Journal of Molecular Diagnostics, Vol. 8, No. 2, May 2006

DOI: 10.2353/jmoldx.2006.040404

Molecular Monitoring of Chronic Myelogenous Leukemia

Identification of the Most Suitable Internal Control Gene for Real-Time Quantification of BCR-ABL *Transcripts*

Y. Lynn Wang,* Joong Won Lee,* Ethel Cesarman,* David K. Jin,† and Balazs Csernus*

From the Department of Pathology and Laboratory Medicine, and the Division of Hematology, Department of Medicine,† Weill Medical College of Cornell University, New York, New York*

Monitoring breakpoint cluster region-Abelson kinase (*BCR-ABL*) levels in patients treated for chronic myelogenous leukemia (CML) has become an integral part of patient management. Real-time reverse transcriptase-polymerase chain reaction is the method of choice for this purpose because of its high analytical sensitivity and reproducibility. Given the variation of RNA quality and quantity in clinical specimens, accurate quantitative assessment of *BCR-ABL* depends on normalization of the *BCR-ABL* signal to an appropriate internal reference. However, the controls used by different laboratories vary, and there is no clear consensus on an ideal reference due to limited investigations. In this study, we compared nine commonly used control genes for three criteria: mRNA abundance, levels in CML and non-CML cells, and their degradation kinetics in comparison with *BCR-ABL*. We found that β-glucuronidase (*GUSB*) is the most suitable among the nine genes tested. Although *ABL* is most widely used, our data suggest that the amount of *ABL* is different in CML and non-CML cells. Moreover, *ABL* levels are regulated by cellular stress. These findings have a direct impact on current clinical laboratory practice and patient care because the use of a proper control gene affects the reported levels of *BCR-ABL* transcripts used for patient management decisions. ***(J Mol Diagn 2006, 8:231–239; DOI: 10.2353/jmoldx.2006.040404)***

Chromosomal translocation t(9;22) is a hallmark of chronic myelogenous leukemia (CML).[1,2] It can be found in 95% of patients with CML. At the molecular level, the translocation joins the 5′ segment of the breakpoint cluster region (*BCR*) gene on chromosome 22 to the 3′ portion of the Abelson kinase (*ABL*) gene on chromosome 9. Reverse transcriptase-polymerase chain reaction (RT-PCR) detection of *BCR-ABL* has long been used to aid in the diagnosis of CML and monitoring of residual leukemia after therapy.[3]

With the recent advent of newer therapies, such as the tyrosine kinase inhibitor Gleevec (also known as STI-571 or imatinib mesylate), molecular monitoring has become indispensable for assessment of patients' therapeutic response and early detection of relapse.[4–8] For patients who develop acquired resistance to Gleevec, therapeutic strategies have been developed to overcome such resistance.[9,10] Conventional RT-PCR that generates only positive or negative results does not allow timely assessment of therapeutic response because many patients remain positive for a long period even after they achieve a cytogenetic response.[11] In contrast, quantitative assessment of *BCR-ABL* transcripts using real-time technology has become the method of choice. It has been proven as a clinically useful test because patients with high or increasing levels of *BCR-ABL* over the disease course have a greater probability of relapse than those with steady-state or decreasing levels of *BCR-ABL*.[3] Essential to accurate determination of *BCR-ABL* is the application of an appropriate internal normalization control because RNA derived from the clinical samples varies a great deal in both quality and quantity.

Review of the literature and a survey conducted by the Association for Molecular Pathology in 2002 showed that internal control genes that are widely used in Europe and North America for *BCR-ABL* quantitative RT-PCR include *ABL*,[12–16] *BCR*,[5,17] glucose 6-phosphate dehydrogenase (*G6PD*),[7,18] and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).[19,20] These were used as internal controls mainly because they had been historically used in conventional RT-PCR assays to assess the quality of cDNA. So far, only one study has been performed by the Europe Against Cancer (EAC) Program to examine suitability of different controls for *BCR-ABL* quantification. The study has proposed using *ABL* as the internal control

Accepted for publication November 4, 2005.

