Leukemia (1999) 13, 1825–1832

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Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real time quantitative RT-PCR

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We sought to establish a rapid and reliable RT-PCR approach for detection and quantification of BCR-ABL fusion transcripts using the LightCycler technology. This device combines rapid thermocycling with online detection of PCR product formation and is based on the fluorescence resonance energy transfer (FRET) between two adjacent hybridization probes carrying donor and acceptor fluorophores. A pair of probes was designed that was complementary to ABL exon 3, thus enabling detection of all known BCR-ABL variants and also normal ABL as an internal control. Conditions were established to amplify less than 10 target molecules/reaction and to detect one CML cell in 10^5 cells from healthy donors. To determine the utility of the assay, we quantified BCR-ABL and ABL transcripts in 254 samples (222 peripheral blood, 32 bone marrow) from 120 patients with CML after therapy with IFN-α ($n = 219$), allogeneic BMT ($n = 17$), chemotherapy ($n = 11$), or at diagnosis ($n = 7$). The level of residual disease in the 245 BCR-ABL positive specimens was expressed as the ratio of BCR-ABL/ABL. This ratio was compared to results obtained by three established methods from contemporaneous specimens. A highly significant correlation was seen between the BCR-ABL/ABL ratios determined by the LightCycler and (1) the BCR-ABL/ABL ratios obtained by nested competitive RT-PCR ($n = 201$, $r = 0.90$, $P < 0.0001$); (2) the proportion of Philadelphia chromosome positive metaphases determined by cytogenetics ($n = 81$, $P < 0.0001$); and (3) the BCR ratio determined by Southern blot analysis ($n = 122$, $P < 0.0001$). We conclude that real-time PCR with hybridization probes is a reliable and sensitive method to monitor CML patients after therapy. The major advantages of the methodology are (1) amplification and product analysis are performed in the same reaction vessel, avoiding the risk of contamination; (2) the results are standardized by the quantification of housekeeping genes; and (3) the complete PCR analysis takes less than 60 min.

Keywords: chronic myelogenous leukemia; real-time PCR; minimal residual disease; quantitative PCR

Introduction

In many ways, chronic myelogenous leukemia (CML) serves as a paradigm for the utility of molecular methods to diagnose malignancy or to monitor patient response to therapy.[1] Several approaches have been introduced that can specifically detect the t(9;22) or its products, such as fluorescent *in situ* hybridization, Southern blotting, Western blotting, and RT-PCR (for reviews, see Refs 2–4). Of these, RT-PCR for BCR-ABL mRNA is by far the most sensitive and consequently has received the most attention in the context of minimal residual disease.

Since non-quantitative RT-PCR analysis gives only limited information, several groups have developed quantitative or semi-quantitative RT-PCR assays that enable the kinetics of residual BCR-ABL transcripts to be monitored over time in patients after allogeneic stem cell transplantation or interferon-α (IFN-α) therapy. Currently, the most common method to quantify BCR-ABL transcripts is by competitive PCR. Although this method has been established in a number of laboratories, it is cumbersome and requires large numbers of individual amplification reactions to be performed for each data point.

Recently, however, novel real-time PCR procedures have been developed that promise to simplify existing protocols. A procedure for quantification of BCR-ABL mRNA using the TaqMan system has been described.[5,6] Here we have established an alternative real-time RT-PCR approach for detection and quantification of BCR-ABL fusion transcripts using the new LightCycler technology,[7,8] which combines rapid thermocycling with online fluorescence detection of PCR product formation as it occurs. Fluorescence monitoring of PCR amplification is based on the concept of fluorescence resonance energy transfer (FRET) between two adjacent dyes. Excitation of a donor fluorophore with an emission spectrum that overlaps the excitation spectrum of an acceptor fluorophore results in nonradioactive energy transfer to the acceptor. Fluorescein is used as a donor fluorophore, LC Red640 (Roche Diagnostics, Mannheim, Germany) as the acceptor fluorophore. Once conditions are established, the amount of fluorescence resulting from the two probes is proportional to the amount of PCR product. We demonstrate that hybridization probes in combination with the LightCycler enables rapid and robust quantification of BCR-ABL and control transcripts in clinical specimens.

Patients and methods

Patients and samples

A total of 254 (222 peripheral blood (PB) and 32 bone marrow (BM) samples from 120 CML patients (76 male; 44 female) were analyzed. Between one and nine samples were investigated from each patient during the course of their disease. Informed consent was obtained as required by the Declaration of Helsinki. At diagnosis, 117 patients were Ph-positive and three Ph-negative/BCR-ABL-positive. 219 samples were obtained on or after therapy with IFN-α, 17 after allogeneic BMT, 11 after chemotherapy, and seven at diagnosis.

Cytogenetic analysis

Cytogenetic studies were performed on BM aspirates according to standard protocols. Metaphases from direct and/or short-term (24-h or 48-h) cultures were examined after Giemsa banding.[9] The cytogenetic response was evaluated as follows: complete, 0% Ph+ metaphases; partial, 1 to 34% Ph+ metaphases; minor, 35 to 94% Ph+ metaphases; and none,

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Received 16 June 1999; accepted 26 July 1999

>94% Ph+ metaphases.[10] A minimum of 10 metaphases were analyzed. A total of 81 RT-PCR results obtained by the LightCycler were available for comparison with contemporaneous (±3 months) cytogenetic analyses.

