

## **A Half-Log Increase in BCR-ABL RNA Predicts a Higher Risk of Relapse in Patients with Chronic Myeloid Leukemia with an Imatinib-Induced Complete Cytogenetic Response**

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**Abstract** **Purpose:** Imatinib induces a complete cytogenetic response (CCR) in most chronic myeloid leukemia patients in chronic phase. Although CCR is usually durable, a minority of patients relapse. Biomarkers capable of predicting those CCR patients with a higher risk of relapse would improve therapeutic management. **Experimental Design:** To assess whether changes in BCR-ABL RNA levels are a prognostic biomarker predictive of relapse, we regularly monitored transcript levels [every 3 months (median)] in 90 patients with CCR during 49 months (median) of imatinib therapy. **Results:** Throughout follow-up, the 20 patients with eventual relapse had higher transcript levels than the durable responders. Major molecular response (MMR;  $>3$ -log reduction of BCR-ABL RNA) was attained by 76 patients (12 with subsequent relapse) and was a significant predictor of prolonged relapse-free survival ( $P = 0.0008$ ). A minimal 0.5-log increase in transcripts (before relapse; experienced by 42 patients, 16 with subsequent relapse) conveyed a significantly shorter relapse-free survival ( $P = 0.0017$ ). Loss of MMR (transcript increase to  $<2.5$ -log reduction, before relapse; experienced by 33 patients, 11 with subsequent relapse) was also predictive of shortened relapse-free survival ( $P = 0.0003$ ). A complete molecular response (undetectable transcripts by nested PCR) was attained by 28 MMR patients (one with subsequent relapse) and conveyed a significantly prolonged relapse-free survival ( $P = 0.0052$ ). **Conclusions:** In chronic myeloid leukemia patients with an imatinib-induced CCR, a minimal half-log increase in BCR-ABL RNA (including loss of MMR) is a significant risk factor for future relapse. The achievement of a complete molecular response imparts longer progression-free survival than the achievement of an MMR.

Chronic myeloid leukemia (CML) is caused by BCR-ABL, a constitutively active tyrosine kinase that results from the t(9;22)(q34;q11), which is cytogenetically evident as the Philadelphia chromosome (Ph). Targeted inhibition of BCR-ABL with imatinib mesylate has become the standard therapy for patients with CML and induces a complete cytogenetic response (CCR) in more than 80% of newly diagnosed patients (1, 2). Patients achieving CCR have prolonged progression-free survival compared with those without CCR (1–3), but 11% to 27% of patients have been shown to subsequently lose CCR (4–9).

Disease burden in patients with CCR is routinely monitored by BCR-ABL real-time quantitative reverse transcription-PCR (RQ-PCR). With RQ-PCR monitoring, the attainment of a 3-log reduction in BCR-ABL RNA [termed a major molecular response (MMR)] identifies a subgroup of CCR patients with significantly lower relapse risk (3, 8–10). The prognostic advantage of achieving MMR was confirmed at several time points, including 12 months after the start of imatinib therapy (3, 10), the time of first achieving CCR (9), and any time at or after achieving CCR (8). In the significant fraction of CCR patients who either never attain MMR (~20–50%; refs. 1, 3, 7, 8) or whose MMR is transient (3, 7, 8), additional prognostic markers would be useful to identify those patients with the highest relapse risk in order to adjust the monitoring frequency and/or the therapeutic strategy.

Because RQ-PCR methods are not yet well standardized, relatively imprecise, and have variable low-level detection limits in different laboratories, the practical clinical value of a single RQ-PCR measurement is limited. In comparison, recurrent, serial monitoring of BCR-ABL RNA to identify time-dependent changes in transcript levels may be a better method for estimating the risk of relapse. For example, a 2-fold increase in BCR-ABL RNA in consecutive samples has been shown to be closely correlated with the presence of BCR-ABL kinase domain mutations and relapse during imatinib therapy (11). Because

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this study included patients who had already relapsed at the time of the RQ-PCR increase, the prognostic value of an increase in BCR-ABL RNA levels for predicting future relapse could not be deduced. Another smaller study failed to confirm the prognostic significance of a single 2-fold transcript increase but found a significant association between two consecutive 2-fold transcript increases and kinase domain mutations in a small cohort ( $n = 5$ ; ref. 12). In addition, in patients with prior allogeneic stem cell transplantation, serial measurements of BCR-ABL RNA were used to accurately stratify relapse risk (13, 14). Whether serial changes in BCR-ABL RNA can be used to specifically predict future relapse risk in patients with an imatinib-induced CCR has not been comprehensively addressed and is the subject of this report.

## Patients, Materials, and Methods

**Patients.** The 90 subjects enrolled in this study represent all patients with CML followed at our institution who fulfilled both of two inclusion criteria: (a) CCR on imatinib (defined as no detectable

Ph-positive metaphases in routine karyotype analysis); (b) at least two BCR-ABL RNA measurements after achievement of CCR and drawn either before relapse or, for patients with stable CCR, before the last cytogenetic assessment. Leukemic relapse was defined as per our previous study (8) and included progression to accelerated or blastic phase, loss of a complete hematologic response, or loss of a CCR. The study protocol was approved by the Institutional Review Board at Oregon Health & Science University and informed consent was provided according to the declaration of Helsinki.

