Comparison of bone marrow high mitotic index metaphase fluorescence *in situ* hybridization to peripheral blood and bone marrow real time quantitative polymerase chain reaction on the International Scale for detecting residual disease in chronic myeloid leukemia

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ABSTRACT

Background
Recently, an International Scale was proposed for standardizing *BCR-ABL* transcript measurements and reporting in the assessment of minimal residual disease by real-time quantitative polymerase chain reaction (RQ-PCR). Here we present the setting up of the International Scale conversion factors for a national laboratory by performing both a cross-analysis of a set of standard samples from a reference laboratory and an analysis of bone marrow and peripheral blood samples at diagnosis (from 32 and 27 patients, respectively).

Design and Methods
A total of 222 bone marrow and 173 peripheral blood mononuclear cell samples from 96 patients with chronic myeloid leukemia were analyzed with RQ-PCR according to Europe Against Cancer protocols. Additionally, 291 bone marrow samples were analyzed with high mitotic index metaphase fluorescence *in situ* hybridization (metaphase FISH).

Results
Major molecular response according to the International Scale in *BCR-ABL/GUS* transcript levels corresponded to a ratio of 0.035% in peripheral blood and 0.034% in bone marrow, yielding the same conversion factor of 2.86 for both types of sample. Based on metaphase FISH, values of 10%/-1.0 log, 1%/-2.0 log and 0.1%/-3.0 log on the International Scale, corresponded to 13%, 2%, and 0.3% of Philadelphia chromosome positive cells in bone marrow, respectively.

Conclusions
In conclusion, conversion factors can be determined either by cross-analyzing a number of samples with a laboratory that has already established the International Scale or utilizing sufficient numbers of reference samples from chronic myeloid leukemia patients at diagnosis, or using the upcoming international standards.

Key words: chronic myeloid leukemia, minimal residual disease, BCR-ABL, real time quantitative PCR, standardization

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The online version of this article contains a supplemental appendix.

Introduction

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder characterized by the Philadelphia chromosome and a disease-specific fusion gene, *BCR-ABL*, whose transcription results in a protein with constitutive tyrosine kinase activity.[1] Imatinib mesylate, developed as a selective BCR-ABL tyrosine kinase inhibitor, is the standard first-line treatment for patients with CML and induces stable minimal residual disease in a majority of patients.[2] The BCR-ABL fusion transcript is a marker for the detection of minimal residual disease during tyrosine kinase inhibitor treatment and after allogeneic hematopoietic stem cell transplantation. A reduction of >3 logarithm units of *BCR-ABL* transcripts compared to a standardized baseline (major molecular response) is associated with a prolonged progression-free survival.[3,4] During imatinib therapy, increasing levels of minimal residual disease herald the development of drug resistance and predict a forthcoming relapse of the disease.[5,6] Similarly, detection of even minute amounts of minimal residual disease after hematopoietic stem cell trasplantation indicates an insufficient response or relapse and often results in therapeutic intervention.[7]

Techniques used for monitoring minimal residual disease should be specific, sensitive and rapid to perform. Different techniques used in such monitoring measure different variables, e.g. the proportion of Philadelphia chromosome-positive cells in mitotic or interphase cells, or the presence and amount of *BCR-ABL* fusion mRNA. Fluorescence *in situ* hybridization (FISH) analyses which monitor the Philadelphia chromosome or *BCR-ABL* fusion gene have a specificity of 100% and, at their best, a sensitivity of 0.1%, when a high number of mitotic cells are analyzed (high mitotic index metaphase FISH).[8]