Address reprint requests to Y. Lynn Wang, Department of Pathology and Laboratory Medicine, Weill Medical College of Cornell University, 1300 York Ave., Box 69, New York, NY 10021. E-mail: lyw2001@med.cornell.edu.

after comparing several commonly used internal control genes.[21] However, the primers/probe for *ABL* used in the study detect not only *ABL* wild-type allele but also the *BCR-ABL* fusion gene. The ratio of *BCR-ABL* to control then becomes *BCR-ABL*/(BCR-*ABL*+*ABL*). Although the authors were concerned that the detected level of *ABL* may be affected by the level of *BCR-ABL* that would lead to an underestimation of the tumor load, the authors stated that the *ABL* assay by this design led to a limited inaccuracy for diagnostic specimens expressing high levels of *BCR-ABL* transcripts. Because the assay is primarily used for CML monitoring after therapy, the impact of the *ABL* primers/probe design on minimal residual disease assessment need to be addressed.

In this study, we investigated nine commonly used control genes for *BCR-ABL* quantification, including β-actin (*ACTB*), β2-microglobulin (*B2M*), *GAPDH*, *G6PD*, *GUSB*, hypoxanthine phosphoribosyltransferase (*HPRT*), phosphoglycerate kinase 1 (*PGK*), TATA-box binding protein (*TBP*), and *ABL*, according to the following criteria: 1) suitable control genes are expressed at similar level to *BCR-ABL*, 2) suitable control genes are expressed in CML cells at similar level to that in non-CML cells, and 3) degradation kinetics of suitable control genes parallels that of the *BCR-ABL* transcripts. We have found that *ABL* does not serve as an appropriate control gene regardless of how primers/probe are designed. Among genes studied, *G6PD* and *GUSB* meet all criteria as appropriate controls for *BCR-ABL* quantification. We recommend using *GUSB* as the control gene of choice because mutations or variations occur at a much rarer frequency in the gene than in the *G6PD* locus.

Materials and Methods

Specimens

A total of 21 patient specimens from 19 patients were studied under an Institutional Review Board protocol. These include 12 specimens from newly diagnosed CML patients, 5 from patients who had been treated, 1 from a patient in blast crisis, 1 from a patient in accelerated phase of disease, and 2 different types of specimens from a patient with minimal residual disease. Specimen types included 15 bone marrow aspirates and 6 peripheral blood samples (Table 1).

RNA Isolation, Quantification, and Reverse Transcription

Total cellular RNA was isolated from patient samples using RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was eluted from the RNeasy column in 30 μl of RNase-free water. The amount of total RNA isolated from the cells was quantified using spectrophotometric measurements. Four micrograms of RNA was reverse-transcribed in an 80-μl reaction volume using a Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's protocol.

Table 1. Sample Description

Specimen	Gender	Age	Specimen type	Stage of disease
1	M	60	BM	CP/diagnosis
2	M	59	BM	CP/diagnosis
3	F	52	BM	CP/diagnosis
4	M	72	BM	CP/diagnosis
5	F	33	BM	CP/diagnosis
6	M	42	BM	CP/diagnosis
7*	F	68	BM	CP/diagnosis
8	M	34	PB	CP/diagnosis
9	M	68	PB	CP/diagnosis
10	M	50	BM	CP/diagnosis
11	M	56	PB	CP/diagnosis
12	M	35	BM	CP/diagnosis
13	F	65	BM	CP/treated
14	M	43	PB	CP/treated
15	F	61	PB	CP/treated
16	F	31	BM	CP/treated
17	M	58	BM	BP
18†	F	55	PB	Post BMT/MRD
19†	F	55	BM	Post BMT/MRD
20*	F	68	BM	CP/treated
21	M	63	BM	AP

*Specimens 7 and 20 were obtained from the same patient at different times.

