Plasma RNA as an alternative to cells for monitoring molecular response in patients with chronic myeloid leukemia

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ABSTRACT

Background and Objectives

Quantitation of BCR-ABL mRNA is emerging as the standard of care to monitor the status of chronic myeloid leukemia (CML). Peripheral blood plasma was analyzed in this study because of previous detection of nucleic acids and proteins from tumor cells in plasma samples.

Design and Methods

Reverse transcriptase polymerase chain reaction was used to establish ratios of BCR-ABL:ABL mRNA in peripheral blood cells and plasma, and absolute levels of BCR-ABL mRNA per unit volume of plasma. Samples from 160 CML patients and 180 control individuals without CML were tested. Cells and plasma samples from 93 of the CML patients were re-analyzed 3-12 months after imatinib treatment.

Results

Ratios of BCR-ABL:ABL mRNA in paired cell and plasma samples of the 160 CML patients correlated significantly ($r=0.83$; $p<0.001$). When results were compared directly using the sign test, the pre-therapy plasma results were significantly different from those from peripheral blood cells ($p=0.028$), but not bone marrow cells ($p=0.119$). Absolute levels of BCR-ABL mRNA in plasma strongly correlated with many laboratory characteristics in pre-therapy CML patients. Higher BCR-ABL: ABL ratios were detected in plasma samples at all time points after treatment, although this was significant only at 3 months ($p=0.0003$). In cases in which results from the assays disagreed, minimal residual disease was detected in plasma samples significantly more frequently than in cell samples ($p<0.001$).

Interpretation and Conclusions

Plasma was a reliable source for monitoring BCR-ABL mRNA levels. Minimal residual disease detection from plasma was more sensitive than from cell samples. Our results suggest that absolute levels of BCR-ABL mRNA per unit volume of plasma may reflect tumor load.

Key words: BCR-ABL, RT-PCR, chronic myeloid leukemia, imatinib, plasma.

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Chronic myelogenous leukemia (CML) is caused by chromosomal translocations resulting in expression of a fusion protein, BCR-ABL, with constitutively active tyrosine kinase activity. Signal transduction pathways downstream of BCR-ABL promote the cell proliferation and inhibition of apoptosis that are characteristic of the disease. Monotherapy with imatinib, a specific inhibitor of BCR-ABL tyrosine kinase activity, is an effective treatment for all stages of CML.[1-3] A high frequency of complete cytogenetic response is seen in imatinib-treated CML patients; however, a molecular response, assessed by detection of BCR-ABL mRNA, is emerging as the standard of care to monitor response to imatinib therapy in CML patients.[4-9] BCR-ABL mRNA in nucleic acids extracted from cells of peripheral blood or bone marrow samples can be amplified and quantitated by reverse-transcription polymerase chain reaction (RT-PCR).[10] However, the number of cells used and conditions for the assays have not been standardized, so quantitation of BCR-ABL mRNA by this approach may not be reliably reproducible from one laboratory to another.

Our previous work has shown that plasma prepared from peripheral blood contains tumor-specific DNA, RNA and proteins.[11-15] In this study, we investigated the use of plasma samples from CML patients for RT-PCR detection of BCR-ABL mRNA.

Design and Methods

Cell lines and CML patient samples

The K562 cell line was obtained from ATCC and was maintained in RPMI 1640 and Iscove's modified Dulbecco's medium with 10% fetal calf serum.

Plasma and cell samples were collected from 160 patients with CML and 180 individuals without CML according to an institutional review board-approved protocol. The diagnoses of CML were confirmed by routine karyotyping and fluorescence *in situ* hybridization (FISH) studies.

RNA extraction and real-time RT-PCR

Total nucleic acid was extracted from 0.5 mL samples of plasma using the NucliSens extraction kit (bioMerieux, Durham, NC, USA). Cell or bone marrow samples were processed to extract mRNA using a Roche MagNAPure extraction kit (Roche Diagnostics, Indianapolis, IN, USA). RNA was extracted from approximately 7×10^6 cells for RT-PCR analysis. After extraction, nucleic acids were dissolved in 50 μL water, of which 20 μL were used in each RT-PCR reaction. Thus, each RT-PCR assay quantitated the mRNA present in 200 μL of plasma. The two types of BCR-ABL fusion transcripts (b2a2/b3a2 and e1a2) were quantitated in a single tube real-time RT-PCR reaction, as was the native ABL transcript to control for sample RNA quality and provide an internal standard for relative quantification. The primers and probes used for the BCR-ABL real-time PCR were: (BCR-P1 F): 5'-CCTCGCAGAACTCGCAACA-3'; (BCR-P2,P3 F) 5'-GAGCTGCAGATGCTGACCAA-3'; (BCR-R): 5'-TCAGACCCTGAGGCTCAAAGTC-3'; (BCR-P1 probe):5'- FAM-ACACGACAACCGGGCAGTGCC-TAMRA-3'; (BCR-P2,3 probe): 5'-FAM-TGCTGTGGACAGTCTGGAGTTTCACACA-TAMRA-3'.