Real time quantitative polymerase chain reaction (RQ-PCR) analyses developed during the last years measure the quantity of *BCR-ABL* mRNA and thus enable more sensitive determination of residual disease. However, the RQ-PCR methodologies and choice of control genes vary between laboratories which complicates the comparison of results obtained from different laboratories. Recently, detailed recommendations on performing RQ-PCR have been presented aiming at world-wide standardization of the technique.[9,10] An International Scale was proposed for expressing RQ-PCR results in a uniform manner so that prognostically significant major molecular response is equivalent to 0.1% of *BCR-ABL*/reference gene transcript ratio as determined in the IRIS study.[10] After determining the laboratory-specific conversion factor, the conversion factor-multiplied results would be comparable world-wide. However, the reference gene to be used was not explicitly defined. *ABL*, *GUS* and *BCR* all remain acceptable alternatives. Laboratories can establish the International Scale conversion factors either (i) by analyzing a sufficiently large reference sample group of pretreatment CML samples or (ii) by analyzing a set of external reference standard samples with known minimal residual disease values obtained from a reference laboratory. Both approaches were employed in this study. For the first mentioned approach we used pretreatment samples from 32 Finnish CML patients. Based on those data, the International Scale conversion factor was determined for two national laboratories with identical methods and close inter-laboratory standardization. For the second approach we analyzed standardization samples prepared by a reference laboratory (Mannheim, Germany) that had determined a conversion factor for direct conversion and traceability of its minimal residual disease values to the values used in the IRIS study. We also compared minimal residual disease measurements performed using bone marrow and peripheral blood. Finally, we set out to assess the relation of the International Scale values to absolute leukemia tumor burden in the bone marrow as assessed by a high mitotic index FISH.

Design and Methods

Patients

A total of 96 CML patients treated in Helsinki and Turku University Hospitals were monitored for minimal residual disease during imatinib treatment or after allogeneic hematopoietic stem cell transplantation. The Philadelphia chromosome status was assessed by standard karyotyping using GTG-banding on 20 metaphases. All except one of the patients were Philadelphia chromosome-positive. The median number of analyzed follow-up samples per patient was two (range, 1-9). All the patients included in this study expressed a b3a2 or b2a2 type of *BCR-ABL* mRNA transcript. Patients expressing more rare b3a3 or b2a3 transcripts could not be followed up with the primer and probe combinations used in these RQ-PCR experiments. The bone marrow aspirate samples were taken according to normal clinical treatment protocols usually every 3 months during imatinib therapy. The study was conducted in accordance with the principles of the Helsinki declaration and was approved by the Helsinki University Central Hospital Ethics committee.

Metaphase FISH

Bone marrow aspirates were taken for metaphase FISH analyses. Cells were cultured according to standard methods,[11] and exposed to colcemid (0.1 μg/mL) for 17 hours to obtain a high number of metaphases. Metaphase-FISH studies were in most cases done using chromosome 22 painting probe (Cambio, Cambridge, UK). In 17 cases, including that of the Philadelphia chromosome-negative patient, follow-up studies were done using a locus-specific, dual color, dual fusion *BCR-ABL* probe mixture (Vysis, Downers Crove, IL, USA). All the hybridizations were performed according to the manufacturers' protocols. In most cases at least 500 to 1000 or more metaphases were analyzed to determine the level of cytogenetic response. If only minor or no response was observed, the number of analyzed cells was lower.

RNA isolation, reverse transcription and RQ-PCR

For RNA isolation the bone marrow or peripheral blood samples were collected into cell preparation tubes (BD Vacutainer® CPT™, Becton Dickinson New Jersey, USA) that enable the separation of mononuclear cells by centrifugation. The volume of blood collected was 2×8 mL and that of bone marrow 2×4 mL. The samples were centrifuged within 30 minutes of collection to separate the mononuclear cells from the granulocytes, including eosinophils containing eosinophil-derived neurotoxin, the major source of leukocyte RNase activity.[12] This enabled standard sample shipment without significant loss in RNA yield. From 6 to 10×10^6 mononuclear cells were used for RNA isolation. RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions. Both the reverse transcription reaction and RQ-PCR, including the used primer and probe sequences, were carried out according to standardized protocols published by the Europe Against Cancer program.[13] Plasmid standards obtained from Ipsogen (Marseille, France) were used for quantification of the sample transcripts. The PCR reactions, consisting of 50 amplification cycles, were performed on an ABI 7700 platform (Applied Biosystems). The baseline was set between cycles 3-15 and the threshold at 0.1 within the exponential growth region of the amplification curve.

β-glucuronidase (*GUS*) was used as the control gene. A cDNA preparation was considered degraded if the cycle threshold (Ct) value of *GUS* was over 28. These cases were excluded from the analysis. Amplification of the plasmids and *GUS* was performed in duplicate. *BCR-ABL* amplification was performed in triplicate. If two of the three triplicate amplifications were positive, the sample was considered positive and the number of transcripts was calculated as the average of the two positive wells. Likewise, if two of the three triplicates amplifications were negative, the sample was regarded as negative. RQ-PCR negativity was defined as no detectable *BCR-ABL* transcripts in a sample having an acceptable level of *GUS* (Ct <28). All samples were processed within 24 h of collection.