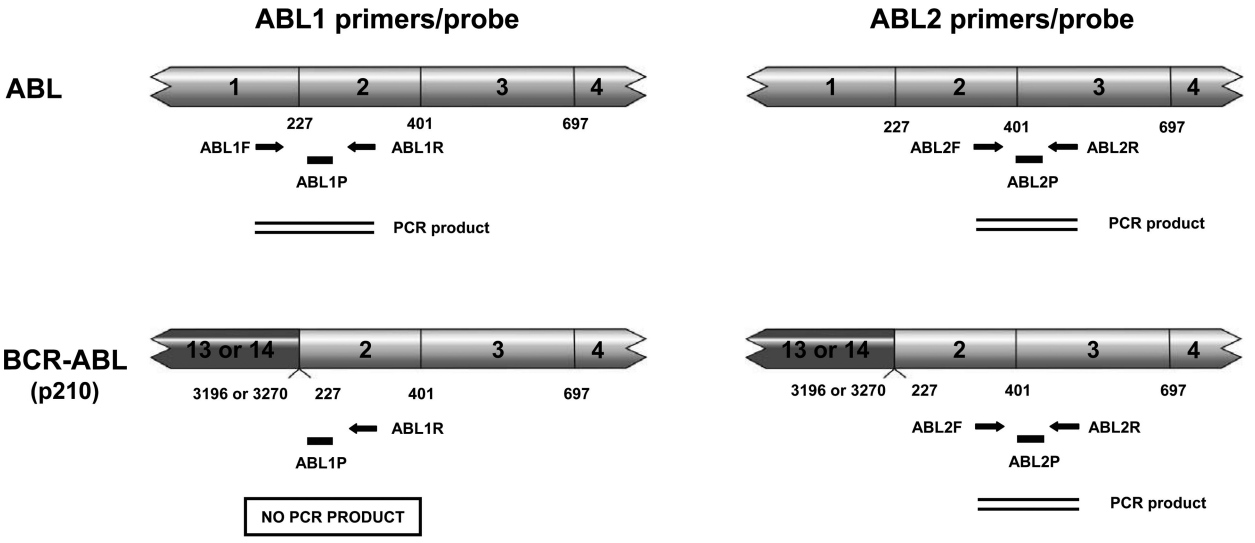
†Specimens 18 and 19 were from the same patient.

M, male; F, female; BM, PB, peripheral blood; bone marrow; BMT/MRD, bone marrow transplantation/ minimal residual disease; CP, chronic phase; BP, blastic phase; AP, accelerated phase.

Real-Time PCR

Real-time PCR was conducted in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems [ABI], Foster City, CA). cDNA made from 100 ng of total RNA was added to 25 μl of 1× Taqman Universal PCR master mix. The reaction contains 300 nmol/L of primers and 200 nmol/L probe. PCR was conducted using the following default TaqMan PCR conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Triplicate PCR reactions were conducted for each sample. Water instead of cDNA was included as a blank sample to control for PCR contamination.

Except for *G6PD* and two *ABL* primer/probe sets, all other primer/probe sets, including *ACTB*, *B2M*, *GAPDH*, *GUSB*, *HPRT*, *PGK*, and *TBP*, were TaqMan Pre-developed Assay Reagents from ABI. *G6PD* was obtained from ABI as an Assay-on-Demand gene expression product. Two *ABL* primer/probe sets were reported previously[21,22] and were custom-made by ABI. Their sequences and relative positions to *ABL* and *BCR-ABL* genes are illustrated in Figure 1. As shown in the left panel of Figure 1, the upstream primer of the ABL1 set hybridizes to exon 1, and the downstream primer and probe hybridize to exon 2 of the *ABL* gene. Because the breakpoints mostly occur in the intron between exons 1 and 2, the ABL1 set therefore detects only the wild-type allele of the *ABL* gene. In comparison, the upstream primer of ABL2 set hybridizes to exon 2, and the downstream primer and probe hybridize to exon 3 of the *ABL* gene (Figure 1, right). It therefore detects both the wild-type *ABL* and translocated *BCR-ABL* messages. Real-time PCR results were analyzed



ABL1F	TCCTCCAGCTGTTATCTGGAAGA	205-227
ABL1R	TGGGTCCAGCGAGAAGGTT	322-304
ABL1P	CCAGTAGCATCTGACTTTGAGCCTCAGGG	241-269
ABL2F	TGGAGATAACACTCTAAGCATAACTAAAGG	372-401
ABL2R	GATGTAGTTGCTTGGGACCCA	495-475
ABL2P	CCATTTTTG GTT TGGGCTTCACACCAT T	467-440