The primers and probe used for the ABL real-time PCR were: (ABL-F): 5' -TCC TCC AGC TGT TAT CTG GAA GA-3'; (ABL-R): 5'-TGG GTC CAG CGA GAA GGT T-3'; (ABL-Probe): 6-FAM-CCA GTA GCA TCT GAC TTT GAG CCT CAG GG-TAMRA-3'.

One-step real-time RT-PCR was performed in a 50 ?L reaction volume using UltraSense™ One-Step Quantitative RT-PCR reagent (Invitrogen, Carlsbad, CA, USA) and the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). RT-PCR reactions included: reaction buffer, 400 nM each of the forward and reverse primers, 100 nM of each of the probes; 2.5 μL of SuperScript III enzyme mix (Invitrogen, Carlsbad, CA, USA), 1 μL of the ROX reference dye (Invitrogen, Carlsbad, CA, USA), and 20 μL of the extracted nucleic acid sample. The thermocycler conditions were: 15 min at 50°C for the reverse transcription step, followed by 1 cycle of 95°C for 2 min, 45 cycles of 95°C for 15 sec, and 60°C for 30 sec. The results are reported as ratios between the quantities of the fused BCR-ABL mRNA and the ABL internal control mRNA, and as mRNA copies per 10 μL plasma, based on standard curves generated in this study.

These standard curves were based on the amplification of dilutions of ABL mRNA obtained from Raji cells. While micrograms of Raji RNA was used in the amplification, we calculated the number of copies of the amplified RNA based on the estimation of 10 copies of RNA/1 picogram of RNA. This is an approximate estimation used here simply to compare samples with each other. We use automation in our RNA extraction and every step in our assay was followed carefully in a consistent fashion to assure the reliability of the absolute values. We also evaluated the internal control and repeated the assays when the value of the internal control was too discrepant from that of the BCR-ABL.

Statistical analysis

The χ^2 of the Kruskal-Wallis test was used for categorical data, and Student's t test for continuous data. Correlations between BCR-ABL:ABL ratios in paired plasma and peripheral blood cell samples were performed using Spearman's correlation coefficients. The sign test, which tests the null hypothesis (no difference) between two groups, was used to compare results obtained by two different methodologies.

Results

Plasma is a reliable source for quantitation of BCR-ABL mRNA

Levels of BCR-ABL mRNA were analyzed by RT-PCR of paired samples of peripheral blood cells and peripheral blood plasma from 160 CML patients. BCR-ABL mRNA was quantitated by normalization to native ABL mRNA in the same samples. Ratios of BCR-ABL: ABL mRNA in plasma samples from CML patients correlated significantly with those from paired peripheral blood cell samples (r=0.83; p<0.0001) (Figure 1). The BCR-ABL fusion transcript was not detected in control plasma samples from 180 individuals without CML.

Of the 160 samples analyzed from CML patients, 100 were from patients who were on therapy with imatinib and 60 were from newly diagnosed patients. Paired plasma and peripheral blood cell samples from patients on therapy showed BCR-ABL: ABL ratios that were not significantly different when the sign test was used to evaluate the comparisons (Table 1). However, the sign test, which tests the null hypothesis (i.e. that the two values are not different), showed a significant difference (p=0.028) between BCR-ABL: ABL ratios from plasma and peripheral blood cells from untreated patients (Table 1). There was no difference between ratios obtained from peripheral blood cells and bone marrow cells or between ratios from bone marrow cells and plasma. The reason for the detected difference in ratios in pre-therapy samples only is not known. However, it is possible that when cells are tested, most of the cells in the samples are leukemic cells and the ratios represent the ratio of the fusion mRNA to the internal control in the leukemic cells. This is supported by the limited variation seen between BCR-ABL: ABL ratios in pre-therapy samples (mean=5.88 and standard deviation=6.78). In contrast, when plasma is analyzed the ratios reflect the turnover of leukemic cells from the entire body. Therefore, more variation in ratios is seen between patients (mean=10.87, standard deviation=13.31). In post-therapy samples, when cells are analyzed, the ratios vary more significantly (Figure 2) reflecting different ratios of leukemic cells in a fashion more similar to that of the plasma.