Southern blot analysis

Southern blotting was performed as reported.[11,12] DNA obtained from PB or BM was digested with *Bgl*II, *Eco*RI, *Bam*HI, *Xba*I, and/or *Hin*dIII, electrophoresed on a 0.7% agarose gel, blotted, and hybridized to the 2 kb *Bgl*II/*Hin*dIII (5′M-bcr) and 1.2 kb *Hin*dIII/*Bgl*II (3′M-bcr) probes. Response was expressed as complete (BCR ratio 0%), partial (1–24%), minor (25–49%) and none (⩾50%).[12]

RNA extraction, cDNA synthesis

Total leukocyte RNA was extracted from 10 to 20 ml of PB and/or from 1 to 5 ml of BM aspirate after lysis of red blood cells.[13] Samples were processed as soon as possible after aspiration, although some spent 1 to 3 days in transit. RNA extraction was performed by CsCl gradient centrifugation[13] or by commercially available extraction kits. RNA was reverse transcribed using random hexamer priming and MMLV reverse transcriptase as described.[13] cDNAs were stored at –20°C.

Competitive PCR

The type of BCR-ABL transcript was determined by multiplex PCR[14] or nested PCR as described.[13,15] An estimate of the number of BCR-ABL transcripts and total ABL transcripts as an internal standard was made by a competitive PCR titration assay.[15–17] The final result of quantification by competitive PCR was expressed as the ratio between BCR-ABL and ABL transcripts in percentage.

Real-time quantitative PCR

PCR was performed using 2 μl mastermix (LightCycler DNA Master Hybridization Probes; Roche Diagnostics, Mannheim, Germany, containing buffer, dATP, dCTP, dGTP, dUTP, and *Taq* polymerase); 4 mM $MgCl_2$, 0.25 μM of each 3′ and 5′ fluorescent hybridization probes (TIB MolBiol, Berlin, Germany), 0.5 μM of each 3′ and 5′ oligonucleotide primer (highly purified salt free (HPSF) grade, MWG, Ebersberg, Germany), 1 U heat labile uracil DNA glycosylase (UDG, Roche Diagnostics), 2 μl cDNA, and water to a final volume of 20 μl. Prior to amplification, mixes were incubated for 5 min at room temperature to allow degradation of specific contaminating PCR products from previous amplifications by UDG. Heat labile UDG was deactivated by an initial denaturation step of 1 min at 95°C. Amplification occurred in a three-step cycle procedure (denaturation, 95°C, 1 s, ramp rate 20°C/s; annealing 64°C, 10 s, ramp rate 20°C/s; and extension 72°C, 26 s, ramp rate 2°C/s) for 45 cycles. The following primers were used for amplification: b2a2, b3a2, b2a3, or b3a3 BCR-ABL, primers B2A and NA4-; e1a2 BCR-ABL, primers PE1+ and NA4–; ABL, primers A2N and NA4-; glucose-6-phosphate dehydrogenase (G6PD), primers G6PD1 and G6PD2 (Figure 1, Table 1). Sequences of the probes have been selected empirically using the recommendations of the manufacturer. Amplification, fluorescence detection, and post-processing calculations were performed using the LightCycler apparatus (Roche Diagnostics).

5′ Hybridization probes were labeled with fluorescein, 3′ probes with LC Red640. Fluorescein is excited by blue light (470 nm) and emits green light at a slightly longer wave length. If the two dyes are in close proximity (ie after co-hybridization of the two oligonucleotide probes to the target sequence), the energy emitted by the fluorescein excites the LC Red640 which then emits red fluorescent light (640 nm). Fluorescence was measured after each annealing step and expressed as the ratio between fluorescence at 640 nm (designated F2) and at 530 nm (background, designated F1).

The fluorescence signal was plotted against the cycle number for all samples and external standards. These standards consisted of serial dilutions (10^1 to 10^7 molecules per reaction) of plasmids pGD210 ($b3a2^{BCR\text{-}ABL}$), pB190 ($e1a2^{BCR\text{-}ABL}$), and pGdBBX (G6PD). Initially, the crossover point was determined for each standard dilution, ie the point at which the signal rose above the background level. The higher the initial number of starting molecules, the earlier the signal appears above the background (Figure 2). A standard curve for each run was constructed by plotting the crossover point against the log (number of standard molecules). The number of target molecules in each sample was then calculated automatically by reference to this curve. Results were expressed initially as the number of target molecules/2 μl cDNA. Normalized levels of disease were calculated as the ratios (expressed as percentage) between BCR-ABL and ABL and between BCR-ABL and G6PD transcripts in 2 μl of cDNA.

Some amplification products performed in the LightCycler were checked by electrophoresis on 1.5% ethidium bromide stained agarose gels. The estimated size of the amplified fragments matched the calculated size[18,19] for b3a2 BCR-ABL (671 bp), b2a2 BCR-ABL (596 bp), b3a3 BCR-ABL (497 bp), b2a3 BCR-ABL (422 bp), e1a2 BCR-ABL (540 bp), ABL (386 bp), and G6PD (343 bp) in all cases.

Statistical analysis

The ratios BCR-ABL/ABL and BCR-ABL/G6PD were compared with the proportion of Ph+ metaphases for all samples that had contemporaneous cytogenetic analyses. The comparison of the BCR-ABL/ABL and BCR-ABL/G6PD ratios between the response groups determined by cytogenetics or Southern blot analysis was made using the Kruskal–Wallis test. The correlation between ABL and G6PD transcript numbers as internal controls, between the ratios BCR-ABL/ABL derived from competitive PCR *vs* real-time PCR, and between paired results from PB and BM was evaluated using Spearman's rank correlation coefficient. The equation of the regression line of ABL and G6PD transcript numbers and results from PB and BM were calculated by least-squares analysis. Results from contemporaneous PB and BM samples were compared by the paired Student's *t*-test.

