estimate,[21] where death without relapse was considered a competing risk.

In order to assess the impact of quantitative *bcr-abl* testing, we considered all *bcr-abl* tests after transplantation among patients who had at least one *bcr-abl*–positive test more than 14 days prior to relapse and 18 months or more following transplantation. To test whether the burden of disease increased at a faster rate among those patients who relapsed compared with those who had not relapsed, 2 approaches were taken. In the first, a generalized estimating equation (GEE) model was fit,[22] and the slope from the regression line of the logarithm of *bcr-abl* level on days after transplantation was estimated among patients who relapsed and among those who had not. The resulting slopes were then compared. In the second approach, a slope was estimated from each individual patient among the groups of patients who relapsed and those who had not. The average slope among those who relapsed was then compared with the average slope among those who did not relapse by means of the Wilcoxon rank-sum test. All *P* values are 2-sided and those from the regression model were estimated from the Wald test.

The data were "locked" for analysis on April 1, 2000.

Results

Patient characteristics

The clinical characteristics of the 379 patients included in these analyses are shown in Table 2. The median age of the patients was 36 years (range, 5-61 years). The majority (204 of 379; 54%) received bone marrow from an allogeneic HLA-matched related donor; in 130 of 379 cases (34%), a matched unrelated donor was used for the transplant. The rest of the patients received transplants from HLA-mismatched or syngeneic donors. The vast majority of transplants (317 of 379; 84%) were performed in first chronic phase.

In sum, 947 informative samples were evaluated for the presence of *bcr-abl* by qualitative RT-PCR. The median number of assays per patient was 2 (range, 1-14). Forty percent of the patients had only one sample taken; 24% had 2 samples taken; 15% had 3; and 21% had 4 or more samples obtained and assayed. The median time from the first *bcr-abl* assay to either relapse or last contact was 12.2 months (range, 0-103.8 months).

Table 2. Patient characteristics

No.	379
Sex	151 F/228 M
Median age, y (range)	36 (5-61)
BMT donor, no. (%)	
Allogeneic matched-related	204 (54%)
Allogeneic mismatched	30 (8%)
Unrelated	130 (34%)
Syngeneic	15 (4%)
Phase, no. (%)	
Chronic	317 (84%)
Accelerated	42 (11%)
Blast crisis	9 (2%)
Blast crisis in remission	11 (3%)

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Test characteristics

Over the study period, the qualitative *bcr-abl* test had a sensitivity and specificity of 91% and 97%, respectively, at a dilution level of $1:10^{-5}$. Samples were quantified by means of the real-time "Taqman" assay. The assay revealed linear detection across 5 logs of input RNA. The detection limit was 1 to 10 pg of Amplichek *bcr-abl* positive in a 1-μg background of Amplichek negative RNA. The quantitative assay for β2μ was also log-linear across 5 logs of input RNA, with a sensitivity limit of 1 pg RNA.

Ten replications of a single dilution series were performed, and the median CV for the b3a2 *bcr-abl* assay across 5 logs of dilutions was 1.4% (range, 1.2%-5.6%). Next, we examined the reproducibility of the assay from day to day by calculating the C_t across all dilutions from 10 different runs. The CVs ranged from 2% to 9% for both the b3a2 and b2a2 *bcr-abl* assays and from 3% to 8% for the β2μ assay. Finally, we examined 67 standard curves from runs used to calculate patient data. Since the C_t was adjusted to optimize the standard curve, we evaluated the CV of the slope and the r^2 of the standard curve. The mean slope was 3.21 (compared with an expected value of 3.32), with a CV of 10%, and the mean r^2 was 0.988 (compared with an expected value of 1.000), with a CV of 2.3%. In addition, we performed experiments to test the stability of *bcr-abl* and β2μ mRNA over time. Aliquots of Amplicheck *bcr-abl* RNA were placed at −80°C, then removed for quantification every 1 to 2 weeks for a total of 13 weeks. The ratio of *bcr-abl* to β2μ was calculated at 7 time points. There was no change in the calculated *bcr-abl* level during the 13 weeks of study (data not shown), suggesting that the quantitative assay was not influenced by freezing time.

