Quantitative real-time RT-PCR monitoring of *BCR-ABL* in chronic myelogenous leukemia shows lack of agreement in blood and bone marrow samples

WENDY STOCK[1], DAOHAI YU, TED KARRISON[1], DORIE SHER[1], RICHARD M. STONE, RICHARD A. LARSON[1] and CLARA D. BLOOMFIELD

[1]University of Chicago Cancer Research Center, Chicago, IL; CALGB Statistical Center, Durham, NC; The Ohio State University Comprehensive Cancer Center, Columbus, OH; Dana Farber Cancer Institute, Boston, MA; Cancer and Leukemia Group B, Chicago, IL, USA

Received October 18, 2005; Accepted November 28, 2005

Abstract. Molecular monitoring of the *BCR-ABL* transcript in chronic myelogenous leukemia (CML) using quantitative RT-PCR provides clinicians with important diagnostic and prognostic information. To determine whether molecular detection and monitoring of CML is comparable using peripheral blood (PB) and bone marrow (BM) aspirate samples, we performed a prospective study using quantitative real-time RT-PCR (QRT-PCR) of paired PB and BM samples from 41 patients with CML entered onto a single Cancer and Leukemia Group B (CALGB) treatment study. QRT-PCR analysis of PB and BM samples was performed prior to initiation of, and during, treatment with homoharringtonine and cytarabine on a CALGB study for previously untreated CML. Statistical analyses demonstrated good agreement of PB and BM pre-treatment samples. However, using the Bland-Altman statistical method that measures true agreement between PB and BM values, we found that there was only modest agreement of *BCR-ABL* measurements in PB and BM for samples obtained during treatment. PB values obtained during treatment tended to be lower than the corresponding BM values [average difference = -0.37 ($p<0.001$) in 36 paired samples] and the 95% limits of agreement ranged from -1.23 to 0.48. Nevertheless, our study demonstrates that BM and PB QRT-PCR values followed a similar trend during treatment (Spearman correlation coefficient, 0.83; 95% CI, 0.70, 0.96). Our data suggest that, quantitatively, PB and BM measurements of *BCR-ABL* are frequently disparate. Since BM values tended to be higher than PB values, BM sampling provides the most accurate assessment of minimal residual disease (MRD). Based on these results, we caution against interchanging BM with PB sampling for MRD monitoring during treatment of CML since this may lead to misinterpretation of treatment results.

Correspondence to: Dr Wendy Stock, University of Chicago Cancer Research Center, 5841 S. Maryland, M/C 2115, Chicago, IL 60637, USA
E-mail: wstock@medicine.bsd.uchicago.edu

Key words: molecular monitoring, quantitative real-time RT-PCR, *BCR-ABL*, chronic myelogenous leukemia, blood and bone marrow analysis

Introduction

During the past decade, molecular diagnostic techniques, including Southern blotting and RT-PCR, have been useful to clinicians for prognostication and evaluation of treatment efficacy for patients with chronic myelogenous leukemia (CML). We and others have reported previously that Southern blot monitoring of the *BCR-ABL* fusion gene rearrangements in peripheral blood (PB) samples correlated with cytogenetic monitoring of bone marrow (BM) aspirate samples, thus providing a less invasive method for the assessment of therapeutic response during treatment of CML (1-4).

Quantitative real-time RT-PCR (QRT-PCR) provides a rapid, automated and highly sensitive means of accurately quantifying *BCR-ABL* transcripts as a surrogate marker of disease that appears to have independent prognostic significance for patients undergoing curative therapy for CML with allogeneic stem cell transplantation (5-9). Recently, molecular monitoring data from the International Randomized Study of Interferon and STI571 (IRIS) phase III trial of patients with previously untreated CML suggested that quantitation of MRD during early treatment time-points is an important marker of response and progression-free survival (10). Since frequent monitoring using BM sampling is inconvenient and costly, the ability to detect and quantify minimal residual disease in PB samples provides several distinct advantages. To determine whether QRT-PCR monitoring of PB is comparable to BM monitoring in patients receiving intensive combination chemotherapy, we measured *BCR-ABL* levels using QRT-PCR of paired PB and BM samples of CML patients enrolled on a CALGB treatment study (CALGB 19804) initiated in 1998, prior to the introduction of imatinib, that tested the combination of homoharringtonine and cytarabine for newly diagnosed chronic phase CML.