Results

Sensitivity of real-time PCR

In repeated tests, we could reliably amplify 10 b3a2 or e1a2 BCR-ABL, 10 ABL, and 10 G6PD molecules per reaction. To

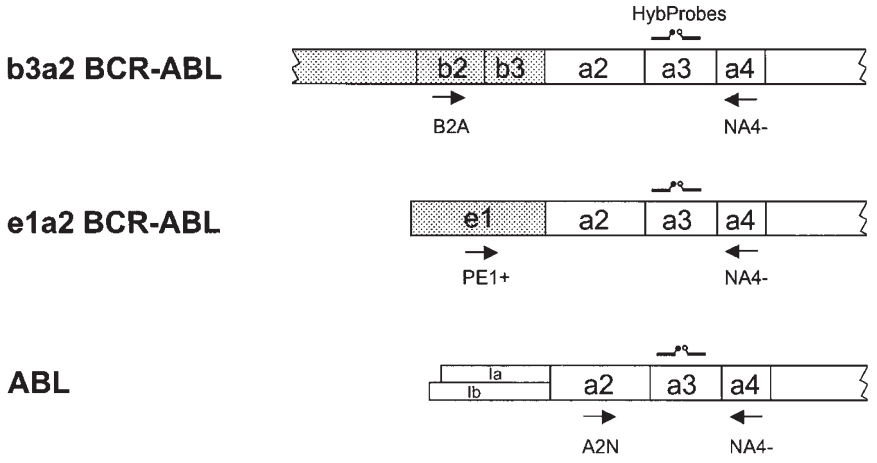

Figure 1 Schematic map of the amplification and detection of BCR-ABL and ABL transcripts. Specific primer pairs were used to amplify various types of BCR-ABL and ABL transcripts. The detection format was uniform using a pair of adjacent fluorescent labeled hybridization probes to ABL exon 3.

Table 1 Oligonucleotides used in the assay

		Mapping
Primers		
B2A	5′ TTCAGAAGCTTCTCCCTGACAT	BCR exon b2[16]
A2N	5′ CCCAACCTTTTCGTTGCACTGT	ABL exon 2[15]
PE1+	5′ CAGATCTGGCCCAACGATGG	BCR exon e1
G6PD1	5′ CCGGATCGACCACTACCTGGGCAAG	G6PD exon 6[17]
G6PD2	5′ GTTCCCCACGTACTGGCCCAGGACCA	G6PD exon 9[17]
NA4–	5′ CGGCTCTCGGAGGAGACGTAGA	ABL exon 4
Hybridization probes		
a3-3′HP	5′ LC Red 640-AATGGGGAATGGTGTGAAGCCCAAA-P	ABL exon 3
a3-5′HP	5′ TGAAAAGCTCCGGGTCTTAGGCTATAATCA-F	ABL exon 3
G6PD-3′HP	5′ LC Red 640-CAAATCTCAGCACCATGAGGTTCTGCAC-P	G6PD exon 7/6
G6PD-5′HP	5′ GTTCCAGATGGGGCCGAAGATCCTGTTG-F	G6PD exon 7

F, fluorescein; P, phosphate; LC Red 640, LightCycler fluorescence dye 640.

Table 2 Quantification of BCR-ABL, ABL and glucose-6-phosphate dehydrogenase (G6PD) transcripts using real-time PCR as compared to the ratio BCR-ABL/ABL determined by competitive PCR

	Real-time PCR					*Competitive PCR*
	BCR-ABL Transcripts/ reaction	*ABL Transcripts/ reaction*	*G6PD Transcripts/ reaction*	*Ratio BCR-ABL/ABL %*	*Ratio BCR-ABL/G6PD %*	*Ratio BCR-ABL/ABL %*
n	254	254	249	254	249	201
Median	10000	540000	630000	4.6	3.7	6.8
Minimum	10 (9 negative)	150	300	0	0	0.0002
Maximum	3700000	11600000	7000000	145	183	133

calculate the maximum sensitivity, serial dilutions of 5, 50, 500, and 5000 myeloid PB cells from a b3a2$^{BCR-ABL}$ positive CML patient at diagnosis in 5×10^7 leukocytes from a healthy donor were processed and analyzed. We routinely achieved a sensitivity of 10^{-5}, ie we could detect one CML cell in 10^5 normal white blood cells. The same approach was performed with a dilution of BV173 (b2a2$^{BCR-ABL}$-positive cell line) and K562 (b3a2$^{BCR-ABL}$-positive cell line) cells in HL60 (BCR-ABL-negative cell line) cells. In this regard, a reproducible sensitivity of 10^{-6} for BV173 and 10^{-7} for K562 was reached.

Reliability of real time PCR

Quantitative analysis of BCR-ABL in 20 identical samples of 500 molecules of plasmid pGD210 (b3a2$^{BCR-ABL}$) in one run (intra-assay comparison) resulted in a coefficient of variation (CV, calculated for the determined concentrations) of 0.18, the respective cycle threshold crossing points resulted in a CV of 0.029. The analysis of 20 identical samples in 20 runs on 20 days (interassay comparison, day-to-day variation with