Table 1 describes the key demographic features of the 90 patients. At the time of starting imatinib, 57 (63%) patients were beyond 6 months from diagnosis. Fifty of these 57 patients had failed previous IFN- $\alpha$  therapy. Nine patients had allogeneic hematopoietic stem cell transplants (four with previous IFN therapy) and two patients had autologous stem cell transplants (both with previous IFN therapy). In patients with chronic-phase CML ( $n = 74$ ), the dose of imatinib was initially 400 mg/d, and in those with accelerated phase, the dose was initially 600 mg/d. Over the entire follow-up period, the average daily imatinib dose was 440 mg in the patients with chronic-phase CML and 580 mg in the patients with accelerated phase or blast crisis. Whereas most patients (79%) received no concomitant antileukemic therapy while taking imatinib, 17 patients were treated with simultaneous

**Table 1.** Demographic characteristics of study patients with CCR

	All patients (N = 90)	Durable CCR (n = 70)	Relapse (n = 20)	P, durable CCR vs relapse
Age, median (range)	48.6 (24-78)	47.8 (24-76)	51.7 (25-78)	NS
Gender (% male)	51	53	45	NS
Months from leukemia diagnosis to start of imatinib, median (range)	14 (0-155)	13 (0-155)	19 (1.4-89)	NS
"Early" CML diagnosis (<6 mo pre-imatinib), %	37	40	25	NS
Disease phase (at the time of starting imatinib) is chronic-phase CML (vs more advanced phases), %	82	87	65	0.03
New CML (Hasford) risk score (Euro score) at diagnosis (21)*	53% low risk; 34% intermediate risk; 13% high risk	59% low risk; 35% intermediate risk; 6% high risk	39% low risk; 31% intermediate risk; 31% high risk	NS
Additional cytogenetic abnormalities at baseline, %	17	16	20	NS
Average imatinib dose, mg/d	440 (chronic phase); 580 (accelerated phase or blast crisis)	440 (chronic phase); 560 (accelerated phase or blast crisis)	450 (chronic phase); 610 (accelerated phase or blast crisis)	NS
Other concomitant therapies (with imatinib)	79% none; 9% IFN; 11% AraC; 1 pt each on Hsp70 vaccine and arsenic trioxide	79% none	80% none	NS
Months of follow-up on imatinib, median (range)	49 (17-79)	49 (17-79)	52 (24-63)	
Months to CCR, median (range)	6.0 (1.9-48)	6.3 (2.5-48)	5.7 (1.9-40)	NS
Post-CCR follow-up (mo), median (range)	41 (8.7-68)	41 (8.7-68)	41 (18-60)	
Log-drop RQ-PCR at the time of first CCR, median (range)	2.2 (0.23-5.3)	2.3 (1.2-5.3)	1.7 (0.23-3.5)	0.03
Months to achieve MMR, median (range; no. patients with MMR)	17 (3-65; n = 76; 63 with CP)	17 (5-65; n = 64; 56 with CP)	18 (3-37; n = 12; 7 with CP)	NS
No. lab monitoring visits per patient, median (range)	10 (4-21)	10 (4-18)	10 (6-21)	NS
Months between monitoring visits, median (range)	3.0 (0.2-58 <sup>†</sup> )	3.1 (0.2-58)	3.0 (0.3-22)	NS

Abbreviations: AraC, 1- $\beta$ -D-arabinofuranosylcytosine (cytarabine); CP, chronic-phase CML; NS, no significant difference (durable CCR patients versus relapse patients).

\*Complete baseline data (at time of diagnosis) available on only 47 patients.

<sup>†</sup>Fifty-eight-month interval before imatinib therapy was initiated.

IFN- $\alpha$  or 1- $\beta$ -D-arabinofuranosylcytosine (cytarabine). Over the entire follow-up interval (median 49 months after starting imatinib), monitoring consisted of peripheral blood counts, bone marrow morphology, metaphase karyotyping and interphase BCR-ABL fluorescence *in situ* hybridization of bone marrow cells, and BCR-ABL RQ-PCR (3.0 month median interval between visits). Cytogenetics and fluorescence *in situ* hybridization were done on bone marrow aspirates as previously described (8).

**Real-time quantitative reverse transcription-PCR.** RQ-PCR for BCR-ABL RNA relative to the glucose-6-phosphate dehydrogenase (G6PDH) reference RNA was done as previously described (8) on 952 samples, 58% from peripheral blood (sample volume of 20-30 mL) and 42% from bone marrow. We have previously shown that the BCR-ABL RNA levels in these two compartments are not quantitatively different (8). G6PDH is one of only two reference genes that meet each of six stringent criteria for an optimized BCR-ABL internal control transcript (15, 16). To determine the relative reduction of transcript levels compared with a standardized baseline value (3, 17), the BCR-ABL to G6PDH transcript ratio was compared with the lab-specific baseline transcript ratio and expressed as a "log-drop" from baseline (on a base-10 log scale). Per the IRIS study (3) and international recommendations (17), the baseline transcript ratio was the median transcript ratio (4.5%) of all of the newly diagnosed chronic-phase CML patients followed at our institution with concomitant 100% Ph-positive metaphases ( $n = 14$ ). Log-drop values were calculated using the instrument-derived  $\Delta$  crossing point numbers [ $\Delta C_p = (BCR-ABL)C_p - (G6PDH)C_p$ ] and routine real-time PCR relative expression analysis methods (18) using the following equation:  $\log\text{-drop} = [(\log 2) \times (\Delta C_{p_e} - \Delta C_{p_b})]$ , where  $C_p$  is the crossing point (PCR cycle threshold) determined by real-time PCR,  $\Delta C_{p_e}$  is the  $\Delta C_p$  value for the experimental sample, and  $\Delta C_{p_b}$  is the median  $\Delta C_p$  of the baseline samples (a constant 4.49).