Patients and methods

Forty-four adults with a confirmed diagnosis of previously untreated CML in chronic phase were treated on a single CALGB treatment study, CALGB 19804, between 1998 and 2002. Homoharringtonine (2.5 mg/m^2/day) and cytarabine (7.5 mg/m^2/day) were given via continuous intravenous infusion for 7 days. Cycles were repeated every 28 days. Patients were scheduled to receive up to 9 monthly cycles of treatment; patients who achieved a major cytogenetic response (>65% normal metaphases) after 9 months were eligible to continue therapy. Of the 44 patients who received treatment, the median number of treatment cycles received was nine (range 1-16). Only 4 patients achieved a major (3) or complete (1) cytogenetic response after 9 cycles of therapy. The clinical and cytogenetic results of this trial have been presented elsewhere and are not the focus of this manuscript (Stone R, *et al*, Blood 100: abs. 785, 2002). All patients also enrolled on a CALGB correlative sciences companion study, CALGB 29801, for the prospective molecular comparison of *BCR-ABL* transcripts using QRT-PCR of matched PB and BM samples prior to treatment and 3 and 9 months after initiation of therapy. Of the 44 treated patients, 41 had paired BM and PB samples available for QRT-PCR analysis.

RNA preparation and cDNA synthesis. Total RNA was extracted from BM and PB mononuclear cells. Total RNA (1-5 μg) was synthesized into cDNA according to standard procedures in the SuperScript™ pre-amplification system (Invitrogen, Rockville, MD).

Quantitative real-time RT-PCR (QRT-PCR). QRT-PCR analyses were performed in the central CALGB laboratory at the University of Chicago. QRT-PCR of patient specimens, standard dilutions, and negative controls were analyzed in triplicate using a LightCycler instrument (Roche Diagnostics, Indianapolis, IN). *BCR-ABL* transcripts were amplified in 20 μl reactions containing 0.1 μl of cDNA; 10 mM Tris-HCl, pH 8.3; 50 mM of KCl; 4 mM $MgCl_2$; 0.2 mM of each dNTP; 5 μg BSA; 1.25 U AmpliTaq Gold DNA polymerase (PE Biosystems, Foster City, CA); 300 nM of each primer; and 100 nM of probe. Similarly, *ABL* transcripts were amplified in order to compensate for differences in RNA integrity and cDNA synthesis efficiency. The final concentrations of primer and probe were 400 nM and 100 nM, respectively. The *BCR-ABL* p210 transcripts were amplified using previously published primer and probe sequences (6). The p190 and *ABL* transcripts were detected using the following primer and probe sequences: (p190 5') GCAGATCTGGCCCAACGAT, (p190 3') TCAGACCCTGA-GGCTCAAAGTC, and (p190 probe) 6FAM-CATGGAGACGCAGAAGCCCTTCAGC-TAMRA; (ABL 5') AAAATGACCCCAACCTTTTCG, (ABL 3') CCA TTCCCCATTGTGATTATAGC, and (ABL probe) 6FAM-TCTAAGCATAACTAAAGGTGAAAAGCTCCGGGT CTT-TAMRA. Standard TaqMan™ PCR parameters (ABI PRISM 7700 SDS) were applied to all *BCR-ABL* and *ABL* amplifications.

Quantification and normalization of BCR-ABL. The absolute quantities of *BCR-ABL* and *ABL* transcripts in patient specimens were determined by reference to standard curves. All standard curves were generated from 5-fold serial dilutions of CML cell line cDNA (ranging from 80 pg to 250 ng) containing the appropriate *BCR-ABL* transcript. Real-time RT-PCR results were reported as a ratio or normalized quotient (NQ) of *BCR-ABL/ABL*. NQ values ≤ 0.0001 are below the level of detection with our assay and would be considered a 'molecular remission'. The sensitivity of each assay was between 10^{-6} and 10^{-7} for the p190 transcript and between 10^{-5} and 10^{-6} for both p210 transcripts.

Statistical considerations. Of the 44 adults with newly diagnosed CML who received protocol treatment, only those with paired PB and BM samples collected at the designated time-points were included in the QRT-PCR data set described below. For analysis purposes, we grouped the paired samples as either pre-treatment samples (34 pairs) or on-treatment samples (36 pairs). Of the 36 paired, on-treatment samples, 20 were studied after 3 months of treatment, 14 were studied after 9 months of treatment, and 2 were obtained from patients who continued treatment for more than 9 months (Table I). To assess the agreement between the two methods (PB vs. BM) of measuring NQ values, we utilized the approach of Bland and Altman (11). Briefly, this method plots the difference between the two measurements against their average. Assuming that the differences are approximately normally distributed, 95% 'limits of agreement' are obtained as $\overline{d} \pm 2s_d$, where $\overline{d}$ is the observed mean difference and s_d is the standard deviation of the differences. The significance of $\overline{d}$, as compared to a null value of 0 (obtained via a one-sample t-test), provides evidence of whether there is a systematic difference between the two types of measurements, whereas the 95% limits of agreement provide an interval in which most (i.e., 95%) of the individual differences can be expected to lie.