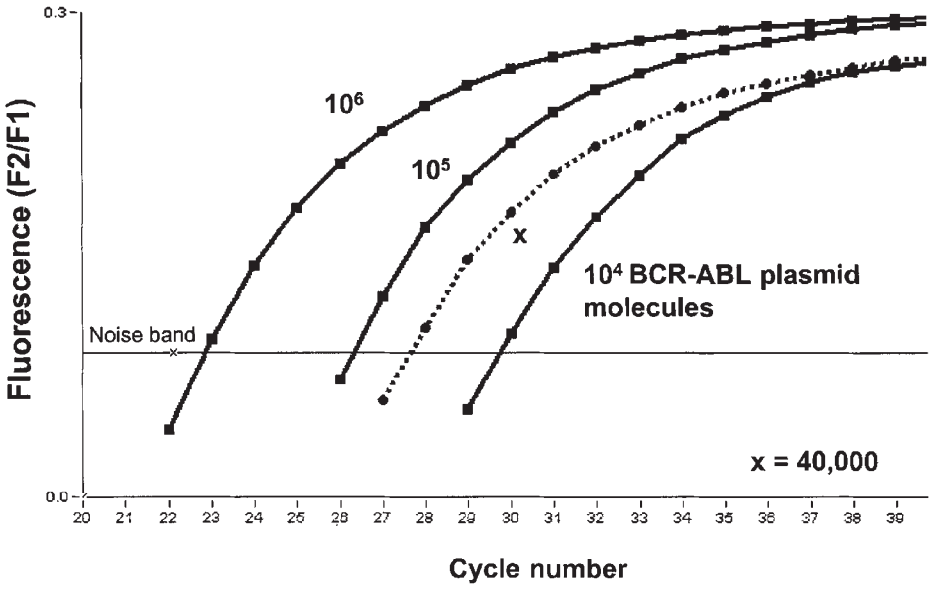

Figure 2 Example of real-time PCR. Three standard dilutions of plasmid pGD210 (b3a2$^{BCR-ABL}$) were compared with a patient's sample of unknown BCR-ABL concentration. Each point represents the fluorescence intensity (F2/F1) measured after each PCR cycle. Plotting the cycle threshold of the unknown sample on the standard curve revealed that 40000 BCR-ABL transcripts were present at the start of the reaction.

new mixtures of reagents) resulted in a CV of 0.17, the cycle threshold crossing points in a CV of 0.028.

Patient samples: qualitative nested RT-PCR

All 254 cDNA samples were BCR-ABL positive by conventional nested PCR. 64 patients expressed b3a2, 40 b2a2, 13 both b3a2 and b2a2, and one each b3a3, b2a3, and e1a2 BCR-ABL transcripts.

Patient samples: real time PCR

Of the 254 samples that were BCR-ABL positive by nested PCR, 245 were also BCR-ABL positive by the LightCycler. Between 10 and 3700000 (median 10000) BCR-ABL transcripts were detected. All 254 samples were positive for total ABL transcripts; between 150 and 11600000 (median 540000) ABL transcripts were detected (Table 2). Nine BCR-ABL negative samples showed ABL transcript levels of 150 to 1390000 (median 119000).

Comparison of ABL and G6PD transcript numbers as internal controls

In 249 samples, G6PD transcripts were quantified as a second internal standard. All samples were positive, between 300 and 7000000 (median 630000) G6PD transcripts were detected (Table 2). The number of G6PD transcripts was at a median of 1.5 times higher than the number of ABL transcripts. G6PD and ABL transcript numbers correlated with $r = 0.74$ ($P < 0.0001$; Figure 3).

Comparison of real-time PCR and competitive PCR

In 201 cDNA samples the ratio BCR-ABL/ABL was determined by nested competitive PCR which compared well with the same ratio determined by real time PCR ($r = 0.90$, $P < 0.0001$, Figure 4). Five of nine samples that were negative on the LightCycler but positive by nested RT-PCR were tested by competitive PCR and found to be positive at a median BCR-ABL transcript level of 0.036% (range 0.0034 to 0.9%).

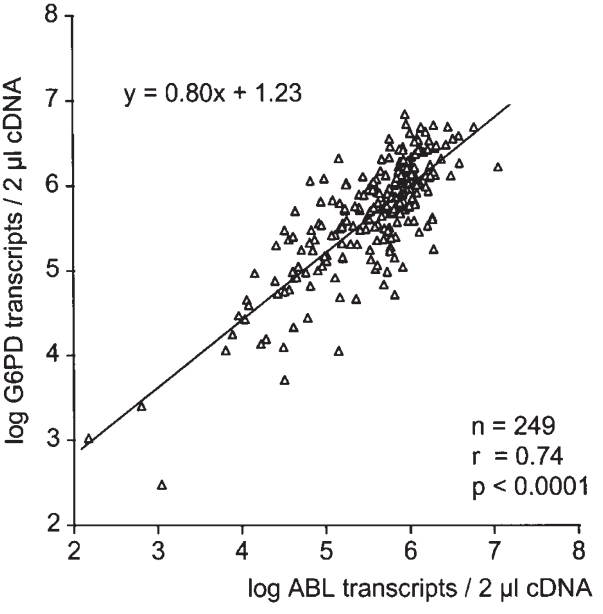

Figure 3 Comparison of the quantification of total ABL and G6PD transcripts as internal controls in 249 consecutive samples. The transcript numbers of both genes correlate with r = 0.74, $P < 0.0001$.

Comparison of real-time PCR and cytogenetics

Contemporaneous metaphase cytogenetic results were available for 81 samples investigated by real-time PCR. They showed a complete response ($n = 17$), partial response ($n = 20$), minor response ($n = 15$), and nonresponse ($n = 29$) and

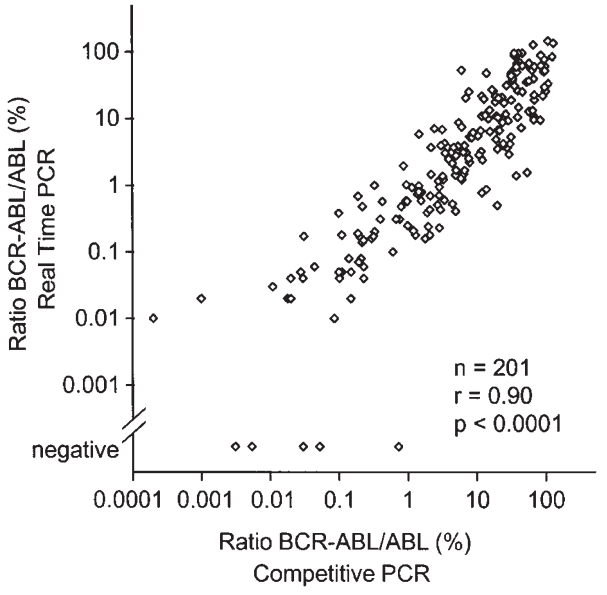

Figure 4 Comparison of the quantification of the ratio BCR-ABL/ABL with real-time PCR *vs* competitive PCR. In five samples positive with competitive PCR no BCR-ABL transcripts were detected by real-time PCR. The ratios derived by both methods correlate with r = 0.90, $P < 0.0001$.

correlated well with the ratios BCR-ABL/ABL ($P < 0.0001$) and BCR-ABL/G6PD ($P < 0.0001$), respectively (Figure 5).