In the two laboratories participating in this study, Helsinki and Turku University Hospitals, the methods and instrumentation used were identical for both FISH and RQ-PCR. Congruence of the analysis methods is ensured by regular exchange of quality control samples.

Determining the conversion factor with standardization samples from the reference laboratory

In order to compare the RQ-PCR results and the level of minimal residual disease in our follow-up cases that corresponds to major molecular response in the International Scale, external standardization samples obtained from the Mannheim reference laboratory were studied. These samples were prepared as follows. Three leukocyte samples from patients expressing b3a2 *BCR-ABL* transcripts were diluted with *BCR-ABL*-negative white blood cells in the reference laboratory. The diluted samples consisted of four different levels of minimal residual disease so that altogether 12 samples were sent for analysis. The samples were shipped frozen in Trizol® RNA stabilization solution. RNA was then isolated from these samples by the Trizol® method according to the manufacturer's instructions. The reverse transcription and the RQ-PCR were done as described above. Both the reverse transcription-reaction and RQ-PCR were repeated at two different time points so that all the variables in the analysis could be controlled. The conversion factor based on these results was calculated in Mannheim according to published procedures.[14]

Statistical analyses

Correlations between different follow-up analyses were defined by calculating the Spearman's rank order correlation (r_s). The statistical significance of differences was assessed with the Mann-Whitney U non-parametric test for continuous variables and with Fisher's exact test for categorical variables. All calculations were done with SPSS version 14.0.1 for Windows software.

Results

The study population consisted of 96 CML patients treated in Helsinki and Turku University Hospitals during 2002-2006 and who were monitored for minimal residual disease during imatinib treatment or after allogeneic hematopoietic stem cell transplantation. The median number of follow-up samples per patient was two (range, 1-9).

Diagnostic samples

Thirty-two patients had a bone marrow sample taken at the time of diagnosis. The median *BCR-ABL/GUS* ratio calculated from these samples was 34% (range, 11-106%). The number of diagnostic peripheral blood samples was 27 and the median *BCR-ABL/GUS* ratio was 35% (range, 8-80%). No statistical difference was observed between the diagnostic *BCR-ABL/GUS* ratios in the bone marrow and in the peripheral blood. At diagnosis the cytogenetic studies were performed with conventional G-banding and metaphase FISH. The proportion of Philadelphia chromosome-positive or *BCR-ABL* fusion positive cells varied between 90-100%. We next studied the relation between the level of *BCR-ABL* transcripts in the bone marrow at diagnosis and selected prognostic factors. The patients were divided into two groups based on the median *BCR-ABL/GUS* ratio at diagnosis (high: ratio >0.34; low: ratio <0.34). No significant differences were found between the two groups and the factors studied (*Table in supplementary data*). No significant associations were either found between peripheral blood transcript levels and the factors described above (*data not shown*). For follow-up samples we calculated the $\log_{10}$-reduction value as compared to the diagnostic sample median using the formula: $0-[\log_{10}(\text{median of diagnostic } BCR\text{-}ABL/GUS \text{ ratio}) - \log_{10}(\text{follow-up } BCR\text{-}ABL/GUS \text{ ratio})]$. Our laboratory median differed significantly from that reported by the Europe Against

Cancer (0.35 *vs.* 0.22 for peripheral blood and 0.34 *vs.* 0.12 for bone marrow). In the following, we only report log-reductions based on our own laboratory baseline data.

Metaphase FISH

For the assessment of the proportion of Philadelphia chromosome-positive cells in bone marrow, we used a highly sensitive FISH analysis performed on a large number of cells (median 540 cells) in metaphase. A total of 291 metaphase FISH studies were performed on follow-up bone marrow samples (Table 1). Complete and near complete cytogenetic responses were determined by the analysis of a median of 792 metaphase cells (range 6-1300).

RQ-PCR on peripheral blood and bone marrow samples

Out of 173 RQ-PCR analyses on peripheral blood 128 were positive (including 27 samples taken at the time of diagnosis). Out of 222 RQ-PCR analyses on bone marrow, 150 were positive (including 32 samples taken at the time of diagnosis). A majority of the RQ-PCR-negative samples were from patients who had undergone allogeneic hematopoietic stem cell transplantation.