Results

Thirty-four paired pre-treatment PB and BM samples and 36 paired on-treatment samples were evaluated using QRT-PCR (Table I). Eighteen patients expressed the b2a2 *BCR-ABL* transcript and 16 patients expressed b3a2 *BCR-ABL*. The median value (range) for pre-treatment BM NQs was 1.3 (0.3-10.2) compared to the median value in PB of 1.2 (0.1-5.0). Following 3 months of treatment, the median values for BM and PB were 0.5 (undetectable 24.0) and 0.4 (undetectable 3.0), respectively. After 9 months of treatment, median BM and PB NQs were 1.2 (0.08-66.0) and 1.0 (0.02-4.23), respectively. Three patients (nos. 4, 7 and 20) achieved a transient major cytogenetic response at 9, 10 and 11 months of treatment, respectively. All three had detectable MRD in both PB and BM at the time of their best cytogenetic response. One patient (no. 42) had a transient complete cytogenetic response following 3 months of treatment but had persistent MRD in a PB sample at that time. Only 1 patient (no. 11) had matched PB and BM samples that were transiently negative (undetectable) for MRD following 3 months of treatment. Cytogenetic analysis was not performed at this time-point.

The scatter plots in Fig. 1 display the raw data comparing PB and BM NQs for all 70 paired samples. In the plots, the difference between the PB and BM values is plotted against

Table I. NQ of *BCR-ABL/ABL* during treatment with homoharringtonine/cytarabine.

Patient no.	Pre-treatment		3 months		9 months		Post 9 months	
	BM	PB	BM	PB	BM	PB	BM	PB
1	1.000	5.000	0.150	0.260	66.000	2.200		
2	1.800	1.300	1.000	0.360	1.700	1.800		
3	1.200	1.600	0.410	0.370	2.900	3.200		
4[a]	1.000	0.300	0.650	0.570	0.082	0.062		
5	2.100	1.100	0.410	0.920	8.400	4.230		
6	0.680	0.530	0.410	0.140	0.700	0.110		
7[a]	1.400	0.940	NA	NA	0.078	0.022	0.099	0.021
8	0.580	0.610	0.150	0.009				
9	0.760	0.300	0.570	0.098				
10	3.300	2.100	24.000	1.100				
11	0.380	0.520	Undetectable	Undetectable				
12	1.600	2.600	0.400	0.076				
13	1.200	0.340	1.000	0.850				
14	2.800	1.200	2.400	0.550				
15	1.800	1.800	3.000	3.000				
16	2.200	4.700	NA	NA	1.700	1.000		
17	1.300	1.100	NA	NA	0.500	0.490		
18	0.800	1.000	NA	NA	0.520	0.460		
19	1.530	1.150	NA	NA	0.820	0.900		
20[a]	1.200	1.600	NA	NA	NA	NA	0.140	0.10
21	NA	NA	0.260	0.052	0.350	0.110		
22	NA	NA	11.000	1.800	1.800	2.000		
23	0.530	0.570						
24	0.470	0.390						
25	2.300	1.700						
26	2.050	1.670						
27	2.900	1.200						
28	1.400	1.500						
29	1.300	1.800						
30	0.580	0.700						
31	10.200	1.280						
32	0.380	1.200						
33	0.300	0.120						
34	0.900	0.740						
35	2.100	3.100						
36	1.900	1.700						
37	NA	NA	1.500	0.450				
38	NA	NA	0.160	0.076				
39	NA	NA	0.470	0.075				
40	NA	NA	1.100	0.690				
41	NA	NA	NA	NA	1.500	1.000		
42[a]	NA	1.300	NA	0.089	NA	1.300		

[a]Patients who achieved a major cytogenetic response. Patient 42 had a complete cytogenetic response after 3 months of treatment. Patients 4, 7 and 20 achieved a major cytogenetic response after 9, 10 and 11 months of treatment, respectively. NA, sample was not available for analysis.