Comparison of real-time PCR and Southern blot analysis

The BCR ratio as determined by quantitative Southern blot analysis in 122 DNA samples extracted from the same PB or BM samples from which RNA was extracted for PCR analysis revealed a complete response ($n = 50$), a partial response ($n = 30$), a minor response ($n = 14$), and a nonresponse ($n = 28$) and correlated with the ratios BCR-ABL/ABL as determined by real time PCR ($P < 0.0001$, Figure 6).

BCR-ABL levels in PB and BM

In eight cases, PB and BM samples obtained on the same day were available for comparison. The ratios BCR-ABL/ABL and BCR-ABL/G6PD in the two tissues were not significantly different. The ratios BCR-ABL/ABL ($r = 0.90$, $P = 0.0046$) and BCR-ABL/G6PD ($r = 0.98$, $P = 0.0004$) correlated well between PB and BM samples (Figure 7).

Discussion

The assessment of the proportion of malignant BM or PB cells is an important prognostic indicator for CML patients after therapy. Cytogenetic analysis is still the standard technique for defining the response of patients to treatment, but this technique suffers from serious limitations. Bone marrow metaphases are required and aspiration and cultivation of proliferating cells is not always sufficient. Furthermore, this technique is relatively insensitive since typically a maximum number of only 20–50 metaphases are analyzed. We and others have

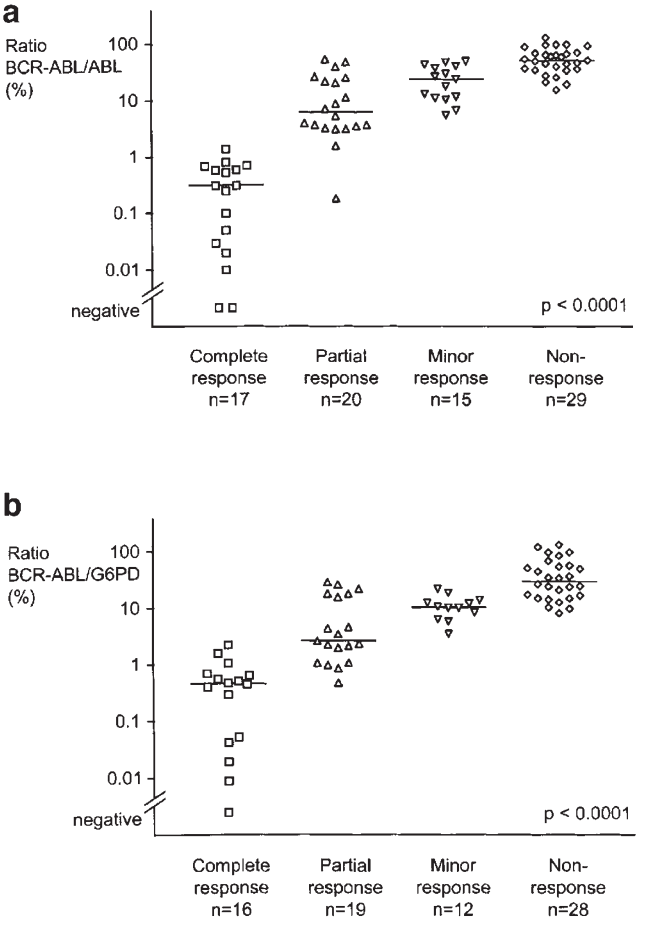

Figure 5 Ratios BCR-ABL/ABL (a) and BCR-ABL/G6PD (b) derived from real-time PCR according to contemporaneous cytogenetic response. The ratios BCR-ABL/ABL and BCR-ABL/G6PD are significantly different between complete, partial, minor and nonresponders ($P < 0.0001$).

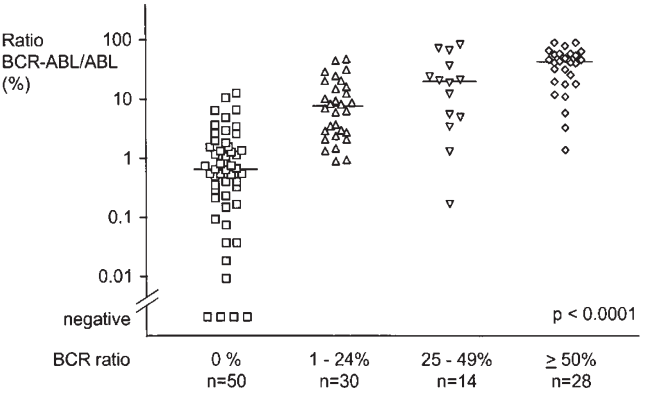

Figure 6 Comparison of the ratio BCR-ABL/ABL derived from real-time PCR *vs* BCR ratio derived from Southern blot analysis of genomic DNA from the same PB or BM samples. The ratios BCR-ABL/ABL are significantly different between the Southern blot response groups ($P < 0.0001$).

previously demonstrated the advantages of quantitative PCR techniques for monitoring CML patients during or after treatment.[2] Competitive PCR results correlate well with the cytogenetic response in individuals treated with IFN-α[17] and moreover, the actual levels of detectable residual disease in