Prevalence of *bcr-abl* molecular relapse

The prevalence of *bcr-abl* positivity is shown in Table 3. Overall, 90 of 379 patients (24%) tested positive at least once. Matched related transplants had a higher prevalence of positive tests (57 of 204; 28%) than patients receiving transplants from an unrelated donor (23 of 130; 18%). The proportion of patients testing positive was similar in those receiving transplants while in chronic phase (81 of 317; 26%), accelerated phase (7 of 42; 17%), and blast crisis or second chronic phase (2 of 20; 10%).

In 605 cases, peripheral blood and bone marrow were simultaneously analyzed, each providing interpretable results. In 91% of cases, the results of blood and marrow were concordant. In 6% of cases, marrow was positive for *bcr-abl* while blood was negative, and in 3% of cases, marrow was negative for *bcr-abl* while blood was positive.

Table 3. *bcr-abl* positivity and patient variables

Variable	No.	*bcr-abl*⁺	Relapse
BMT donor			
Matched related	204	57	8
Mismatched related	30	5	1
Unrelated	130	23	2
Syngeneic	15	5	2
Phase			
Chronic phase	317	81	11
Accelerated phase	42	7	1
Blast crisis	9	0	0
Blast crisis in remission	11	2	1
Total	379	90	13

Risk of relapse in *bcr-abl*–positive patients

The relapse experience of these patients is also shown in Table 3. Overall, 16 of 379 patients relapsed. Of the 90 patients who ever had a positive *bcr-abl* assay, 13 relapsed (14%). Of the 289 patients with persistently negative *bcr-abl* assays, 3 relapsed (1.0%). The median time of relapse in *bcr-abl*–positive patients was 916 days after their first positive assay (range, 251-2654 days). The median time to relapse in the 3 *bcr-abl*–negative patients who later relapsed was 250 days (range, 105-3011 days). Overall, the unadjusted hazard ratio (HR) of relapse for *bcr-abl* positivity compared with negativity was 19.2 (95% confidence interval [CI], 5.4-68.3; $P < .0001$).

The cumulative incidence of relapse for *bcr-abl*–positive patients is shown in Figure 1. The probability of relapse 3 and 5 years following first *bcr-abl* positivity was 19% (95% CI, 6%-32%) and 29% (95% CI, 12%-46%), respectively (Figure 1). The probability of relapse among patients undergoing a related versus an unrelated transplant was similar (Figure 2).

Multivariable analysis

Clinical features potentially associated with relapse were added individually to *bcr-abl* qualitative status (Table 4). Phase of disease (chronic vs other), donor type (matched related vs mismatched or unrelated), and chronic GVHD (presence vs absence) were each added to the regression model containing only *bcr-abl* status. As noted above, the unadjusted HR of relapse for *bcr-abl* patients compared with *bcr-abl* patients was 19.2 ($P < .0001$). The addition of phase, donor type, and chronic GVHD did not appreciably alter the HR of relapse associated with *bcr-abl*, and none of these factors were statistically significantly associated with relapse after the *bcr-abl* status was factored in.

Quantitation of *bcr-abl* and relapse

In total, 344 samples from 85 *bcr-abl* patients were available for quantification. The correlation of *bcr-abl* levels (copies per μg RNA) between 150 pairs of peripheral blood and bone marrow samples was good (Pearson correlation coefficient, 0.65; Spearman rank correlation coefficient, 0.91).

Seventy-six of the 85 patients had at least 2 quantitative tests available, and 10 of these patients ultimately relapsed. The logarithm of PCR was regressed on days after transplantation for each patient, and the resulting regression line estimated. Included in these curves were all *bcr-abl* values, including those before 18 months after BMT. Nine of 10 patients (90%) who relapsed had a

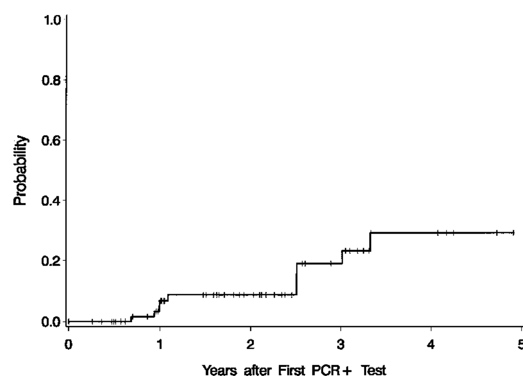

Figure 1. The cumulative incidence of relapse for *bcr-abl* positive patients. Ninety of 379 CML patients tested *bcr-abl* positive "late" after transplantation, and the cumulative incidence of relapse was 19% and 29% at 3 and 5 years, respectively, from the first positive assay.