Comparison of simultaneous bone marrow metaphase FISH and peripheral blood RQ-PCR minimal residual disease analyses

Out of 132 simultaneous bone marrow metaphase FISH and peripheral blood RQ-PCR samples, 62 gave positive results in both analyses. Results correlated well with each other (rs 0.769, $p<0.001$) (Figure 1). In 30 metaphase FISH-negative cases *BCR-ABL* transcripts were still detectable by RQ-PCR. There were no pairs of samples that were RQ-PCR-negative and FISH-positive. In 40 samples both metaphase FISH and peripheral blood RQ-PCR gave negative results. The patients in complete cytogenetic remission had *BCR-ABL/GUS* values of 0.37% or less which corresponds to >2.0 units of log reduction from the laboratory median baseline value. Most (94%) patients in complete cytogenetic remission had values >2.3 logs below the baseline value, which therefore may be regarded as the peripheral blood RQ-PCR molecular counterpart of metaphase FISH negativity. A ≥3 log reduction from the laboratory median baseline corresponded to 0.035% of *BCR-ABL/GUS* transcript ratio in peripheral blood (Table 2).

Table 1. Cytogenetic results based on bone marrow metaphase FISH analyses in 291 follow-up samples from 82 patients.

Cytogenetic results (% of Ph positive cells)	*Number of samples (%)*	*Median number of analyzed metaphases (range)*
0%	167 (57)	804 (6-1300)
<1%	35 (12)	792 (178-1028)
1-34.9%	48 (17)	200 (41-1013)
35-95%	24 (8)	50 (20-360)
>95%	17 (6)	40 (10-102)
Total	291 (100)	540 (6-1300)
Minimal residual disease*	202 (62)	792* (6-1300)

The median number of analyzed metaphases in samples with none or <1% of residual cells (minimal residual disease setting) is marked with an asterisk.

Comparison of concurrent bone marrow metaphase FISH and RQ-PCR minimal residual disease analyses

A total of 181 metaphase FISH analyses and RQ-PCR assays were performed on concurrent bone marrow samples. Bone marrow metaphase FISH and RQ-PCR analyses were consistently positive in 68 cases. The Spearman's rank correlation coefficient was 0.91 ($p<0.001$) between these samples (Figure 1). RQ-PCR detected *BCR-ABL* transcripts in 44 cases which were in complete cytogenetic remission by FISH analyses. Both complete cytogenetic and molecular responses were observed in 69 cases. The number of analyzed cells in metaphase FISH studies and median reductions in *BCR-ABL* expression in each response group are shown in Table 3. All patients in complete cytogenetic remission had *BCR-ABL/GUS* values of 0.25% or less, which correspond to >2.2 units of log reduction from the laboratory median pretreatment baseline of 34%. Most (97%) patients in complete cytogenetic remission had values >2.6 logs below the baseline value. Major molecular response (≥3 log reduction of *BCR-ABL* transcript) corresponded to 0.034% of bone marrow *BCR-ABL/GUS* transcript ratio.

Comparison of concurrent RQ-PCR in bone marrow and peripheral blood

A total of 104 concurrent bone marrow samples and peripheral blood RQ-PCR samples were available for comparison (Table 4). Altogether 66 follow-up sample pairs gave positive results for both samples with a correlation of 0.82 ($p<0.001$, Figure 2, Panel A). Twenty-five pairs were negative for both samples, and all of these were also negative in metaphase FISH (median 979 metaphase cells analyzed; range, 6-1300).