Figure 1. Schematic diagram of the two different sets of primers/probes for *ABL* quantification (**left**, ABL1; **right**, ABL2). **Lightly shaded boxes** represent *ABL* cDNA and **darkly shaded boxes** represent *BCR* cDNA with exons indicated. Numbers below cDNAs indicate nucleotide positions at exon boundaries. **Arrows** represent PCR primers and their relative positions to *ABL* and *BCR-ABL* cDNAs. **Black bars** represent the TaqMan probes and their positions. Sequences of primers and probes and their locations are shown under each diagram.

with ABI Prism 7000 SDS software, and autothresholds and autobaselines determined by the software for each individual gene target were applied to generate values of corresponding threshold cycles (Ct).

Degradation Kinetics

To study the degradation kinetics of *BCR-ABL* and control genes, mononuclear cells from the bone marrow of two CML patients were placed on the bench to let cells die and RNA degrade. Aliquots of cells were collected at different time points, followed immediately by RNA extraction. RNA was reverse-transcribed, and levels of *BCR-ABL* and control genes were determined simultaneously by real-time RT-PCR in triplicate reactions. Levels of *BCR-ABL* and control genes at each time point after day 0 are expressed as a percentage of the initial time point calculated by the ΔCt method using the following formula: $\% = 2^{-\Delta Ct} \times 100\%$, where $\Delta Ct = Ct_{day\ x} - Ct_{day\ 0}$. For example, if on day *x*, Ct is one cycle higher than on day 0, then $\Delta Ct = 1$ and $2^{-\Delta Ct} \times 100\% = 50\%$.

Results

mRNA Abundance of Commonly Used Control Genes Compared with BCR-ABL

Suitable normalization control genes should be expressed at similar levels to the *BCR-ABL* gene so that they would be similarly sensitive to variations in the amount of RNA in test samples. To select for such control genes, we compared the expression levels of nine commonly used control genes, including *ABL*, *ACTB*, *B2M*, *G6PD*, *GAPDH*, *GUSB*, *HPRT*, *PGK*, *TBP*, and *BCR-ABL* in patient samples using TaqMan technology on an ABI PRISM 7000 Sequence Detection System. Except for *ABL*, none of the selected genes are located on chromosomes 8, 17, 19, or 22, which are frequently subject to rearrangements in CML. Among them, *ABL* was assessed using two sets of differently designed primer/probe sets (see Material and Methods; Figure 1). ABL1 primers flank the major breakpoint region in the *ABL* gene; they therefore only amplify cDNA derived from the wild-type *ABL* transcript. In contrast, both ABL2 primers are located downstream from the translocation breakpoints; ABL2 therefore amplified cDNA from both fusion

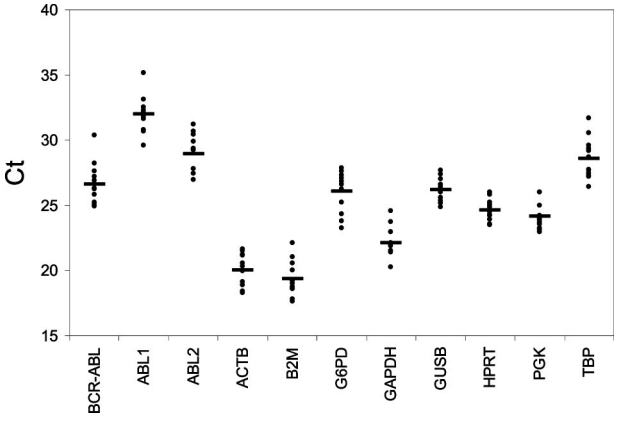


Figure 2. Threshold cycles of the *BCR-ABL* and control genes in 12 pretreatment patient samples. Triplicate PCR reactions were performed for each patient sample. The mean of Ct values of 12 samples are represented by **horizontal bars**.