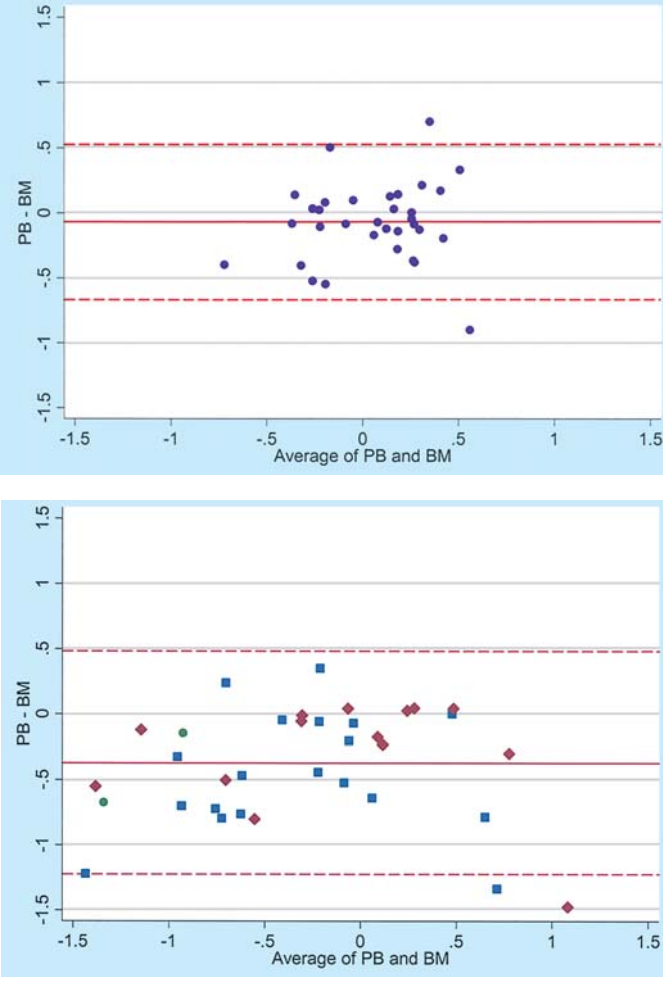

Figure 1. Scatter plot of difference (y-axis) vs. average (x-axis) for samples from 34 patients prior to initiation of treatment for CML (top) and from 27 patients (36 paired samples) during treatment of CML with homoharringtonine/cytarabine (bottom). The one case with undetectable levels for both PB and BM is omitted from the figure. QRT-PCR measurements are expressed as a NQ values. PB and BM values were first transformed to log (base 10) scale. 95% agreement intervals are represented by the dashed lines falling on either side of the mean difference (middle solid line). In the bottom plot, squares represent 3-month values; diamonds, 9-month values; and circles, post 9-month values.

their average. Since the variability in the raw differences increased with the mean, a logarithmic transformation (base 10) was first applied to the individual values. Thus, a difference of 1 in Fig 1. represents a 10-fold difference between PB and BM values. The solid horizontal line is the mean difference and the dotted lines above and below the mean are the 95% limits of agreement. In the pre-treatment samples, there is no evidence for a systematic difference between the PB and BM values (mean difference = -0.07, p=0.19), but the 95% limits of agreement are broad (-0.67 to 0.53). For the on-treatment data, the PB values were significantly lower than BM (mean difference = -0.37, p<0.001) and the 95% limits of agreement range from -1.23 to 0.48. Thus, although there appears to be good agreement between PB and BM Q-PCR in pre-treatment samples, the agreement between PB and BM values during treatment was modest at best.

If the samples are dichotomized as molecularly detectable residual disease vs. those achieving molecular remission (NQ ≤ 0.0004), there is no discordance between the PB and BM measurements. Specifically, among 69 samples in which the BM exhibited the presence of disease, all 69 PB measurements also did so; and in the single sample in which the BM level was undetectable, the PB yielded an undetectable reading also. Thus the 'sensitivity' of the PB readings is 100% (69/69) and the 'specificity' is 100% (1/1), although the latter estimate obviously suffers from a lack of adequate numbers. For the purpose of comparison to other published studies comparing BM and PB PCR monitoring, we also measured the association of PB and BM pairs based on their ranked values using the Spearman correlation coefficient. The Spearman statistic showed a strong correlation of 0.83 (0.70, 0.96) for paired PB and BM NQ values.