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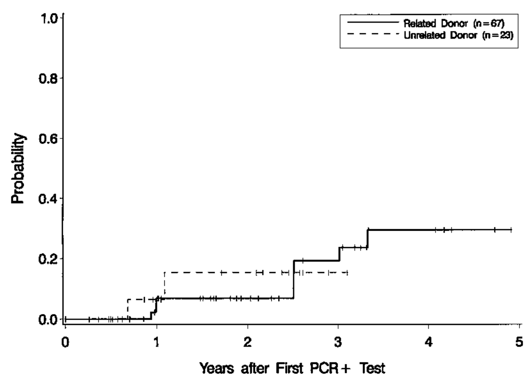

Figure 2. The effect of donor status on relapse in *bcr-abl* positive patients. The cumulative incidence of relapse for patients testing *bcr-abl* positive after receiving a transplant from a matched related (solid line) versus an unrelated or mismatched related donor (hatched line) was not statistically significantly different.

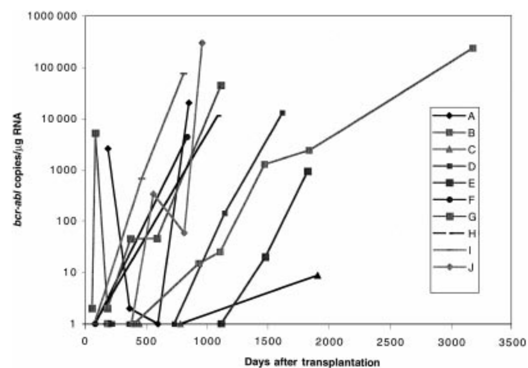

Figure 3. Quantitative RT-PCR results in patients who relapsed. Individual *bcr-abl* curves (copies/μg RNA versus time from transplantation) for 10 patients who were *bcr-abl* positive more than 18 months after transplantation, had multiple *bcr-abl* tests, and ultimately relapsed.

positive *bcr-abl* slope, compared with 25 of 66 patients (38%) who did not relapse. The slope of the patients who relapsed was, on average, higher compared with the slope among patients who did not relapse ($P = .002$, Wilcoxon rank-sum test).

The association of *bcr-abl* kinetics and relapse was also explored by averaging *bcr-abl* slopes over the entire population rather than across individual patients. This analysis used GEEs, which allow one to estimate the statistical significance of the difference observed between the slope of the regression lines among patients who relapsed and those who did not, taking into account the (potential) multiple measurements per patient. This analysis does not require that each patient have at least 2 measurements, as did the analysis that calculated a slope for each individual. Eleven of the 85 patients ultimately relapsed, and 74 have not. The slope calculated among the 11 patients who relapsed is statistically significantly higher than the slope calculated among the remaining 74 patients who did not relapse ($P = .03$).

Figure 3 shows the individual *bcr-abl* levels from the time of transplantation for 10 patients who relapsed. The median *bcr-abl* level at the time of cytogenetic relapse was 40 443 copies per μg RNA. The lowest level of *bcr-abl* found at cytogenetic relapse was 960 copies per μg RNA. The curves demonstrate a fairly steady progression of increasing *bcr-abl* level prior to relapse with 2 exceptions. Patient A was an unrelated donor patient who underwent his transplantation for blast crisis. This patient had a *bcr-abl* level of 2595 *bcr-abl* copies per μg RNA on day 187 after transplantation. The course was then complicated by an extramedullary relapse, which was treated with 6 cycles of chemotherapy. During this time, the *bcr-abl* fell. Approximately 9 months after finishing chemotherapy, the patient experienced both a medullary and an extramedullary relapse. A second patient, "G," developed cytogenetic evidence of the Ph at day 80, which disappeared, with an accordant decrease in *bcr-abl* level, with the discontinuation of immunosuppression and the appearance of GVHD. At the 1-year follow-up, the GVHD had quieted, and *bcr-abl* increased over the next 2 years, until relapse.