The BCR-ABL mRNA detected in cell-free plasma samples must have originated from lysed tumor cells. In order to estimate the minimum number of tumor cells that could have been the source of the BCR-ABL mRNA detected in plasma, we added varying amounts of lysate from BCR-ABL-positive K562 cells to control plasma from individuals without CML. Immediately after addition of the K562 lysate to plasma, nucleic acids were extracted by the same protocol used for plasma samples. The RT-PCR assay results showed that BCR-ABL mRNA was detectable when lysate from as few as ten K562 cells was diluted in 1 mL of non-CML plasma.

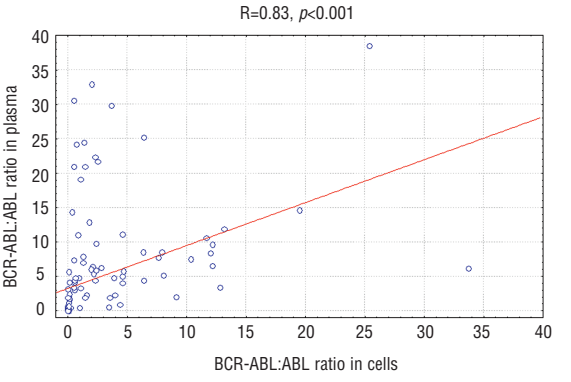

Figure 1. Good overall correlation (r=0.83) between cells and plasma BCR-ABL:ABL ratios.

Table 1. Comparison of BCR-ABL:ABL ratios obtained from plasma with those obtained from cells using the sign test.

	Number of samples	Percent v < V	Z	p value
PB cells vs. plasma (untreated)	60	65	2.19	0.028
PB cells vs. plasma (at 3 months)	43	61.9	1.389	0.165
PB cells vs. plasma (at 6 months)	22	42.9	0.436	0.663
PB cells vs. plasma (at ≥ 9 months)	28	63.2	0.918	0.359
PB cells vs. BM cells (untreated)	50	60	1.272	0.203
BM cells vs. plasma (untreated)	50	38	1.555	0.119

K562 cells do not accurately represent average CML cells and are known to have amplification of the *BCR-ABL* gene. Most likely more primary CML cells are needed to give similar results. The stability of the detected RNA in blood samples before isolating plasma samples and testing was also evaluated. Blood samples kept at room temperature for up to 48 hours showed less than 25% reduction, which is within the coefficient of variability of the RT-PCR assay. After 48 hours, the quality of the RNA in the blood sample deteriorated significantly (>50%).

Use of plasma samples to monitor residual disease in imatinib-treated CML patients

Paired samples of peripheral blood cells and plasma were obtained from 93 imatinib-treated CML patients at various times after completion of treatment. Ratios of BCR-ABL: ABL mRNA decreased significantly from baseline in both plasma and peripheral blood cell samples (Figure 2). At every time point after treatment, the median levels of BCR-ABL mRNA found in plasma were greater than those in peripheral blood cells (Figure 2). This difference was statistically significant at 3 months (Wilcoxon's matched pairs test, p=0.0003), but not at 6, 9, or 12 months after treatment. The detection of minimal residual disease (positive results in the RT-PCR assay for BCR-ABL mRNA) in plasma and peripheral blood cell

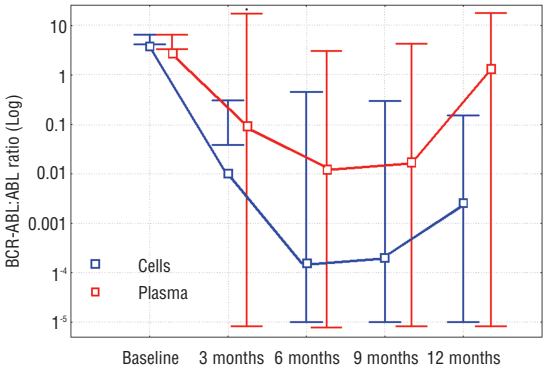

Figure 2. **BCR-ABL:ABL ratios in peripheral blood cells and plasma samples of 93 CML patients at various times after treatment with imatinib. The median and 95% confidence interval of the BCR-ABL:ABL ratio in peripheral blood cells and plasma are shown in pre-therapy samples and after 3, 6, 9, and 12 months of therapy.**