Table 2. Threshold Cycles of the Control Genes and *BCR-ABL* in CML Samples (n = 12)

Genes	Ct (mean ± 2SD)*	ΔCt (*BCR-ABL* − control)
BCR-ABL	26.58 ± 3.25	—
ABL1	31.96 ± 2.75	−5.38
ABL2	28.94 ± 3.07	−2.36
ACTB	20.02 ± 2.43	+6.56
B2M	19.39 ± 2.65	+7.19
G6PD	26.07 ± 3.09	+0.51
GAPDH	22.12 ± 2.30	+4.46
GUSB	26.32 ± 1.71	+0.26
HPRT	24.66 ± 1.62	+1.92
PGK	24.14 ± 2.05	+2.44
TBP	28.57 ± 3.11	−1.99

*The Ct values for individual specimen are calculated based on six replicate reactions from two experiments. Data shown are means ± 2SD for 12 CML samples.

and wild-type *ABL* transcript.[23] Ct values of the *BCR-ABL* and the nine control genes were determined in 12 pretreatment patient samples (Figure 2; Table 1, patients 1 to 12). For each gene, mean Ct values of 12 samples and their differences from that of *BCR-ABL* are listed in Table 2. The mean Ct value of *BCR-ABL* in the 12 patients was 26.58. Among the 10 control genes, *ACT, B2M,* and *GAPDH* had mean Ct values more than four cycles lower than *BCR-ABL*. On the other hand, *ABL* level as assessed by ABL1 primer/probe set was more than five cycles higher than *BCR-ABL*. mRNA levels of the remaining genes including *G6PD, GUSB*, *HPRT*, *PGK*, *TBP,* and *ABL* by ABL2 primers/probe were less than 2.5 cycles different from the *BCR-ABL*; they therefore meet these criteria as suitable control genes.

mRNA Levels of the Control Genes in CML and Non-CML Cells

Because quantitative *BCR-ABL* is primarily used in patients who have been treated for CML to monitor their response to therapy, the bone marrow or peripheral blood from these patients typically contains normal hematopoietic cells in addition to residual CML cells. A key criterion for a suitable control gene should be that it is expressed in CML cells at a comparable level to that in non-CML cells, so that the level of the control gene ultimately reflects the amount of total RNA being analyzed irrespective of the CML-to-non-CML cell ratio in the mixture. To study which control genes meet these criteria, we first analyzed the control genes in well-characterized leukemic cell lines. To mimic samples from treated patients, we mixed one part of K562 cells, a CML cell line bearing *BCR-ABL*, in nine parts of HL60 cells, a promyelocytic cell line that lacks *BCR-ABL*. We compared the levels of the control genes in K562 cells with no dilution and K562 diluted with HL60. It is expected that the Ct values of *BCR-ABL* differ by 3.32 (equivalent to a 10-fold difference in the amount of initial *BCR-ABL* cDNA). It is also expected that Ct values of a suitable control gene are essentially the same in equal amounts of pure K562 and mixed K562/HL60 cells if the control gene is expressed at comparable levels in both cell types. As shown in Table 3, among the nine genes tested by 10 primer/probe sets, there was a difference of less than 0.5 cycles in the Ct values of *ACTB*, *GAPDH*, *GUSB*, and *PGK* between K562 and the K562/HL60 mixture (ΔCt column in Table 3), suggesting that their levels are similar in both CML and non-CML cells. The Ct of *B2M*, *G6PD*, *HPRT*, *TBP,* and *ABL* levels by both ABL1 and ABL2 differ between the two samples from 0.76 cycle for *HPRT* to 2.51 cycles for ABL2, suggesting that K562 and HL60 cells contain significantly different amounts of the respective control gene mRNA. *B2M*, *HPRT,* and *TBP* were not further studied for this reason as well as the fact that they are not widely used controls for *BCR-ABL* quantification. Besides having a Ct that is more than six cycles lower than *BCR-ABL*, the amplification plot of *ACTB* is shaped differently from other control genes, because the plateau phase was reached much earlier, suggesting that some PCR components are limiting in the reaction (data not shown). *ACTB*, therefore, was also not chosen for further study.