**Molecular response thresholds.** A MMR was defined as a log-drop from baseline of  $\geq 3.0$ . If BCR-ABL RNA was initially undetectable, a larger volume preparation of cDNA (200  $\mu$ L) was volume reduced and reamplified, as previously described (8), and if BCR-ABL RNA was still undetectable, nested PCR (in triplicate) was done (8). Samples were classified as complete molecular response (CMR) only if all three nested PCR replicates were negative and if the RNA was of adequate quality (G6PDH  $C_p < 22.7$ ) to ensure that a minimal 4.0-log-drop in BCR-ABL RNA levels, if present, would have been detectable (8). This 4.0 log-drop threshold for defining unsatisfactory (typically degraded) samples was chosen because, of the first 1,092 BCR-ABL-positive samples randomly referred to our laboratory, 10% had detection thresholds below 4.0 log, and a sample rejection rate of 10% was targeted for future studies. If the acceptability stringency had been further increased, to a 4.5-log cutoff, the predicted sample rejection rate would have increased to a burdensome 39%. In this study, without long-distance sample transportation, 32 (3.4%) samples had substandard RNA quality using the 4.0 log-drop cutoff.

**ABL kinase domain mutation analysis.** Screening for mutations in the ABL kinase domain was done by bidirectional direct DNA sequencing of BCR-ABL PCR products. Amplicons for sequencing were generated with forward primer BCRF and reverse primer ABLKinaseR as previously described (19). If no visible amplicons were seen after the first round of PCR (1,579 bp for b3a2 and 1,504 bp for b2a2), 5  $\mu$ L of the first-round PCR reaction were reamplified (using the same primers) in a second-round PCR. This method detects mutant alleles with a low-level sensitivity of 20%.

**Statistics.** Samples with undetectable BCR-ABL RNA (by all methods) were assigned a real-time PCR crossing point 1 log lower than the low-level detection limit of the assay to generate a numerical log-drop value for quantitative analyses. Continuous "time" variables were rounded to the nearest 6-month integer to allow categorization for comparing BCR-ABL RNA levels at various time points and between different groups. Continuous RQ-PCR levels in different groups were compared with either the Mann-Whitney rank sum test (for comparing two groups) or

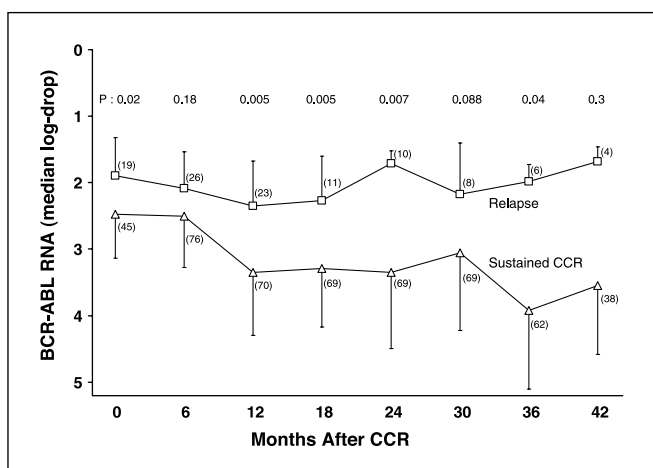
the nonparametric Kruskal-Wallis test (for comparing three or more groups). The Kaplan-Meier method was used to determine the time-related cumulative probabilities of relapse-free survival and major and complete molecular responses. For the "landmark" survival analyses, the landmark time was chosen so that 84% (median + 1 SD) of the patients with an RQ-PCR increase or CMR first achieved this "grouping event" before the landmark time. Differences in relapse-free survival were compared using the log-rank test. A Cox proportional hazard regression model was used to determine relative hazard ratios and to define the time-dependent relapse risk for all variables. All reported  $P$  values are two sided, and  $P < 0.05$  was considered significant. The Statview software program (SAS Institute) was used for all statistical calculations.

## Results

**Patients.** The 90 subjects were monitored for a median of 49 months following the initiation of imatinib and a median of 41 months after achieving CCR (Table 1). The RQ-PCR monitoring was begun 7.2 months (median; range, -1.6-34 months) after the initiation of imatinib therapy, and the subsequent interval between successive molecular monitoring visits (median of 10 samples per patient) was 3.0 months (median). Twenty (22%) patients relapsed over the course of follow-up [after 19 months (median; range, 8.5-54 months) of prior CCR]. Residual samples for sequence analysis were available for 17 of the 20 relapsing patients, and 10 of 17 (59%) had detectable kinase domain point mutations. These residual sequencing samples were drawn 24 months (median; range, 12-61 months) after imatinib therapy was begun and 0 months (median; range, -12-7 months) after relapse. The detected mutations included L248V, G250E, G251D, Y253H (twice), E282G, E292V, E352G, F359V, and H396R. Mutations were first detectable at the same time as the relapse event in three patients and before the relapse event (by a median of 10 months; range, 3-17 months) in seven patients. Of the 20 patients with relapse, six developed blast crisis CML, four of whom subsequently died. As of the last follow-up, none of the other 14 relapse patients had lost a complete hematologic response, as previously defined (8).

The 20 relapsing patients did not significantly differ from the 70 patients with durable CCR with respect to any of the variables listed in Table 1 with the exception of (a) baseline CML stage (chronic phase conferring a better prognosis than advanced disease stages) and (b) the molecular response at the time of first achieving CCR (8). In comparison, other variables that have been shown to be significant prognostic factors in patients treated with imatinib (2, 20)—duration of disease before imatinib initiation and pretreatment clinical and hematologic parameters (as reflected in the Hasford risk score; ref. 21)—were not significant risk factors in this study restricted to patients with CCR.