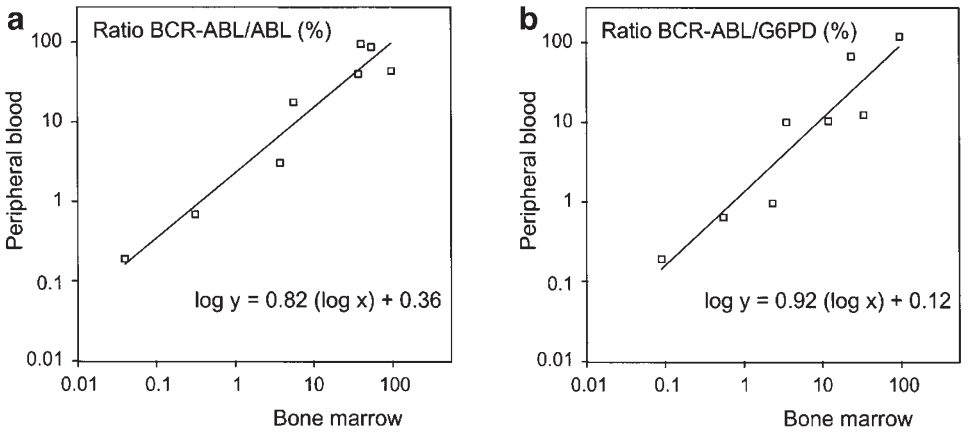

Figure 7 Correlation of the $\log_{10}$ ratio BCR-ABL/ABL (a) and BCR-ABL/G6PD (b) obtained from eight pairs of contemporaneous PB and BM samples. The coefficient of regression is r = 0.90 ($P = 0.0046$) for the ratios BCR-ABL/ABL and r = 0.98 ($P = 0.0004$) for the ratios BCR-ABL/G6PD.

complete cytogenetic responders may vary by as much as four orders of magnitude.[20] In patients after BMT, rising or persistently high levels of BCR-ABL mRNA can be detected prior to cytogenetic or hematologic relapse.[16,21–24] Quantitative PCR data has been used to initiate donor lymphocyte transfusions for treatment of relapse[25] and to monitor its response.[26]

The advent of real-time PCR enables the direct measurement of the amount of PCR product during the amplification process and therefore reflects the dynamics of the reaction. Although the final amount of PCR product at the plateau phase usually does not correlate well with the number of starting molecules in the reaction, the time-point (cycle number) at which the fluorescence for a particular sample rises above the background (crossover point or threshold cycle) is a much more accurate indicator of the initial number of PCR targets. Amplification and specific detection of fusion transcripts by real-time PCR is possible by simultaneous amplification and fluorescence melting curve analysis using non-specific labeling by the double strand DNA specific dye SYBR Green I.[27] However, if specific detection and accurate quantification is required, it is necessary to use adjacent fluorescence labeled hybridization probes. Real-time PCR does not require post-PCR sample handling and therefore considerably reduces the risk of contamination. In addition, the possibility of contamination is further reduced by the use of heat labile UDG prior to amplification.

Real-time PCR for the detection and quantification of BCR-ABL transcripts using the TaqMan system with a double fluorescence labeled probe[28,29] has been described recently.[5,6] In this study we have developed a related system using fluorescent hybridization probes in the LightCycler.[8,30] One advantage of this system is the rapid cycling time (<40 s/cycle) due to amplification being performed in glass capillaries and being driven by forced air heating. This system enables the temperature in each reaction to be changed very rapidly. By minimizing denaturation and annealing times, specificity, speed and yield of the reaction are significantly improved.[8]

We have optimized and validated this technique in samples from 120 CML patients. The use of probes matching to ABL exon 3 sequences in combination with appropriate primers allows the detection of all known BCR-ABL variants (for reviews, see Refs 31 and 32), including e1a2,[33] e6a2,[32] c3a2 (e19a2),[34] and BCR-ABL transcripts lacking ABL exon 2 (b2a3[35] or b3a3[36,37]). The sensitivity of the single step real-time PCR with hybridization of fluorescent probes is almost as high as standard nested PCR. The reason why the real-time assay is slightly less sensitive is almost certainly due to the fact that we used 2 μl of cDNA in the LightCycler, but 5 μl of cDNA for standard nested PCR.

To standardize BCR-ABL mRNA levels for variability in RNA and cDNA quality, we amplified transcripts of housekeeping genes as internal controls. Appropriate control sequences should be expressed at similar levels as the target genes in nonresponders, and should be cDNA specific, ie they should span long introns, and pseudogenes in genomic DNA must not exist.[38–41] We therefore decided to use the amplification of total ABL cDNA sequences between exons a2 and a4. The advantage of using ABL is that the same pair of hybridization probes used to quantify BCR-ABL may be applied. We tested the variability of the ABL quantification by comparing ABL and G6PD transcript levels in samples of different qualities and different levels of BCR-ABL. The levels of ABL and G6PD were significantly correlated and similar results were obtained when either BCR-ABL/ABL or BCR-ABL/G6PD ratios were compared with cytogenetic results. These data suggest that either gene may be used as an internal standard. For individual samples, however, significant differences in the G6PD/ABL ratio are seen occasionally and it remains to be determined which is the most appropriate control gene for use in quantitative RT-PCR analysis.

The reliability of this system has been demonstrated by comparison with an existing method of PCR quantification, nested competitive PCR. Virtually identical results were determined with both methods, with a coefficient of correlation of $r = 0.90$ ($P < 0.0001$). The degree of concordance with metaphase cytogenetics and Southern blot analysis was also high. However, some overlap between the conventional cytogenetic response groups may be explained at least in part by the statistical significance of sampling small numbers of metaphases.[42] Results with PB and BM were concordant in all cases indicating that either tissue may be used for residual disease studies in CML. This result confirms similar data that have previously been described using competitive PCR.[17,43]

We conclude that quantitative real-time PCR with hybridization probes is a rapid, sensitive and reliable method for monitoring CML patients after therapy. The method offers the opportunity to standardize the assay and to develop rigorous standards and controls. It is likely that real-time PCR will enable the quantitative analysis of residual disease to become more widely available, not only for patients with CML but

also for other malignancies that are characterized by specific molecular markers.