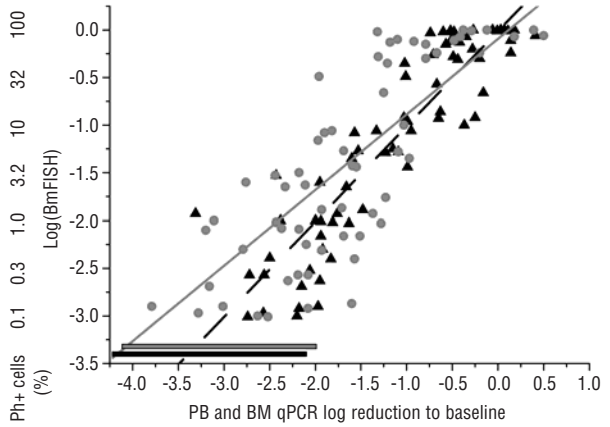

Figure 1. Correlation in positive follow-up pairs of sample between bone marrow (BM) metaphase FISH and RQ-PCR in peripheral blood (PB) (solid gray line and circles) and BM (dashed black line and triangles). The horizontal lines represent the range of log_{10} (*BCR-ABL/GUS*) ratios in PB (gray) and BM (black) in cases which concomitant metaphase FISH analysis indicated complete cytogenetic remission. The equations and the non-parametric Spearman's rank correlation coefficients obtained from these data are for metaphase FISH *vs.* PB RQ-PCR y=0.79x –0.09, rS 0.76 ($p<0.001$) and for metaphase FISH *vs.* BM RQ-PCR y=0.9996x –0.01, rS 0.90 ($p<0.001$).

Table 2. Comparison of cytogenetic response in bone marrow and molecular response in peripheral blood (PB) defined as log reduction to laboratory or Europe Against Cancer (EAC) median values.

Cytogenetic response	*Samples*	*Median number of analyzed cells (range)*		*Median PB BCR-ABL/GUS ratio log reduction (range)*		*Median PB BCR-ABL/GUS –ratio % (range)*
				Laboratory median ratio 0.35	*EAC median ratio 0.22*	
Complete (0%)	40	1000	(6–1300)		no detectable transcripts	0.000
	30	743	(43–1011)	-2.99 (-4.31 – -1.98)	-2.79 (-4.11 – -1.78)	0.036 (0.002–0.368)
Near complete (<1%)	22	792	(178–1028)	-2.24 (-3.79 – -1.28)	-2.04 (-3.59 – -1.08)	0.201 (0.006–1.819)
Partial (1–34.9%)	22	238	(50–1013)	-1.86 (-3.51 – -0.97)	-1.66 (-3.31 – -0.76)	0.487 (0.011–3.791)
Minor (35–95%)	13	50	(20–155)	-0.78 (-1.31–0.5)	-0.58 (-1.11–0.70)	5.741 (1.723–109.515)
None (>95%)	5	40	(10–52)	-0.29 (-1.32–0.39)	-0.09 (-1.12–0.59)	18.000 (1.676–84.997)
Total	132	556	(6–1300)	-2.13	-1.93	0.067

rs: Spearman's rank correlation coefficient for positive metaphase FISH and PB RQ-PCR follow-up samples was 0.77 (p<0.001). The log reduction was calculated to the laboratory or EAC median BCR-ABL/GUS ratio, respectively.

Table 3. Comparison of cytogenetic and molecular response in bone marrow (BM) defined as log reduction to laboratory or Europe Against Cancer (EAC) median values.

Cytogenetic response	*Samples*	*Median number of analyzed cells (range)*		*Median BM BCR-ABL/GUS ratio log reduction (range)*		*Median BM BCR-ABL/GUS –ratio % (range)*
				Laboratory median ratio 0.34	*EAC median ratio 0.12*	
Complete (0%)	69	902	(6–1300)	no detectable transcripts		0.000
	44	794	(51–1015)	-3.12 (-5.31 – -2.13)	-2.67 (-4.85 – -1.68)	0.026 (0.0002–0.251)
Near complete (<1%)	17	756	(314–1028)	-2.06 (-2.74 – -1.63)	-1.61 (-2.29 – -1.18)	0.294 (0.061-0.789)
Partial (1–34.9%)	26	175	(41–1013)	-1.19 (-3.31 – -0.16)	-0.74 (-2.86–0.30)	2.217 (0.017–23.709)
Minor (35–95%)	16	57	(20–360)	-0.46 (-1.02–0.41)	-0.01 (-0.57–0.87)	11.836 (3.263–88.076)
None (>95%)	9	50	(10–102)	-0.01 (-0.60 – 0.18)	0.45 (-0.15–0.63)	33.086 (8.564–51.475)
Total	181	500	(6–1300)	-2.03	-1.58	0.018

rs: Spearman's rank correlation coefficient for positive metaphase-FISH and BM RQ-PCR follow-up samples was 0.91 (p<0.001). The log reduction was calculated to the laboratory or EAC median BCR-ABL/GUS ratio, respectively.