**Kinetics of BCR-ABL RNA after CCR.** At the time of first achieving CCR (median of 6.0 months after the start of imatinib therapy), the median BCR-ABL RNA level was significantly lower in the patients with sustained CCR (2.3 log-drop; 25th-75th percentile, 1.9-2.9) compared with those with future relapse (1.7 log-drop; 25th-75th percentile, 1.3-2.6), and was a significant predictive marker of subsequent relapse-free survival ( $P = 0.03$ ; Table 1). After CCR, the kinetics of serial BCR-ABL RNA levels showed a notably different pattern in the patients with subsequent relapse compared with



**Fig. 1.** BCR-ABL RNA levels in patients with sustained CCR versus those with future relapse. Plotted are the median BCR-ABL RNA levels (log-drop from a median baseline) at specific time points (0-42 mo) after first achieving CCR in patients with sustained CCR (triangles) versus those with subsequent leukemic relapse (squares). Bars reflect half of the 25th to 75th percentile interquartile range. *P* values reflect comparisons (Cox regression model) between these two groups of patients at each time point. Parentheses enclose sample number values for each time point. Samples drawn at or after relapse were excluded.

those with sustained CCR (Fig. 1). In particular, even though samples drawn at or after the time of relapse were excluded, the median BCR-ABL RNA level was higher in the patients with future relapse at every time point during CCR (Fig. 1). Furthermore, these higher levels of BCR-ABL RNA were associated with significantly shortened relapse-free survival at every time point from 12 to 36 months (Fig. 1). Among only the 33 patients with new diagnosis CML, BCR-ABL RNA levels were also higher at every time point in patients with future relapse as compared with those with durable CCR (data not shown).

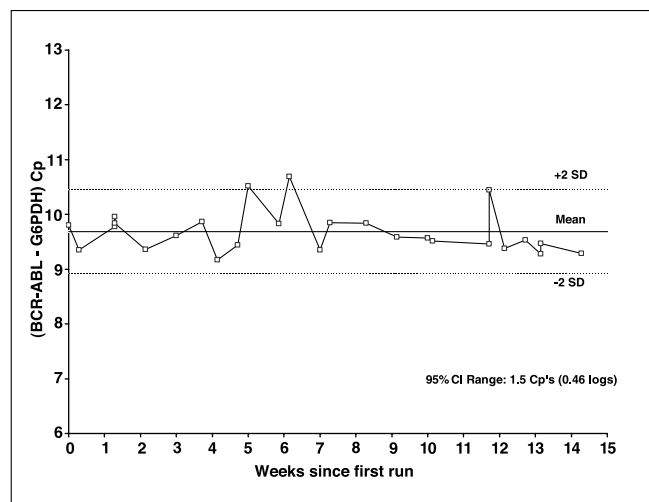
**Major molecular responses.** MMR was attained by 76 of 90 (84%) patients after a median of 17 months of imatinib (Table 1). After 12 months of imatinib therapy, a cumulative 29% of the patients had achieved MMR (31% of the chronic-phase patients), and at 24 months, 62% had achieved MMR (64% of the chronic-phase patients). Of the 76 patients with MMR, 12 (16%) patients subsequently relapsed, compared with 8 relapses among the 14 patients (57%) who never attained MMR. The relapse-free survival was significantly shorter for the patients who never attained MMR (median of 46 months on imatinib) as compared with those who did attain MMR (median not reached;  $P = 0.0008$ ), and the hazard ratio for relapse was 4.1 [95% confidence interval (95% CI), 1.7-10;  $P = 0.002$ ; data not shown].

**Increasing BCR-ABL RNA levels precede and predict relapse.** An initial step toward assessing the prognostic relevance of serial changes in BCR-ABL RNA was a determination of the interassay analytic variability of the RQ-PCR assay. Without this knowledge, it would be impossible to define the threshold BCR-ABL RNA level change, between successive samples, which could be confidently attributed to biologic, rather than analytic, variability. A frozen aliquot of a guanidinium thiocyanate lysate of a preparation of CML cells diluted 1:10,000 into a non-CML cell line was thus assayed in each batch run of BCR-ABL RQ-PCRs. Repeating only the downstream reverse transcriptase and/or PCR steps would, in comparison, falsely underestimate

total assay variability by excluding the considerable imprecision of the nucleic acid extraction procedure (22). Among 26 identical aliquots from a single preparation of this "low positive" quality control reagent, the distribution of  $\Delta$  crossing point ( $C_p$ ) values (from which log-drop values are mathematically determined) defined a 95% CI (3.9 SD) of 1.5  $C_p$  and an analytic interassay coefficient of variation (SD/mean) of 4.0% (Fig. 2). The equation that defines BCR-ABL log-drop values [ $(\log 2) \times (\Delta C_{p_e} - \Delta C_{p_b})$ ] then predicts a 95% CI of [ $(\log 2) \times (10.4 - 8.9)$ ] = 0.46 log (2.9-fold) at this low transcript level. This  $\sim 0.5$ -log threshold reflects the "worst-case scenario" of analytic assay variability, as it was measured at a low level of BCR-ABL RNA. Analytic precision will be improved at higher expression levels (11, 12). A 0.5-log (3.2-fold) or greater change in BCR-ABL RNA levels between successive samples is then quite unlikely (<5% chance) to be due to analytic assay imprecision and thus very likely to be due to underlying biological processes. Conversely, transcript ratio changes of <0.5 log cannot be confidently excluded from being attributable to nothing more than analytic assay imprecision.

The minimal 0.5-log threshold for distinguishing analytic versus biological variability was then applied to the practical question of whether serial changes in BCR-ABL RNA were a significant predictive marker of future relapse risk. For this analysis, an "increasing" RQ-PCR (loss of MMR) was defined, in patients that achieved the 3-log MMR level, as any BCR-ABL RNA level that was <2.5 log below the median baseline after the achievement of MMR. In the patients that never achieved MMR, increasing RQ-PCR values were defined as a >0.5-log increase between any two consecutive samples. Half-log RQ-PCR changes that were first detected at the same time as relapse (or thereafter) could not, by definition, be predictive of future relapse and were thus not coded as increasing.