Acknowledgements

The study was subsidized by the Deutsche José Carreras Leukämiestiftung eV. and the Forschungsfonds der Fakultät für Klinische Medizin Mannheim der Universität Heidelberg, Germany. We thank Drs H Leying and G Betzl (Roche Diagnostics, Mannheim/Penzberg, Germany) for continuous technical advice and support. The M-bcr probes for Southern blot analysis were provided by Prof CR Bartram (University of Heidelberg, Germany). Standard plasmid pGD210 (b3a2$^{BCR-ABL}$) was provided by Dr R van Etten (Harvard Medical School, Boston, MA, USA), plasmid pB190 (e1a2$^{BCR-ABL}$, GenBank accession number AF113911) by Dr T Burmeister (Free University Berlin, Germany), and plasmid pGdBBX by Dr Ph Mason (Imperial College School of Medicine, London, UK). We wish to thank the hematologists and cytogeneticists participating in the German CML trials and in the UK Medical Research CML trials for sending samples and providing clinical and cytogenetic data.

References

1 Sawyers CL. Chronic myeloid leukemia. *New Engl J Med* 1999; **340**: 1330–1340.
2 Cross NCP. Assessing residual leukaemia. *Baillières Clin Haematol* 1997; **10**: 389–403.
3 Lion T. Monitoring of residual disease in chronic myelogenous leukemia: methodological approaches and clincal aspects. *Leukemia* 1996; **10**: 896–906.
4 Morley A. Quantifying leukemia. *New Engl J Med* 1998; **339**: 627–629.
5 Mensink E, van de Locht A, Schattenberg A, Linders E, Schaap N, Guerts van Kessel A, de Witte T. Quantitation of minimal residual disease in Philadelphia chromosome positive chronic myeloid leukaemia patients using real-time quantitative RT-PCR. *Br J Haematol* 1998; **102**: 768–774.
6 Preudhomme C, Révillion F, Merlat A, Hornez L, Roumier C, Duflos-Grardel N, Jouet JP, Cosson A, Peyrat JP, Fenaux P. Detection of BCR-ABL transcripts in chronic myeloid leukemia (CML) using a 'real time' quantitative RT-PCR assay. *Leukemia* 1999; **13**: 957–964.
7 Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* 1997; **22**: 130–138.
8 Wittwer CT, Ririe KM, Andrew RV, David DA, Gundry RA, Balis UJ. The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques* 1997; **22**: 176–181.
9 ISCN. *An International System for Human Cytogenetic Nomenclature*. In: Harnden DG, Klinger HP (eds). Karger: Basel, 1985.
10 Kantarjian HM, Smith TL, O'Brien S, Beran M, Pierce S, Talpaz M, and the Leukemia Service. Prolonged survival in chronic myelogenous leukemia after cytogenetic response to interferon-α therapy. *Ann Intern Med* 1995; **122**: 254–261.
11 Grossman A, Silver RT, Szatrowski TP, Gutfriend A, Verma RS, Benn PA. Densitometric analysis of Southern blot autoradiographs and its application to monitoring patients with chronic myeloid leukemia. *Leukemia* 1991; **5**: 540–547.
12 Reiter A, Skladny H, Hochhaus A, Seifarth W, Heimpel H, Bartram CR, Cross NCP, Hehlmann R. Molecular response of CML patients treated with interferon-α monitored by quantitative Southern blot analysis. *Br J Haematol* 1997; **97**: 86–93.
13 Cross NCP, Feng L, Bungey J, Goldman JM. Minimal residual disease after bone marrow transplant for chronic myeloid leukaemia detected by the polymerase chain reaction. *Leuk Lymphoma* 1993; **11** (Suppl. 1): 39–43.
14 Cross NCP, Melo JV, Feng L, Goldman JM. An optimized multiplex polymerase chain reaction (PCR) for detection of BCR-ABL fusion mRNAs in haematological disorders. *Leukemia* 1994; **8**: 186–189.
15 van Rhee F, Marks DI, Lin F, Szydlo RM, Hochhaus A, Treleaven J, Delord C, Cross NCP, Goldman JM. Quantification of residual disease in Philadelphia-positive acute lymphoblastic leukemia: comparison of blood and bone marrow. *Leukemia* 1995; **9**: 329–335.
16 Cross NCP, Feng L, Chase A, Bungey J, Hughes TP, Goldman JM. Competitive polymerase chain reaction to estimate the number of BCR-ABL transcripts in chronic myeloid leukemia patients after bone marrow transplantation. *Blood* 1993; **82**: 1929–1936.
17 Hochhaus A, Lin F, Reiter A, Skladny H, Mason PJ, van Rhee F, Shepherd PCA, Allan NC, Hehlmann R, Goldman JM, Cross NCP. Quantification of residual disease in chronic myelogenous leukemia patients on interferon-α therapy by competitive polymerase chain reaction. *Blood* 1996; **87**: 1549–1555.
18 Chissoe SL, Bodenteich A, Wang YF, Wang YP, Burian D, Clifton SW, Crabtree J, Freeman A, Iyer K, Jian L, Ma Y, McLaury HJ, Pan HQ, Sarhan OH, Toth S, Wang Z, Zhang G, Heisterkamp N, Groffen J, Roe BA. Sequence and analysis of the human ABL gene, the BCR gene, and regions involved in the Philadelphia chromosomal translocation. *Genomics* 1995; **27**: 67–82.
19 Chen EY, Cheng A, Lee A, Kuang WJ, Hillier L, Green P, Schlessinger D, Ciccodicola A, D'Urso M. Sequence of human glucose-6-phosphate dehydrogenase cloned in plasmids and a yeast artificial chromosome. *Genomics* 1991; **10**: 792–800.
20 Hochhaus A, Lin F, Reiter A, Skladny H, van Rhee F, Shepherd PCA, Allan NC, Hehlmann R, Goldman JM, Cross NCP. Variable numbers of BCR-ABL transcripts persist in CML patients who achieve complete cytogenetic remission with interferon-α. *Br J Haematol* 1995; **91**: 126–131.
21 Lin F, Kirkland MA, van Rhee F, Chase A, Coulthard S, Bungey J, Goldman JM, Cross NCP. Molecular analysis of transient cytogenetic relapse after allogeneic bone marrow transplantation for chronic myeloid leukaemia. *Bone Marrow Transplant* 1996; **18**: 1147–1152.
22 Lin F, van Rhee F, Goldman JM, Cross NCP. Kinetics of increasing BCR-ABL transcript numbers in chronic myeloid leukemia patients who relapse after bone marrow transplantation. *Blood* 1996; **87**: 4473–4478.
23 Gaiger A, Lion T, Kalhs P, Mitterbauer G, Henn T, Haas O, Fodinger M, Kier P, Forstinger C, Quehenberger P, Hinterberger W, Jäger U, Linkesch W, Mannhalter C, Lechner K. Frequent detection of BCR-ABL specific mRNA in patients with chronic myeloid leukemia (CML) following allogeneic and syngeneic bone marrow transplantation (BMT). *Leukemia* 1993; **7**: 1766–1772.
24 Lion T, Henn T, Gaiger A, Kalhs P, Gadner H. Early detection of relapse after bone marrow transplantation in patients with chronic myelogenous leukaemia. *Lancet* 1993; **341**: 275–276.
25 van Rhee F, Lin F, Cullis JO, Spencer A, Cross NCP, Chase A, Garicochea B, Bungey J, Barrett J, Goldman JM. Relapse of chronic myeloid leukemia after allogeneic bone marrow transplant: the case for giving donor leukocyte transfusions before the onset of hematologic relapse. *Blood* 1994; **83**: 3377–3383.
26 Raanani P, Dazzi F, Sohal J, Szydlo R, van Rhee F, Reiter A, Lin F, Goldman JM, Cross NCP. The rate and kinetics of molecular response to donor leucocyte transfusions in chronic myeloid leukaemia patients treated for relapse after allogeneic bone marrow transplantation. *Br J Haematol* 1997; **99**: 945–950.
27 Bohling SD, King TC, Wittwer CT, Elenitoba-Johnson KSJ. Rapid simultaneous amplification and detection of the MBR/JH chromosomal translocation by fluorescence melting curve analysis. *Am J Pathol* 1999; **154**: 97–103.
28 Gibson UEM, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. *Genome Res* 1996; **6**: 995–1001.
29 Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996; **6**: 986–994.
30 Higuchi R, Dollinger G, Walsh PS, Griffith R. Simultaneous amplification and detection of specific DNA sequences. *Biotechnology* 1992; **10**: 413–417.
31 Melo JV. The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype. *Blood* 1996; **88**: 2375–2384.
32 Hochhaus A, Reiter A, Skladny H, Melo JV, Sick C, Berger U, Guo JQ, Arlinghaus RB, Hehlmann R, Goldman JM, Cross NCP. A novel BCR-ABL fusion gene (e6a2) in a patient with Philadelphia