Table 2. **Detection of minimal residual disease in plasma and peripheral blood cell samples of imatinib-treated CML patients.**

BCR:ABL mRNA detection in:		*Number (%) of patients at various times after completion of treatment*			
Cells	*Plasma*	*3 months (n=43)*	*6 months (n=22)*	*9 months (n=19)*	*12 months (n=9)*
Results agreed					
Positive	Positive	29 (67%)	12 (54%)	9 (47%)	3 (33%)
Negative	Negative	8 (19%)	7 (32%)	6 (32%)	3 (33%)
Results did not agree					
Positive	Negative	0	2* (9%)	1* (5%)	0
Negative	Positive	6 (14)	1° (5%)	3° (16%)	3° (33%)

**Later testing (at 12 months) of cell samples from these patients was negative; °later testing of cell samples from two of the 12-month cases became negative by plasma testing while the rest of these samples became positive by cell testing.*

samples of CML patients after treatment with imatinib is presented in Table 2. Results of testing plasma and peripheral blood cell samples for minimal residual disease agreed for 86% of the patients at 3 months, 86% at 6 months, 79% at 9 months, and 67% at 12 months. In those cases for which the results from the different types of sample did not agree, minimal residual disease was detected in plasma samples and not in cell samples from a significantly greater number of patients than the converse (i.e. detection in cell samples and not in plasma samples) ($p<0.001$). For three patients, minimal residual disease was detected in peripheral blood cell samples only, but all three cases became negative in subsequent samples. In one patient at 6 months and three patients at 9 months, minimal residual disease was not detected in the peripheral blood cell sample, but later all cell samples were positive. Three cells samples were negative at 12 months and for two of these the plasma samples became negative at 15 months while the third patient's cell sample became positive.

These results show that assays of minimal residual disease in plasma and peripheral blood cells sample agree in a large percentage of patients, and in most of the cases in which the results of the assay are discordant, minimal residual disease was detected in plasma but not in cell samples, suggesting that testing plasma is a more sensitive method for detecting minimal residual disease. Further studies are needed to explore the clinical value of this increased sensitivity.

The plasma- and cell-based assays are comparable in their ability to detect residual leukemic cells. The sensitivity of the cell-based assay is one K562 cell per 100,000, which corresponds to 3 cells/reaction, and this is comparable to that of plasma (10 cells/mL, which corresponds to 2.5 cells/reaction).

Absolute levels of BCR-ABL mRNA in plasma

Since we used equal amounts of extracted RNA, representing equal amounts of plasma, in all our assays, we were able to express the approximate absolute levels of BCR-ABL mRNA as mRNA copies per 10 μL of plasma. While the exact copy of mRNA is difficult to quantify, we estimated the number of copies of BCR-ABL mRNA using dilutions from Raji cell lysates to construct a standard curve for the amplification of the ABL transcript. The generated numbers of copies were estimated based on the assumption of the presence of 10 copies of mRNA per 1 picogram of total RNA. Comparison of the pre-therapy BCR-ABL:ABL mRNA ratios and approximate absolute levels of BCR-ABL mRNA with various patients' characteristics revealed significant correlations between absolute values of BCR-ABL per 10 μL of plasma and these characteristics, but rarely with BCR-ABL:ABL mRNA ratios from either cells or plasma (Table 3).

Significant direct correlations were found between absolute levels of BCR-ABL mRNA and levels of white blood cells (r=0.72; p=0.00004), percent blasts (r=0.52; p=0.0045), levels of serum GOT (r=0.45; p= 0.011), percentages of myelocytes (r=0.46; p=0.014) and metamyelocytes (r=0.43; p=0.024), levels of lactate dehydrogenase (r=0.68; p=0.000077), and FISH findings (r=0.58; p=0.0000001) (Table 3). A significant negative correlation was observed between absolute levels of BCR-ABL mRNA and percent lymphocytes (r=-0.63; p= 0.00013). Correlations between BCR-ABL:ABL mRNA ratios and these variables were less strong than those found for absolute plasma levels of BCR-ABL mRNA in every case, and in most cases, were so weak as to be statistically not significant (Table 3).