Of the 90 patients with CCR, 42 had increasing BCR-ABL RNA levels (before relapse), with the increase first occurring

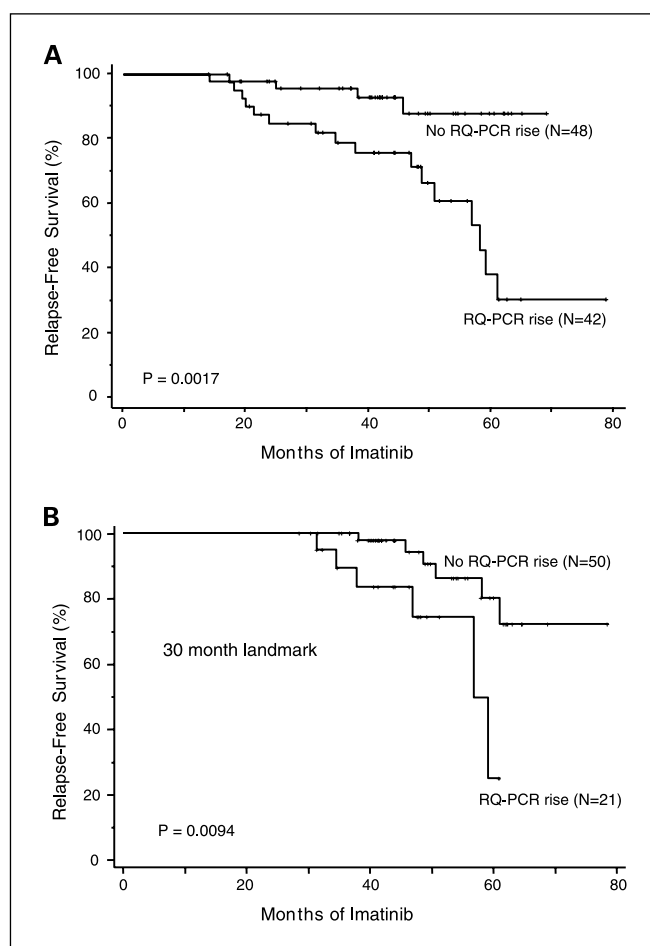


**Fig. 2.** Interassay analytic variability for BCR-ABL RQ-PCR. Aliquots of a cell lysate containing a very low leukemia cell burden (1:10,000 dilution of CML cells into non-CML cells) were assayed for BCR-ABL RNA (including a repeat RNA prep) with each of 26 batch runs of patient samples. The  $\Delta C_p$  values [(BCR-ABL)  $C_p$  - (G6PDH)  $C_p$ ], from which log-drop values are directly calculated, are plotted. The mean  $\Delta C_p$  value (9.7) and upper (10.4) and lower (8.9) 95% CIs ( $\pm 2$  SD) are indicated. Given the equation that defines BCR-ABL log-drop values [ $(\log 2) \times (\Delta C_{p_e} - \Delta C_{p_b})$ ], the 95% CI of  $C_p$  values (1.5) translates into a 0.46 log range [(0.301)  $\times$  (10.4 - 8.9)].

15 months (median; range, 5-47) after CCR. Thirty-three of these 42 patients had achieved MMR, and 9 patients never achieved MMR but had a 0.5-log RQ-PCR increase. Of the 42 patients with increasing transcript levels, 26 (62%) remained in durable CCR throughout follow-up (median of 21 months after the RQ-PCR increase; range, 3-39 months), whereas 16 (38%) patients subsequently relapsed (median of 9.2 months after the RQ-PCR increase; range, 1-34 months). In 13 of 42 patients, the sample type analyzed at the time of the RQ-PCR increase was different from the immediately preceding sample type (blood versus bone marrow). However, the rate and risk of relapse in these 13 patients [5 of 13 (38%)] were not different from the rate and risk of relapse in the 29 patients with a concordant sample type at the time of the RQ-PCR increase ( $P > 0.2$ ). BCR-ABL kinase domain mutations were detected in 10 of the 16 relapsing patients with prior increases in BCR-ABL RNA (a median of 11 months after the increase; range, -8-34 months). Although this increase in BCR-ABL RNA triggered a subsequent imatinib dose escalation in 11 patients [4 (36%) of whom later relapsed], this dose escalation was not significantly protective of subsequent relapse ( $P > 0.6$ ).

Of the 48 patients with stable or decreasing transcript levels, only 4 (8%) later relapsed (none of whom had kinase domain mutations). Three of the four relapsing patients without a prior RQ-PCR increase did have a  $>0.5$ -log increase in BCR-ABL RNA, but at a time point at or after the date of relapse. The relapse-free survival was significantly shortened for the 42 patients with a half-log increase in BCR-ABL RNA (median of 58 months after imatinib therapy was initiated) as compared with the 48 patients with stable or decreasing RNA (median not reached;  $P = 0.0017$ ), and the hazard ratio for relapse was 4.9 (95% CI, 1.6-15;  $P = 0.0046$ ; Fig. 3A). In a landmark analysis, of the 21 patients who had had a half-log increase in BCR-ABL RNA at 30 months after the initiation of imatinib treatment, 6 later relapsed, with a median relapse-free survival of 57 months. In comparison, of the 50 CCR patients without a half-log transcript increase by 30 months, 6 later relapsed, and the median relapse-free survival was significantly longer (median not reached;  $P = 0.0094$ ; Fig. 3B). The 19 patients with a relapse or last follow-up visit before 30 months were excluded from this landmark analysis.