The median levels of *BCR-ABL/GUS* transcript levels in both bone marrow and peripheral blood were very similar both at diagnosis and at follow-up (Figure 2, Panel A) facilitating the use of the same conversion factor irrespective of the type of sample. There did, however, appear to be two groups of patients with a consistent difference between bone marrow and peripheral blood values in repeated follow-up samples: one group had higher transcript concentrations in peripheral blood than in bone marrow (Figure 2, Panel B, red circle) while in the other group it was the other way round (Figure 2, Panel B, blue circle). Examples of individual patients' data are shown in *Supplementary data, Figure 1*. For many patients, the difference in bone marrow and peripheral blood transcript

Table 4. Sensitivity comparison between parallel bone marrow and peripheral blood RQ-PCR analyses.

	PB negative	*%*	*PB positive*	*%*	*Total*	*%*
BM negative	25	24	3 (-3.47)	2.9	28	26.9
BM positive	10 (-3.86)	9.6	66 (BM RQ-PCR -1.95) (PB RQ-PCR -2.10)	63.5	76	73.1
Total	35	33.7	69 r_s 0.816 (p<0.001)	66.3	104	100

The median log reduction to laboratory baseline is indicated below in parentheses. PB: Peripheral blood; BM: Bone marrow

ratios remained constant during follow-up and thus may imply a difference in disease biology. However, no apparent discrepancy in prognostic variables or response to tyrosine kinase inhibitor therapy was observed in these two groups of patients (*data not shown*). This disparity was due to differences in *BCR-ABL* transcript values, not due to differences in *GUS* control gene expression (*data not shown*). As regards PCR negativity, discordant results between bone marrow and peripheral blood RQ-PCR analyses occurred in 13 cases. All of them had only a marginal amount of minimal residual disease, as the log reduction varied between -2.97 and -4.04. In 10/35 (29%) peripheral blood RQ-PCR negative samples, bone marrow RQ-PCR showed a positive signal. The opposite situation (bone marrow negative – peripheral blood positive), was observed in 3/28 (11%) pairs of sample. Concomitant metaphase FISH results, when available (n=12) were all negative (median 838 metaphase cells analyzed; range, 73-1012). These results imply that bone marrow RQ-PCR may be more sensitive in detecting minimal residual disease, but the difference is small.

Conversion of RQ-PCR data to the International Scale

We next wanted to convert the RQ-PCR data to the International Scale, as recently proposed[10] and determine the link between International Scale values and disease tumor burden as assessed by FISH on the bone marrow. As we had sufficient numbers of both baseline diagnostic and follow-up samples, we could reliably calculate the log-reduction from the baseline for follow-up samples. The International Scale has two anchor points: the baseline value is defined as International Scale 100% and a 3 log reduction (major molecular response) is defined as International Scale 0.1% (*Supplementary data Figure 2*). In our data, a 3 log reduction in *BCR-ABL/GUS* transcript levels corresponded to a ratio of 0.035 in peripheral blood and 0.034 in bone marrow, yielding a conversion factor of 2.86. Furthermore, our *BCR-ABL/GUS* ratios were also compared to the *BCR-ABL/BCR* and *BCR-ABL/ABL* ratios on the International Scale using standardization samples prepared at the reference laboratory in Mannheim. The conversion factor based on these samples was calculated in Mannheim. The correlation of the results on these standardization samples was linear, R^2 0.9947, and the derived preliminary conversion factor to the International Scale was 1.8446.

By using the high mitotic index FISH analysis based on several hundred analyzed metaphases, we constructed a regression model to predict the percentage of Philadelphia positive cells in bone marrow from values on the International Scale (*Table 5 and Supplementary Data, Figure 3*). Overall, the International Scale percentage values corresponded closely to tumor burden as assessed by bone marrow FISH. Based on regression analysis, values of 10%/-1.0 log, 1%/-2.0 log and 0.1%/-3.0 log on the International Scale, corresponded to 13%, 2%, and 0.3% of Philadelphia chromosome-positive cells in bone marrow, respectively.