Notably, the Ct of *ABL* assayed by ABL1 or ABL2 was significantly different in pure K562 and K562/HL60 mixtures (Table 3, *P* value), suggesting that ABL amounts are different in K562 and HL60 cell lines. Intriguingly, *ABL* detected by ABL1 and ABL2 changed unexpectedly in the opposite direction comparing K562/HL60 cell mix-

Table 3. Threshold Cycles of the Control Genes and *BCR-ABL* in K562 and K562/HL60 Mixtures

Genes	Ct (mean ± 2SD)*		ΔCt†	*P* value‡
	K562	K562/HL60		
BCR-ABL	21.50 ± 0.34	24.97 ± 0.08	3.47	<0.001
ABL1	29.45 ± 0.92	27.56 ± 0.94	−1.89	<0.001
ABL2	22.90 ± 0.26	25.41 ± 0.50	2.51	<0.001
ACTB	18.90 ± 0.16	18.98 ± 0.20	0.08	0.176
B2M	20.93 ± 0.18	18.52 ± 0.20	−2.41	<0.001
G6PD	22.87 ± 0.07	24.98 ± 0.02	2.12	<0.001
GAPDH	18.99 ± 0.24	18.96 ± 0.08	−0.03	0.618
GUSB	23.84 ± 0.26	23.72 ± 0.12	−0.12	0.062
HPRT	21.59 ± 0.80	22.35 ± 0.72	0.76	0.006
PGK	21.97 ± 0.06	21.50 ± 0.50	−0.47	0.001
TBP	23.61 ± 0.14	24.99 ± 0.08	1.38	<0.001

*The Ct values are calculated based on six replicate reactions from two experiments.
†ΔCt = Ct (K562/HL60) − Ct (K562).
‡Analyzed by Student's *t*-test.