Unlike BCR-ABL:ABL mRNA ratios in cells, absolute levels of BCR-ABL mRNA in plasma may provide a means to quantify tumor load. As shown in Figure 3, there was marginal correlation between absolute levels of BCR-ABL in plasma and BCR-ABL:ABL ratios in cells from peripheral blood cells (Figure 3A) or bone marrow (Figure 3B) (r =0.37 and r =0.33, respectively). The correlation between BCR-ABL:ABL ratios in cells from peripheral blood and bone marrow was also marginal

Table 3. Spearman's correlations (r values) between various patients' characteristics and BCR-ABL:ABL mRNA ratios in peripheral blood (PB) cells, plasma, and absolute BCR-ABL levels per 10 μL of plasma.

Patients' characteristic	*PB cell BCR-ABL:ABL ratio*		*Plasma BCR-ABL:ABL ratio*		*Plasma BCR-ABL/10 μL*	
	r value	*p value*	*r value*	*p value*	*r value*	*p value*
White blood cells	0.63	0.00013	0.27	0.15	0.72	0.000004
% Blasts	0.12	0.54	0.08	0.70	0.52	0.0045
Uric acid	0.35	0.076	0.27	0.17	0.18	0.37
Total protein	0.41	0.022	0.13	0.49	0.24	0.20
Total bilirubin	0.35	0.056	0.11	0.55	0.29	0.12
SGOT	0.26	0.16	0.26	0.16	0.45	0.011
% promyeloctes	0.26	0.18	0.18	0.35	0.35	0.065
% Myelocytes	0.35	0.072	0.03	0.89	0.46	0.014
% Metamyelocytes	0.14	0.49	0.09	0.65	0.43	0.024
% Lymphocytes	-0.47	0.0077	-0.26	0.15	-0.63	0.00013
Absolute lymphocytes	0.49	0.0051	0.09	0.64	0.29	0.11
Lactate dehydrogenase	0.56	0.0021	0.32	0.095	0.68	0.000077
% Eosinophils	-0.45	0.015	-0.13	0.50	-0.19	0.33
% FISH-Ph$^+$ positive cells	0.46	0027	0.11	0.40	0.58	0.000001
% Ph$^+$ cells by cytogenetics	0.27	0.059	0.16	0.25	0.23	0.10
BM cell BCR-ABL:ABL ratio	0.41	0.0033	0.33	0.021	0.33	0.020
PB cell BCR-ABL:ABL ratio			0.16	0.21	0.37	0.0032

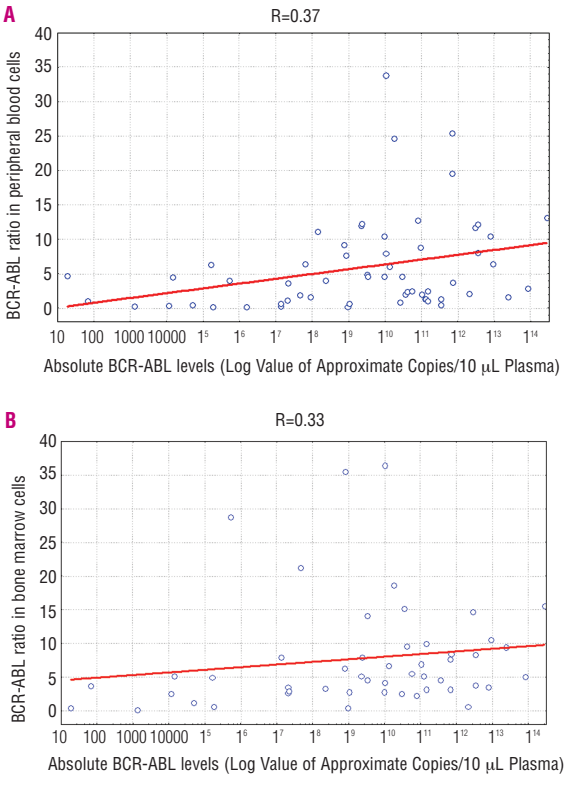

Figure 3. Absolute copies of BCR-ABL mRNA per 10 μL plasma and BCR-ABL:ABL ratios in peripheral blood cells (A) and bone marrow cells (B) from 60 newly diagnosed CML patients. There is a marginal correlation between absolute BCR-ABL mRNA levels in plasma and BCR-ABL: ABL ratios in peripheral blood cells (r=0.37, *p*=0.003) and bone marrow cells (r=0.33, *p*=0.02).