Collectively, these results suggest that, in quantitative terms, the PB analysis does not agree strongly with BM readings but may be an acceptable alternative to BM if only an indication of the trend in molecular response to CML therapy is sought. We also evaluated whether there was any relationship between the type of *BCR-ABL* transcript and the level of expression. No significant difference in mean transcript number, prior to initiation or on treatment, was noted for patients with b3a2 *BCR-ABL* when compared to the mean transcript number for patients with the b2a2 *BCR-ABL* transcript (data not shown).

Discussion

As the treatment paradigm for CML has changed with the introduction of imatinib to frontline therapy, accurate evaluation of MRD states is becoming an important surrogate for response and for prognosis (12). A number of recent studies suggest that the degree of change in the NQ value using QRT-PCR during the first months of treatment with imatinib is predictive of subsequent cytogenetic response and also appears to correlate with clinical outcome for patients with both early chronic phase and advanced CML (10,12-18; Odenike O, *et al*, Blood 98: abs. 763, 2001). The failure of imatinib as a single agent to completely eradicate MRD in the majority of CML patients, as well as the emergence of imatinib resistance has led to the exploration of novel tyrosine kinase inhibitors and of imatinib-based combination therapies (19). While not shown to induce significant cytogenetic or molecular responses on this CALGB trial, both homoharringtonine and cytarabine have activity in CML and trials are either underway or in planning stages to test these agents in combination with imatinib to evaluate their ability to further reduce or eradicate MRD (20).

This analysis demonstrates that PB and BM quantification of *BCR-ABL* transcripts using QRT-PCR exhibit only fair agreement with one another in absolute terms. During treatment, BM values tended to be higher than those in PB. However, no cases were found in which BM had detectable MRD using our QRT-PCR assay and the PB did not, although this finding must be tempered by the fact that only one patient on this trial became PCR-negative. To our knowledge, this is the first comparison of blood and marrow *BCR-ABL* transcript values that has utilized a statistical test (Bland-Altman) that measures the agreement between paired samples. Previous comparisons of matched blood and marrow PCR monitoring in CML and in other leukemias, such as acute promyelocytic

leukemia (APL), have utilized the Spearman correlation co-efficient that measures the 'rank order' relationship of the paired samples but doesn't measure the true agreement between PB and BM values (5,12). For example, if a paired PB (NQ=2.2) and BM (NQ=22) have NQ values that are both ranked the second highest PB and BM NQ values, respectively, in a series of 30 paired samples, the Spearman correlation co-efficient will be high despite the fact that the paired samples have a 10-fold difference in absolute copy number. Likewise, the ordinary Pearson correlation coefficient also measures the strength of the relationship between two variables, not their absolute agreement (11). Therefore, the Bland-Altman statistic is a more accurate test for measuring actual agreement between BM and PB *BCR-ABL*.

Based on our results, we suggest that QRT-PCR analysis of BM provides more accurate information about true MRD status in patients receiving treatment for CML. Although our data demonstrate that similar trends occurred in matched PB and BM values allowing clinicians to gauge molecular response using PB sampling, BM B*CR-ABL* levels during treatment were consistently higher than those in PB. Periodic BM examinations during CML treatment also remain important for assessment of other disease parameters, including morphology and cytogenetics. Subtle changes in marrow morphology (e.g. fibrosis) and the acquisition of additional cytogenetic abnormalities that may be detected in Philadelphia chromosome positive or negative cells may also influence clinical decisions (20). It is also critical to consider that the most important goal of quantitative MRD monitoring is its correlation with a clinical endpoint. Others have demonstrated that sequential reduction in *BCR-ABL* transcript using QRT-PCR of PB during treatment (10) correlates with survival in CML patients receiving imatinib. Taken in the context of our results showing a lack of true agreement between BM and PB, we caution against the interchange of BM and PB QRT-PCR values during MRD monitoring of CML since it may lead to misinterpretation of treatment results. Furthermore, as MRD evaluation becomes an important clinical surrogate in CML, it is essential to establish a universal standard for MRD measurement and reporting of *BCR-ABL*.

Acknowledgements

This work was supported by U10-CA101140 to the Cancer and Leukemia Group B, Leukemia Correlative Sciences Committee, CA16058, CA77658, CA31946 and the Coleman Leukemia Research Foundation.