To directly assess the prognostic relevance of increasing BCR-ABL RNA levels after achieving MMR, we carried out a subset analysis of only the 76 MMR patients. Of the 33 patients who later lost their MMR [6.1 months (median; range, 1-31 months) after MMR was first achieved], a minority ( $n = 11$ ) subsequently relapsed [33%; 14 months (median; range, 4-34 months) after loss of MMR]. In comparison, only one relapse occurred in the 43 MMR patients without a prior "loss of MMR"—in a patient whose BCR-ABL RNA first increased above 2.5 log at the same time as, not before, his relapse. The relapse-free survival was significantly shortened for the 33 patients who lost their MMR (median of 57 months after imatinib therapy was initiated) as compared with the 43 patients without loss of MMR (median not reached;  $P = 0.0003$ ), and the hazard ratio for relapse was 16 (95% CI, 2.1-120;  $P = 0.0079$ ; Fig. 4A). A large multisite clinical trial (IRIS) has shown that patients with an MMR after 12 or 18 months of imatinib therapy have prolonged relapse-free survival (1, 3). We used an analogous landmark analysis, after 30 months of imatinib, to assess the prognostic relevance of a durable versus transient MMR. Of the 20 MMR patients

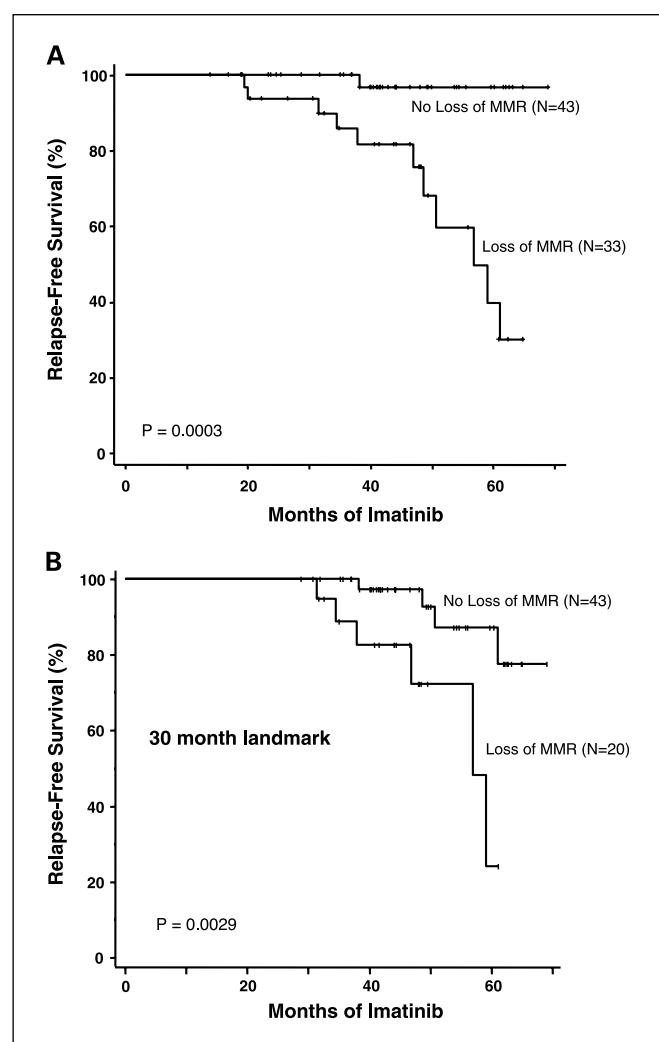


**Fig. 3.** A half-log increase in BCR-ABL RNA levels predicts shorter relapse-free survival. **A.** cumulative rate of relapse-free survival (Kaplan-Meier method) for those subjects with increasing BCR-ABL RNA levels ( $>0.5$  log;  $n = 42$ ) versus those with stable or declining BCR-ABL RNA ( $n = 48$ ). **B.** analogous landmark survival analysis of patients with ( $n = 21$ ) and without ( $n = 50$ ) an increasing BCR-ABL RNA level by 30 mo after imatinib therapy was initiated. The 19 patients with a relapse or last follow-up visit before 30 mo were excluded from this landmark analysis.

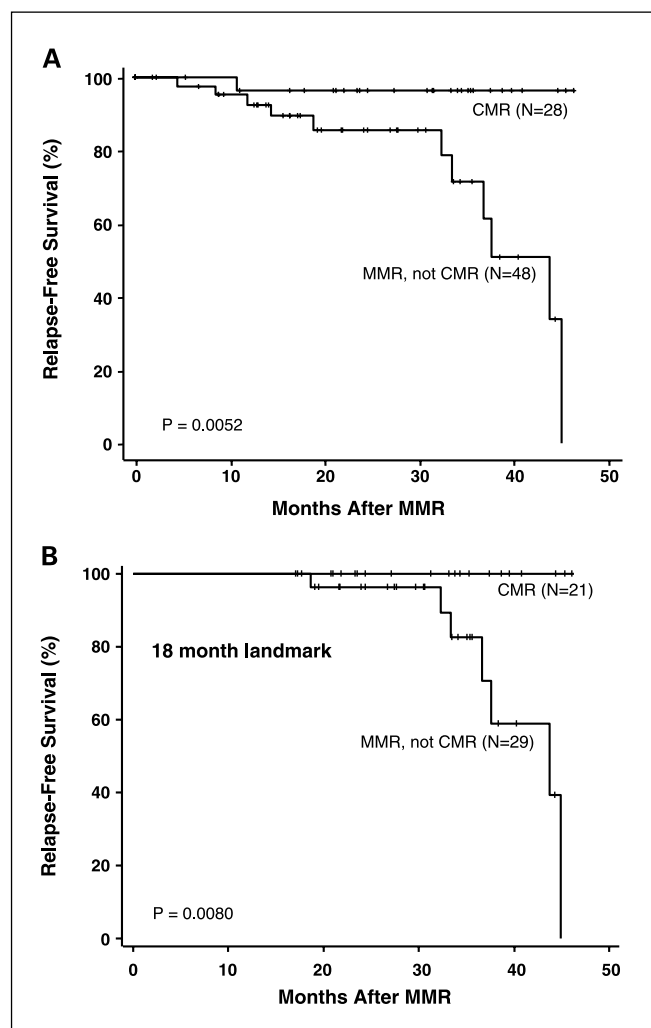
who had had a loss of MMR at 30 months, 6 later relapsed, with a median relapse-free survival of 57 months. In comparison, of the 43 MMR patients with a durable MMR at 30 months, 4 later relapsed, and the relapse-free survival was significantly longer (median not reached;  $P = 0.0029$ ; Fig. 4B). The 13 MMR patients with a relapse or last follow-up visit before 30 months were excluded from this landmark analysis.