(r=0.41; *p*=0.003). In contrast there was significant correlation (r= 0.83) between ratios obtained from cells and plasma (Figure 1). This suggests that absolute levels, as standardized to a specific volume of plasma, represent a parameter that is different from that provided by the ratios. The demonstration that absolute plasma levels of BCR-ABL in pre-therapy samples correlated with known prognostic indicators and white cells, as shown in Table 3, suggests that absolute values provide a better measurement, but further studies correlating these levels to response to therapy and outcome are needed.

Discussion

Quantitation of BCR-ABL mRNA levels by RT-PCR for diagnosis and monitoring disease is rapidly becoming the standard of care for CML patients. The results of this study show that quantitation of BCR-ABL mRNA in plasma and peripheral blood samples from CML patients correlated well overall. RT-PCR of plasma samples was a sensitive method for detecting BCR-ABL mRNA. Additionally, assays of minimal residual disease in paired plasma and cell samples from the same imatinib-treated CML patients showed concordance of results in 67-86% of cases depending on the time during therapy at which the samples were obtained. In nearly all cases in which the results of the assay methods did not agree, minimal residual disease was detected in plasma but not in cell samples. Although these data need confirmation by studies with larger numbers of imatinib-treated CML patients, they suggest that minimal residual disease can be detected with greater sensitivity in plasma samples. Using plasma rather than cell samples provides a new dimension by potentially measuring tumor load through quantifying the number of BCR-ABL fusion mRNA in a unit volume of plasma, which most likely reflects the tumor load in the entire body rather than the relative percentage of tumor cells in the sample. As shown in Table 3, only absolute levels of BCR-ABL transcript in plasma (tumor load) showed strong correlations with various important laboratory findings (white blood cells, blasts, serum GOT, myelocytes, metamyelocytes, lymphocytes, and lactate dehydrogenase). These correlations indicate that quantitation of absolute levels of BCR-ABL mRNA in plasma provides information that the conventional cell-based assay does not.

Based on our previous work, we speculate that the source of BCR-ABL mRNA in cell-free plasma samples is most likely the dying tumor cells. This tumor-specific mRNA appears to be complexed with other proteins, because it is protected from RNase in plasma, whereas BCR-ABL mRNA purified from the K562 cell line is rapidly degraded when added to normal plasma (our unpublished experiments). By contrast, nucleic acids in normal cells undergoing apoptosis are mostly degraded

within the cells, and the apoptotic cells are cleared without lysis and release of their contents. If BCR-ABL mRNA in plasma is spilled from dying tumor cells, then its concentration should reflect tumor mass more accurately than BCR-ABL mRNA extracted from cells in peripheral blood samples, which may include both cells that are aggressively multiplying and those that are not aggressively multiplying. Consistent with this idea, we found a poor correlation between percentages of cells carrying the Philadelphia chromosome and BCR-ABL mRNA levels in plasma samples in untreated patients. We also found greater variability in BCR-ABL levels from plasma of untreated CML patients than from peripheral blood cell samples of the same patients, and a poor correlation between BCR-ABL levels from these two types of samples. Once patients are treated with imatinib, tumor mass decreases, and we observed that BCR-ABL mRNA levels detected in plasma and cells samples from imatinib-treated patients correlated well. Thus, results in this study are consistent with the hypothesis that plasma is enriched in tumor-specific RNA spilled by dying tumor cells, and that BCR-ABL mRNA levels measured in plasma samples reflect tumor mass. This could be clinically useful, for example, if patients with higher levels of BCR-ABL mRNA in plasma are found to respond better to higher doses of imatinib.

Finally, the use of plasma for RT-PCR analysis offers a simple means of standardization and reporting of test results. Potentially, all laboratories could express the results as relative BCR-ABL copy number per 10 μL of plasma. This would allow ready comparison of results among different studies, and provide clinicians with consistent test results upon which to base their diagnoses and clinical management of CML patients.

Authors Contributions

WM: performed testing; RT, MG and IJ: helped in performing testing; MK, HK, JC, SO'B and FG: provided clinical data and analyzed these data; MA: analyzed data, designed the experiments and wrote the article.

Conflict of Interest

The authors reported no potential conflicts of interest.

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