Table 4. Regression analysis of hazard of relapse

Variable	RR	95% CI	*P*
Univariate model			
bcr-abl	19.2	5.4-68.3	< .0001
Multivariable models			
A. *bcr-abl*	20.1	5.6-72.0	< .0001
phase (CP vs other)	0.6	0.2-1.9	.41
B. *bcr-abl*	19.0	5.4-67.6	< .0001
donor type*	0.7	0.2-2.2	.51
C. *bcr-abl*	27.3	6.1-123.0	< .0001
cGVHD	1.0	0.3-2.7	.94

CI indicates confidence interval; CP, chronic phase, cGVHD, chronic graft-versus-host disease.

*Indicates mismatched or unrelated donor versus matched related.

Seventy-three patients had at least one *bcr-abl*–positive assay but never relapsed. In total, 308 additional assays were performed on these patients (median, 4 assays per patient; range, 1-12), and 109 (35%) were positive at least once for *bcr-abl*. Fifty of 73 patients had only one positive assay (69%); 14 of 73 (19%) had 2 positive assays; and 9 of 73 (12%) had 3 or more assays positive for *bcr-abl.* Quantitative values for these patients who did not relapse tended to be quite low. In these samples, 62% had fewer than 100 copies of *bcr-abl* per μg RNA; 23% between 100 and 1000 copies; and 15% more than 1000 copies. In retrospect, these high values were caused by normalization of the *bcr-abl* levels by very low amounts of the control β2μ gene, which may have greatly artificially increased the calculated *bcr-abl* level. However, in all of the analyses above, these values were nevertheless used to avoid issues of bias. In those patients who had a single positive result and who had negative *bcr-abl* assays at all other time points, the median *bcr-abl* level was 24 copies per μg RNA.

Discussion

The detection of *bcr-abl* mRNA following transplantation is common and is most strongly associated with relapse shortly after transplantation and in the T-cell–depleted setting.[6,8,10] However, a significant number of patients have evidence of molecular relapse years after transplantation. The significance of late molecular relapse in these CML patients is unclear. Since therapeutic intervention with interferon or donor leukocyte infusion can induce remission in patients who undergo a cytogenetic relapse,[23-25] but at the cost of morbidity and mortality, it is of interest to determine the relationship of molecular relapse and outcome. Should all patients who are in molecular relapse be treated? Should these patients be followed closely, but without therapeutic intervention? Or should intervention be based on quantification of *bcr-abl* level? To address these issues, we studied 379 patients who had survived relapse-free at least 18 months after BMT. We found that *bcr-abl* positivity occurred in a quarter of all patients, and the relative risk of relapse for *bcr-abl*–positive patients compared with *bcr-abl*–negative patients was approximately 20. Clinical factors, such as phase of

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disease, type of transplant, and GVHD, did not influence the association of *bcr-abl* positivity with relapse. However, the absolute risk of relapse in those patients with molecular relapse was modest, with a 19% 3-year cumulative incidence estimate following their first positive *bcr-abl* assay. The application of a quantitative assay appeared powerful in distinguishing patients destined to relapse from those with persistent disease displaying more dormant *bcr-abl* growth kinetics.

In our previous study, we noted that in patients tested 6 to 12 months after BMT, recipients of related and unrelated marrow had a similar prevalence of positive *bcr-abl* assays (approximately 25%), yet related transplant recipients had a much higher rate of relapse (60% vs 10%). In the current study, which focused on patients who survived without relapse for at least 18 months, the situation is apparently different, as donor type and GVHD had no statistically significant impact on relapse. It may be that this finding reflects the time needed for the unrelated donor patients to develop immune tolerance, which, while obviating GVHD, might also diminish any graft-versus-leukemia effect. Thus with immune tolerance, the risk of relapse associated with molecular relapse in the unrelated donor patients mirrors that of the related donor transplants.