Discussion

During imatinib therapy more than 70% of patients achieve complete cytogenetic remission according to conventional karyotyping.[15] However, many patients remain Philadelphia chromosome-positive according to FISH and particularly RQ-PCR analyses.[16-18] A major molecular response, exceeding 3 logs of *BCR-ABL* transcript reduction from the reference baseline in RQ-PCR studies, has been associated with an excellent progression-free survival.[3,4] Increasing levels of *BCR-ABL* transcripts may indicate loss of response to imatinib or relapse after allogeneic hematopoietic stem cell transplantation.[5,19] Thus, sensitive monitoring of minimal residual disease during tyrosine kinase inhibitor therapy or after transplantation is of major importance for CML patients. We found an excellent correlation between bone marrow metaphase FISH and bone marrow or peripheral blood RQ-PCR. In particular, the bone marrow RQ-PCR reflected closely reflected the percentage of Philadelpha chromosome-positive cells in bone marrow as assessed by FISH (r_S=0.91, p<0.001). Significant concordance between BM and peripheral blood assays has also been reported in previous studies.[17,20,21] Schoch *et al.* compared several techniques for detecting minimal residu-

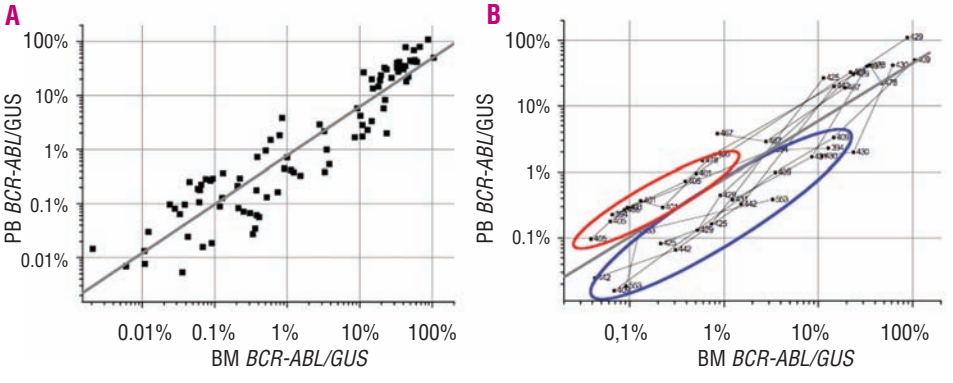

Figure 2. Correlation between positive follow-up pairs of bone marrow (BM) and peripheral blood (PB) samples assessed with RQ-PCR. The equation obtained from these data is: y= 0.74x – 0.70 (gray line) and the Spearman's rank correlation coefficient r_S 0.84 (p<0.001). A: all data points. B: individual patients' data (with the patient number shown) with at least three samples available. In panel B, individuals with PB RQ-PCR ratios consistently above or below the regression line are marked with red and blue, respectively.

Table 5. Correspondence of the International Scale to the percentage of Philadelphia chromosome positive cells in bone marrow as assessed by FISH.

	International Scale	*Ph-positive metaphases in Bone marrow*
Log	%	%
0.0	100	81.1
-0.3	50	46.8
-1.0	10	13.0
-1.3	5	7.5
-2.0	1	2.1
-2.3	0.5	1.2
-3.0	0.1	0.33
-3.3	0.05	0.19
-4.0	0.01	0.05

Bone marrow percentage values were calculated according to the equation shown in Figure 3 in the supplementary data.

dal disease and found a highly significant correlation between all of them.[22] Hypermetaphase FISH analysis was found to be more sensitive as it detected Philadelphia chromosome-positive cells in 20% of cases in complete cytogentic remission according to standard karyotyping or in 16% of interphase FISH negative cases. PCR-based techniques were even more sensitive. In confirmation, our analyses indicated that 43% of bone marrow metaphase FISH-negative cases were positive by RQ-PCR, with a median of 3.0 log reduction of *BCR-ABL/GUS* ratio from the reference baseline in these patients. Bone marrow metaphase FISH-negative, bone marrow RQ-PCR positive samples constituted 39% of metaphase FISH negative cases and had a 3.1 log decline in transcript levels from the reference baseline. Three reference (housekeeping) genes have been deemed suitable for RQ-PCR analyses: *ABL*, *BCR,* and *GUS*.[10] We chose GUS as the control gene for our laboratories, in line with a recent recommendation.[23] Primers for the most commonly used control gene, *ABL*, also amplify *BCR-ABL*, which prevents a meaningful determination of high *BCR-ABL/ABL* ratios at diagnosis. Because of ABL and BCR-ABL co-amplification, linearity of the assay is affected at residual disease levels >10%. Using GUS as the reference gene enables more precise evaluation of the initial transcript ratio in pretreatment samples and the median BCR-ABL/GUS ratio in a reference population of untreated CML patients can be determined in a similar way as the BCR-ABL/BCR ratio was determined in the IRIS study.[3] Our analysis of diagnostic samples showed *BCR-ABL/GUS* expression ratios at least two times higher than those reported by the Europe Against Cancer from a slightly lower number of untreated reference patients.[13] We, therefore, propose that the median *BCR-ABL/GUS* ratio at diagnosis for inter-laboratory and intra-laboratory use should be revised and based on a sufficiently large number of patients. No evident correlation of the individual baseline transcript ratio with any known prognostic factors or therapy response was detected.