References

1. Stock W, Westbrook CA, Peterson B, *et al*: Value of molecular monitoring during the treatment of chronic myeloid leukemia: a Cancer and Leukemia Group B study. J Clin Oncol 15: 26-36, 1997.
2. Ayscue LH, Ross DW, Ozer H, Rao K, Gulley ML and Dent GA: Bcr/abl recombinant DNA analysis versus karyotype in the diagnosis and therapeutic monitoring of chronic myeloid leukemia. Am J Clin Pathol 94: 404-409, 1990.
3. Grossman A, Silver RT, Szatrowski TP, Gutfriend A, Verma RS and Benn PA: Densitometric analysis of Southern blot autoradiographs and its application to monitoring patients with chronic myeloid leukemia. Leukemia 5: 540-547, 1991.
4. Reiter A, Skladny H, Hochhaus A, *et al*: Molecular response of CML patients treated with interferon-alpha monitored by quantitative Southern blot analysis. German chronic myeloid leukaemia (CML) Study Group. Br J Haematol 97: 86-93, 1997.
5. Radich JP, Gooley T, Bryant E, *et al*: The significance of bcr-abl molecular detection in chronic myeloid leukemia patients 'late', 18 months or more after transplantation. Blood 98: 1701-1707, 2001.
6. Eder M, Battmer K, Kafert S, Stucki A, Ganser A and Hertenstein B: Monitoring of BCR-ABL expression using real-time RT-PCR in CML after bone marrow or peripheral blood stem cell transplantation. Leukemia 13: 1383-1389, 1999.
7. Preudhomme C, Revillion F, Merlat A, *et al*: Detection of BCR-ABL transcripts in chronic myeloid leukemia (CML) using a 'real-time' quantitative RT-PCR assay. Leukemia 13: 957-964, 1999.
8. Martinelli G, Montefusco V, Testoni N, *et al*: Clinical value of quantitative long-term assessment of bcr-abl chimeric transcript in chronic myelogenous leukemia patients after allogeneic bone marrow transplantation. Haematologica 85: 653-658, 2000.
9. Olavarria E, Kanfer E, Szydlo R, *et al*: Early detection of BCR-ABL transcripts by quantitative reverse transcriptase-polymerase chain reaction predicts outcome after allogeneic stem cell transplantation for chronic myeloid leukemia. Blood 97: 1560-1565, 2001.
10. Hughes TP, Kaeda J, Branford S, *et al*: Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. N Engl J Med 349: 1423-1432, 2003.
11. Bland JM and Altman DG: Statistical methods for assessing agreement between two methods of clinical measurement. Lancet i: 307-310, 1986.
12. Gallagher RE, Yeap BY, Bi W, *et al*: Quantitative real-time RT-PCR analysis of PML-RAR alpha mRNA in acute promyelocytic leukemia: assessment of prognostic significance in adult patients from intergroup protocol 0129. Blood 101: 2521-2528, 2003.
13. O'Brien SG, Guilhot F, Larson RA, *et al*: Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med 348: 994-1004, 2003.
14. Lee WI, Kantarjian H, Glassman A, Talpaz M and Lee MS: Quantitative measurement of BCR/abl transcripts using real-time polymerase chain reaction. Ann Oncol 13: 781-788, 2002.
15. Merx K, Muller MC, Kreil S, *et al*: Early reduction of BCR-ABL mRNA transcript levels predicts cytogenetic response in chronic phase CML patients treated with imatinib after failure of interferon alpha. Leukemia 16: 1579-1583, 2002.
16. Wang L, Pearson K, Pillitteri L, Ferguson JE and Clark RE: Serial monitoring of BCR-ABL by peripheral blood real-time polymerase chain reaction predicts the marrow cytogenetic response to imatinib mesylate in chronic myeloid leukaemia. Br J Haematol 118: 771-777, 2002.
17. Wu CJ, Neuberg D, Chillemi A, *et al*: Quantitative monitoring of BCR/ABL transcript during STI-571 therapy. Leuk Lymphoma 43: 2281-2290, 2002.
18. Shah NP, Tran C, Lee FY, Chen P, Norris D and Sawyers CL: Overriding imatinib resistance with a novel ABL kinase inhibitor. Science 305: 399-401, 2004.
19. Marin D, Kaeda JS, Andreasson C, *et al*: Phase I/II trial of adding semisynthetic homoharringtonine in chronic myeloid leukemia patients who have achieved partial or complete cytogenetic response on imatinib. Cancer 103: 1850-1855, 2005.
20. O'Dwyer ME, Gatter KM, Loriaux M, *et al*: Demonstration of Philadelphia chromosome negative abnormal clones in patients with chronic myelogenous leukemia during major cytogenetic responses induced by imatinib mesylate. Leukemia 17: 481-487, 2003.