The data suggest that a qualitative positive test alone should be interpreted with caution, rather than automatically triggering a therapeutic intervention. For example, if peripheral blood is routinely monitored, a positive qualitative test could signal an opportune time to test bone marrow for cytogenetic evidence of relapse. Alternatively, quantification of *bcr-abl* might be performed for those patients with a qualitative positive test to further define burden of disease and risk of subsequent cytogenetic or hematologic relapse. In this study, the slope of *bcr-abl* change was significantly steeper in those patients destined to relapse compared with those who did not. This observation is in keeping with prior published work demonstrating the power of PCR quantification.[13-15] Indeed, although our method of Q-PCR was technically different from that used in other methods demonstrating the association of increasing copy number and relapse, the rate of kinetic *bcr-abl* increase and the levels of *bcr-abl* at relapse in this present study were quite similar to those in previous studies.[13] In addition, the sensitivity and reproducibility of the real-time Q-PCR employed in our study are consistent with those previously published.[26-28] Finally, recently Olavarria et al[29] studied 138 CML patients "early" (3 to 5 months) after transplantation and showed that the *bcr-abl* level was highly correlated with relapse. Patients with no evidence of *bcr-abl* had a 9% risk of subsequent cytogenetic or hematologic relapse, whereas patients defined as having a low burden of disease (fewer than 100 *bcr-abl* transcripts per μg RNA) or a high level of transcripts (more than 100 copies per μg) had a cumulative relapse rate of 30% and 74%, respectively. These results are consistent with the high risk of relapse associated with early qualitative *bcr-abl* positivity at 6 months after BMT formerly reported.[5,8] The results from Olavarria et al on *bcr-abl* quantification in early posttransplantation patients thus nicely complement the data presented in this manuscript on the risks associated with late *bcr-abl* detection, painting a more complete landscape of the prevalence and significance of minimal residual disease detection in this setting.

Our study admittedly is potentially flawed in the sense that not all patients provided assays at the same time point after transplantation. The upshot of this imperfect sampling is that the observed association of *bcr-abl* positivity with an increased hazard of relapse may be biased. The precise nature of this bias is impossible to know, but one might suspect that patients doing well would be less likely to be tested as frequently as patients thought to be doing poorly. Although we cannot discount the potential for such bias, our data do not suggest an obvious effect of this type. Among patients who have not relapsed, 40% received only one PCR test, 23% received 2 tests, 15% received 3 tests, and the remaining 22% received 4 or more tests. Among the patients who relapsed, 44% received only 1 test, 19% received 2 tests, 25% received 3 tests, and 13% had 4 or more tests. The average number of tests among patients who have and have not relapsed was not statistically significantly different ($P = .64$), and in fact, the average number among those who have not relapsed was slightly higher.

Our current study, coupled with previous work by us and others, demonstrates that many patients continue to have molecular evidence of disease for years, yet fail to relapse. This persistence of molecular disease (dormancy) has also been seen in acute myelogenous leukemia (AML), and acute lymphoblastic leukemia (ALL).[30-32] Indeed, most patients with t(8;21) AML have been found to have molecular evidence of the AML/ETO translocation after years in remission.[30,31] In addition, pediatric ALL patients who relapsed after more than 10 years of remission have been found to relapse with a leukemia possessing the same clonal immunoglobulin heavy chain VDJ gene rearrangement as initially present at diagnosis.[33] What is the mechanism of dormancy? One can imagine several nonmutually exclusive mechanisms, including the following: (1) prolonged immune surveillance; (2) the persistence of preleukemic clonal disease without the full complement of genetic lesions required for the development of frank malignancy; or (3) the presence of a secondary genetic lesion that may render the leukemic progenitor cells relatively quiescent. Regardless, it is likely that a functional "cure" of leukemia is not equivalent to the total eradication of all leukemic or preleukemic cells. The discovery of the mechanisms of dormancy may lead to inroads in defining what it takes to cure leukemia.

There are obstructions to using *bcr-abl* testing to guide therapy, since technical issues of contamination, sensitivity, and specificity are hardly trivial.[34] In addition, *bcr-abl* rearrangements have been described in normal adults, although at an abundance not detectable by standard RT-PCR assays used for MRD testing.[35,36] Despite these caveats, it appears that *bcr-abl* detection is a significant clinical event. The rationale for determining MRD is the hope that early intervention may preclude eventual clinical relapse and improve survival. Proper intervention for *bcr-abl* molecular disease in CML is unknown, and such intervention should be offered only in a research setting. Only such careful study will tell whether the concept of early intervention for molecular relapse fulfills that promise.

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