The preliminary conversion factor of 1.8446 based on the analysis of the Mannheim standardization samples is 35% lower than the conversion factor of 2.86 which we obtained using our own reference samples from untreated CML patients. A difference of 35% is still within the analytical imprecision limits of single RQ-PCR analyses, but significant when based on the analysis of several samples. It is possible that the reference CML patients in this study differed slightly from the reference group of untreated CML patients in the IRIS study. Some additional factors may also have contributed to the observed difference, such as the use of the mononuclear cell fraction instead of total leukocytes after red blood cell lysis, and differences in the methods used for RNA isolation. Such factors may also affect the sensitivity of the RQ-PCR assay. The reference group of untreated CML patients was about 30 patients both in this study and in the IRIS study, but the set of standardization samples consisted of dilutions from only three CML patients. If these patients had persistent differences in their *ABL, GUS* or *BCR* expression, it may have affected the conversion factors between different reference genes. In the latter case the ongoing further validation using 25 samples from different CML patients may bring the conversion factor closer to the factor derived from data of local untreated CML patients. Overall, the transcript levels were very similar in bone marrow and peripheral blood. However, when comparing individual transcript data, we curiously found that the relation of bone marrow to peripheral blood transcript ratio remained remarkably constant during follow-up: some patients had constantly higher levels of *BCR-ABL* transcripts in peripheral blood than in bone marrow and in other patients the reverse was true. Similar results were found when comparing peripheral blood RQ-PCR to bone marrow FISH reflecting a true difference in relative tumor burden. In a few patients, the difference was substantial (>2 logs, *data not shown*), but for most patients it was in the range of ±0.5 log. The dependence of the transcript level on the cell source has been noted earlier[9] but the biological cause remains obscure. However, in selected patients this difference may have an effect on estimates of minimal residual disease and should be taken into account. Additionally, in these patients the follow-up should preferably be based on one type of sample only. Further studies focusing on this inter-individual variation in *BCR-ABL* transcript level in different cellular compartments is warranted, as it may reflect significant disparities in CML pathobiology between patients.

As major molecular response, a ≥3 log reduction from a standard reference baseline, has become a widely accepted clinical landmark, it is important that clinical laboratories can assess and report their RQ-PCR results in a similar fashion as in the original studies.[3,4] Recently, an important consensus effort was reported aiming at harmonizing different methodologies used in assessing minimal residual disease by RQ-PCR.[9,10] Central to the recommendation was the establishment of the International Scale, which has two anchor points: the reference (diagnostic) baseline level represents 100% on the International Scale, and a major molecular response is fixed at 0.1%. With these two refer-

ence points, each laboratory can calculate a conversion factor to convert *BCR-ABL*/reference gene transcript ratios to the International Scale. As three different reference genes are allowed, it would be important to examine the median expression level of each reference gene from a sufficiently large patient population and use this knowledge when converting *BCR-ABL* RQ-PCR results between different reference genes.

We conclude that a significant overall correlation between results of concomitant peripheral blood and bone marrow samples enables peripheral blood to be the first choice as the source of sample for minimal residual disease analysis. These analyses may be complemented with metaphase FISH and conventional cytogenetics from bone marrow samples. We strongly support the use of the International Scale for standardizing and reporting minimal residual disease results as assessed by RQ-PCR.

Authorship and Disclosures

TLu, VK, SK and KP contributed to the conception and design of the study. TLu, VJ, MM, TL and KP analyzed and interpreted the data, TLu wrote the article which was critically revised for important intellectual content by VJ, SM, VK, AH, SK and KP. All authors were approved the version to be published. The authors reported no potential conflicts of interest.

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