chromosome negative chronic myelogenous leukemia. *Blood* 1996; **88**: 2236–2240.
33 Melo JV, Myint H, Galton DA, Goldman JM. P190$^{BCR-ABL}$ chronic myeloid leukaemia: the missing link with chronic myelomonocytic leukaemia? *Leukemia* 1994; **8**: 208–211.
34 Pane F, Frigeri F, Sindona M, Luciano L, Ferrara F, Cimino R, Meloni G, Saglio G, Salvatore F, Rotoli B. Neutrophilic-chronic myelogenous leukemia (CML-N): a distinct disease with a specific marker (BCR-ABL with c3a2 junction). *Blood* 1996; **88**: 2410–2414.
35 van der Plas DC, Soekarman D, van Gent AM, Grosveld G, Hagemeijer A. bcr-abl mRNA lacking abl exon a2 detected by polymerase chain reaction in a chronic myelogeneous leukemia patient. *Leukemia* 1991; **5**: 457–461.
36 Paldi Haris P, Barta A, Lengyel L, Batai A, Masszi T, Remenyi P, Denes R, Paloczi K, Kelemen E, Foldi J. Molecular background of a new case of chronic myelogenous leukemia with bcr-abl chimera mRNA lacking the A2 exon (letter). *Leukemia* 1994; **8**: 1791.
37 Iwata S, Mizutani S, Nakazawa S, Yata J. Heterogeneity of the breakpoint in the ABL gene in cases with BCR/ABL transcript lacking ABL exon a2. *Leukemia* 1994; **8**: 1696–1702.
38 Lion T. Control genes in reverse transcriptase-polymerase chain reaction assays. *Leukemia* 1996; **10**: 1527–1528.
39 Soutar RL, Dillon J, Ralston SH. Control genes for reverse-transcription polymerase chain reaction: a comparison of beta actin and glyceraldehyde phosphate dehydrogenase. *Br J Haematol* 1997; **97**: 247–248.
40 Cross NCP, Lin F, Goldman JM. Appropriate controls for reverse transcription polymerase chain reaction (RT-PCR). *Br J Haematol* 1994; **87**: 218.
41 Taylor JJ, Haesman PA. Control genes for reverse transcriptase/polymerase chain reaction (RT-PCR). *Br J Haematol* 1994; **86**: 444–447.
42 Hook EB. Exclusion of chromosomal mosaicism: tables of 90%, 95%, and 99% confidence limits and comments on use. *Am J Hum Genet* 1977; **29**: 94–97.
43 Lin F, Goldman JM, Cross NCP. A comparison of the sensitivity of blood and bone marrow for the detection of minimal residual disease in chronic myeloid leukaemia. *Br J Haematol* 1994; **86**: 683–685.