**Prognostic significance of a CMR.** CMR (the failure to detect BCR-ABL transcripts) has been reported in 4% to 41% of patients on imatinib (3, 6–8, 10, 23, 24). This wide variability in reported response rates may reflect the duration and dose of imatinib therapy and/or the heterogeneous analytic detection limits in individual laboratories using nonstandardized PCR methods. As we have shown that MMR patients with a half-log increase in BCR-ABL RNA have a shorter relapse-free survival than those without a significant transcript increase (Fig. 4), we hypothesized that a BCR-ABL RNA change in the opposite direction—the achievement of a CMR—might be a significant predictive marker of a yet more durable remission. To assess this hypothesis, we evaluated outcomes in the 28 (37%) patients with MMR who achieved CMR, compared with the

48 patients with MMR who failed to attain CMR. A CMR was attained 8.9 months (median; range, 0-31 months) after MMR was achieved and 23 months (median; range, 7-45 months) after imatinib was initiated. In the entire group of 90 patients, the cumulative rate of achieving a CMR was 3% after 12 months of imatinib therapy, 18% after 24 months, 27% after 36 months, and 34% (maximal) after 45 months. Relapses occurred in only 1 of the 28 (4%) patients with CMR (11 months after MMR) compared with 11 of the 48 (23%) patients without CMR [32 months (median; range, 4-45 months) after MMR]. The relapse-free survival was significantly shortened for the MMR patients who failed to attain CMR (median of 44 months after MMR) compared with the CMR patients (median not reached;  $P = 0.0052$ ), and the hazard ratio for relapse was 11 (95% CI, 1.4-83;  $P = 0.024$ ; Fig. 5A). In a landmark analysis, of the 29 patients who had not converted their MMR to CMR by 18 months, 7 later relapsed, and the median relapse-free survival was 44 months after MMR. In



**Fig. 4.** Loss of MMR predicts shorter relapse-free survival. **A**, cumulative rate of relapse-free survival (Kaplan-Meier method) for the subset of patients ( $n = 76$ ) that achieved MMR, split into those with ( $n = 33$ ) and without ( $n = 43$ ) subsequent loss of MMR (to  $<2.5$  log-drop). **B**, analogous landmark survival analysis of patients with ( $n = 20$ ) and without ( $n = 43$ ) a loss of MMR by 30 mo after imatinib therapy was initiated. The 13 MMR patients with a relapse or last follow-up visit before 30 mo were excluded from this landmark analysis.



**Fig. 5.** The achievement of a CMR predicts prolonged relapse-free survival. **A**, cumulative rate of relapse-free survival (Kaplan-Meier method) for those MMR subjects with a CMR ( $n = 28$ ) versus those with MMR but without a CMR ( $n = 48$ ). **B**, analogous landmark survival analysis of patients who had achieved ( $n = 21$ ) or not achieved ( $n = 29$ ) a CMR by 18 mo after MMR was first attained. The 26 MMR patients with a relapse or last follow-up visit before 18 mo were excluded from this landmark analysis.

comparison, the 21 patients who had attained a CMR by 18 months had a significantly prolonged relapse-free survival (none with relapse;  $P = 0.008$ ; Fig. 5B). The 26 MMR patients with a relapse or last follow-up visit before 18 months were excluded from this landmark analysis.

## Discussion

Although the majority of chronic-phase CML patients treated with imatinib attain durable CCR, a minority later relapse (2, 6-8). The predominant mechanism of this acquired imatinib resistance is point mutations in the BCR-ABL kinase domain that impair the optimal binding of imatinib to its target (25-27). From a practical perspective, early recognition of patients with a higher risk of relapse could trigger more intense monitoring and help expedite necessary changes to the therapeutic strategy, such as dose escalation or second-line ABL kinase inhibitors (nilotinib or dasatinib; refs. 28, 29). A key component of risk stratification is the identification and

validation of biomarkers of increased risk. Toward this goal, we now report that an increase in BCR-ABL RNA levels of greater than a half-log (3.2-fold) is a significant predictor of relapse in patients with an imatinib-induced CCR. With a median follow-up of 49 months, CCR patients with a minimal half-log increase in BCR-ABL RNA thus had a 4.9-fold increased risk of subsequent relapse compared with patients without such an increase. Although the BCR-ABL RNA levels in our study were expressed as a log reduction from a median baseline, the increased relapse risk associated with a half-log (3.2-fold) transcript increase should be equally applicable in laboratories not reporting transcript levels as log-drops. Despite the significantly increased relative risk conferred by an RQ-PCR increase, relapse occurred in only a minority (16 of 42) of these patients (38% positive predictive value). Nevertheless, this information should be clinically useful, leading either to more intense monitoring or to consideration of alternative therapeutic strategies, before overt relapse occurs. The analogy with early intervention for relapse after allogeneic transplants for CML suggests that this approach may lead to improved efficacy and outcomes (30).

Despite the high diagnostic sensitivity (80%) of increasing RQ-PCRs for identifying patients with subsequent relapse, the relatively high false-positive rate (62%) was a mathematical certainty, given our observation of 0.46 log (95% CI) of RQ-PCR interassay analytic measurement variability (Fig. 2). This 2.9-fold assay imprecision parameter is comparable to the 2- to 4.5-fold range of similarly calculated assay imprecision estimates from other laboratories (11, 12). Thus, by chance, 2.5% (half of 5%) of the 952 total RQ-PCRs in this study (24 samples) would be predicted to have false-positive "increases" >0.5 log above expected levels due solely to random assay imprecision. Our observation of 26 false-positive RQ-PCR increases in patients without subsequent relapse—almost identical to the theoretical prediction of 24 such events—validates the accuracy of our statistical model for estimating assay imprecision. This high false-positive rate could, of course, be reduced by increasing the 0.5-log threshold value for defining "increasing" BCR-ABL RNA, but only at the cost of reduced sensitivity for predicting relapse.

In an analogous study of serial changes in BCR-ABL RNA levels in imatinib-treated patients with CML, Branford et al. (11) reported that a 2-fold (0.3 log) serial increase in BCR-ABL RNA was 96% sensitive for the detection of "acquired resistance" (loss of cytogenetic or hematologic response), with a positive predictive value of 79%. In comparison, the seemingly lower sensitivity (80%) and positive predictive value (38%) parameters in our study are likely the result of a major difference in the analysis definitions used by these two studies. In particular, as our goal was to assess increasing RQ-PCR levels as a *predictive* marker of future (not concomitant) relapse, we defined patients with an increasing RQ-PCR that was first detected at the same time as (not before) relapse as "falsely negative" for this predictive biomarker, whereas Branford et al. defined these patients as "true positives." Despite these definitional differences, both of these studies support the practical value of routinely evaluating serial *changes* in BCR-ABL RNA levels, in addition to assessing the achievement of static single time point response thresholds. Similarly, after allogeneic stem cell transplantation, although the change in BCR-ABL RNA levels between consecutive time points was not specifically

assessed, the relapse risk was higher for patients either with transcript levels above a specific threshold value (13, 14) or with an increase in the slope of the regression line of transcript levels versus time (14).

A second major conclusion from this study is the prognostic benefit of achieving the lowest possible BCR-ABL RNA level, below the detection limit of the most sensitive nested PCR assay. Patients achieving a CMR thus had a significantly longer relapse-free survival and an 11-fold lower relapse risk than those that achieved an MMR, but not a CMR. Thus, even though the achievement of an MMR is a good prognostic marker (3, 8, 10) that imparts a low absolute relapse risk, an additional degree of protection is afforded by achieving molecular negativity. In this study, CMR was defined by the absence of detectable BCR-ABL RNA by nested PCR in a sample of sufficient quality to ensure that a minimal 4.0-log reduction in BCR-ABL RNA, if present, would have been detectable. This additional 1-log (or greater) reduction in BCR-ABL RNA levels is associated with the significantly prolonged relapse-free survival in CMR patients, as compared with patients with the 3-log reduction defined as MMR. The significant prognostic advantage of achieving CMR suggests that samples with "undetectable" BCR-ABL RNA by routine RQ-PCR should be reanalyzed by the most sensitive analytic method, typically nested PCR, with a detection limit of at least 4 log. Alternatively, the requirement for reflex nested PCR testing would be obviated if the lab RQ-PCR method was as sensitive as nested PCR. In our laboratory, nested PCR is slightly more sensitive than nonnested real-time PCR (by ~0.5 log), but in other laboratories, this may not be the case (13).

Given that the achievement of each progressively lower threshold level of minimal residual disease—CCR, then MMR—has been associated with a progressively better outcome, the proven extension of this biomarker-disease link to the next convenient threshold level (CMR) should not be surprising. Conversely, this same prognostic relationship also predicts that BCR-ABL RNA level changes in the opposite direction, seen as an MMR that is achieved and then lost, should impart a relatively poorer prognosis. Our finding of a significantly higher future relapse rate in MMR patients that lost (versus maintained) this 3-log level of response directly confirms this hypothesis. These data also suggest that increasing BCR-ABL RNA levels indicate an increased relative relapse risk even when the level of minimal residual disease (and thus the absolute relapse risk) is very low. Branford et al. (11) have analogously shown that a 2-fold increase in BCR-ABL RNA was associated with a significantly higher incidence of ABL kinase domain mutations (33%) in patients with "very low levels of BCR-ABL RNA".

Despite the major limitation of this study—the rather small and heterogeneous patient cohort—we conclude that, in patients with CML receiving imatinib therapy, serial changes in BCR-ABL RNA levels can be used as a sensitive prognostic marker of relative relapse risk. In particular, a half-log increase in BCR-ABL RNA is a significant risk factor for subsequent relapse, even when the level of residual disease is below the 3-log MMR threshold. Conversely, BCR-ABL RNA levels that continue to decrease, reaching undetectable levels by the most sensitive available assay (CMR), impart a beneficial prognostic effect significantly greater than that of reaching MMR alone. Definitive confirmation of the prognostic usefulness of increasing BCR-ABL RNA levels should now be undertaken in

a prospective trial with a more homogeneous study group. These new prognostic markers may allow more accurate stratification of relapse risk and thus better clinical management of patients with CML on imatinib therapy.

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## A Half-Log Increase in BCR-ABL RNA Predicts a Higher Risk of Relapse in Patients with Chronic Myeloid Leukemia with an Imatinib-Induced Complete Cytogenetic Response

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