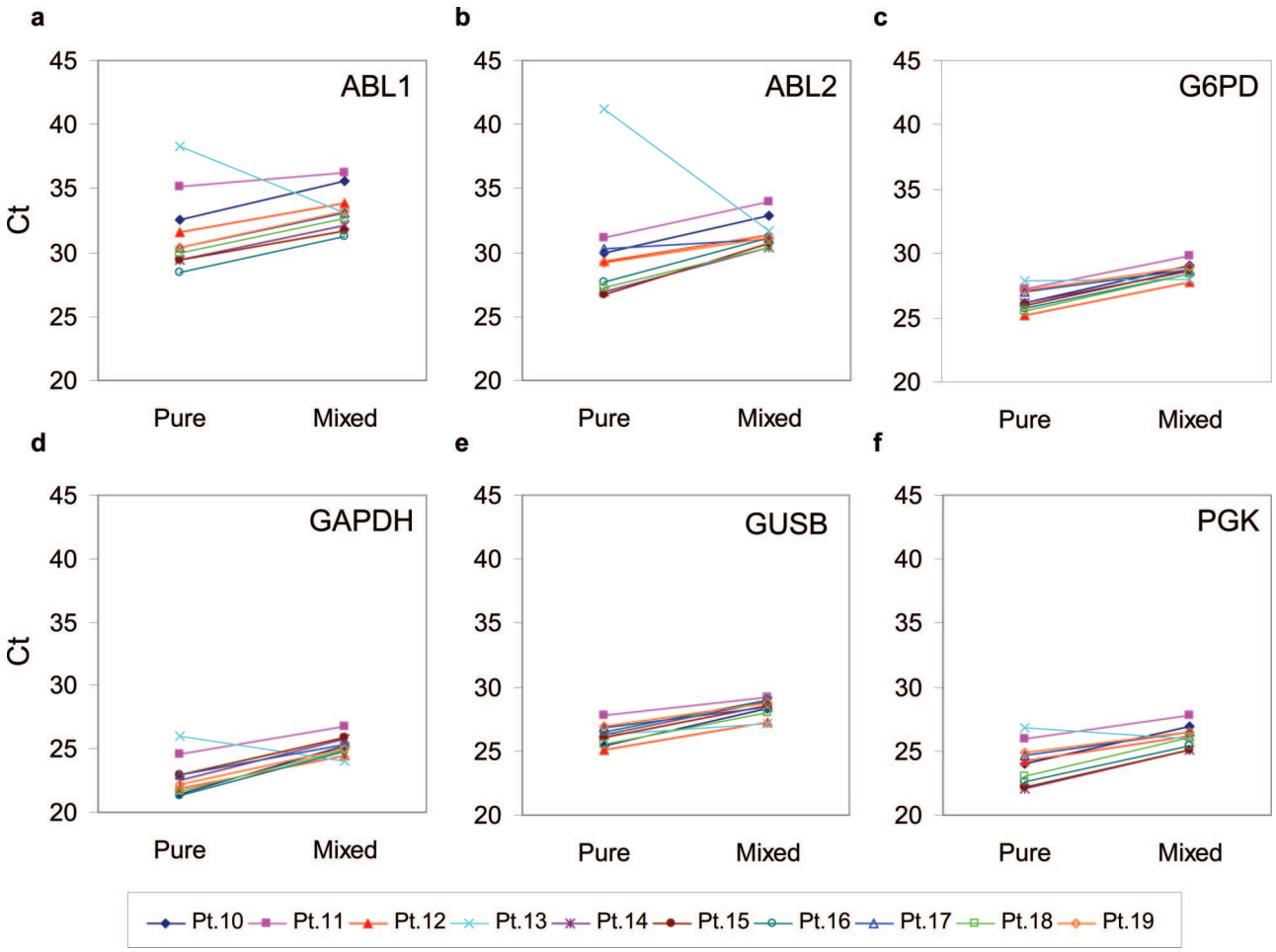


Figure 3. Threshold cycle difference of six internal control genes in 10 pairs of pure patient samples and mixed samples. cDNA from 10 patient specimens (Table 1, patients 10 to 19) were mixed with cDNA from normal peripheral blood mononuclear cells at 1:16 ratio. Levels of six different control genes as indicated in the graphs were determined in the 10 pairs of pure and mixed samples. Mean Ct values of triplicate real-time PCR reactions were plotted. Lines connect pure and mixed patient samples to show pairwised relationship.

tures with pure K562 cells. Specifically, the Ct by ABL1 in the cell mixture was 1.89 cycles lower than pure K562 cells, whereas Ct by ABL2 was 2.51 cycles higher (Table 3). These apparently paradoxical changes probably pertain to the distinct design of the two *ABL* primer/probe sets (Figure 1; see Discussion).

To further examine whether mRNA levels of the control genes vary in CML and non-CML cells taken from patients and to exclude that differences in expression were due to peculiarities of the cell lines tested, we mixed CML patient and normal specimens and compared Ct of mixed samples with pure patient samples. As with the study on cell lines, we expected no significant change in Ct on mixing if CML and normal peripheral blood mononuclear cells have similar levels of a control gene. Among six commonly used controls, Ct values of *G6PD* (Figure 3c) and *GUSB* (Figure 3e) had the least change between pure and mixed samples for all 10 specimens, although levels of *G6PD* appeared to be different in K562 and HL60 cells. *GAPDH* (Figure 3d) and *PGK* (Figure 3f) had intermediate differences between the pair of pure and mixed samples and were therefore not further analyzed. Notably, both ABL1 and ABL2 varied to a large degree between the sample pairs (Figure 3, a and b). The most dramatic change occurred in paired samples of patient 13. The Ct of both ABL1 and ABL2 dramatically decreased on mixing pure patient samples with the normal. Apparently, in this patient, using *ABL* as a normalization control for *BCR-ABL* quantification would definitely lead to erroneous results. Taken together with the study on cell lines, we conclude that *ABL* is not expressed at a similar level in CML and other hematopoietic cells and that ABL levels change as the ratio of CML to non-CML cells changes, even when total numbers of cells remain constant. In serial follow-up of treated patients, the *BCR-ABL*-to-*ABL* ratio would be misleading when used as a parameter to monitor residual disease.

Degradation Kinetics of BCR-ABL *and Control Genes*

To serve as a legitimate control, the degradation kinetics of a gene should ideally parallel that of *BCR-ABL* transcripts so that the *BCR-ABL*-to-control gene ratio remains the same even when RNA is partially degraded. To study

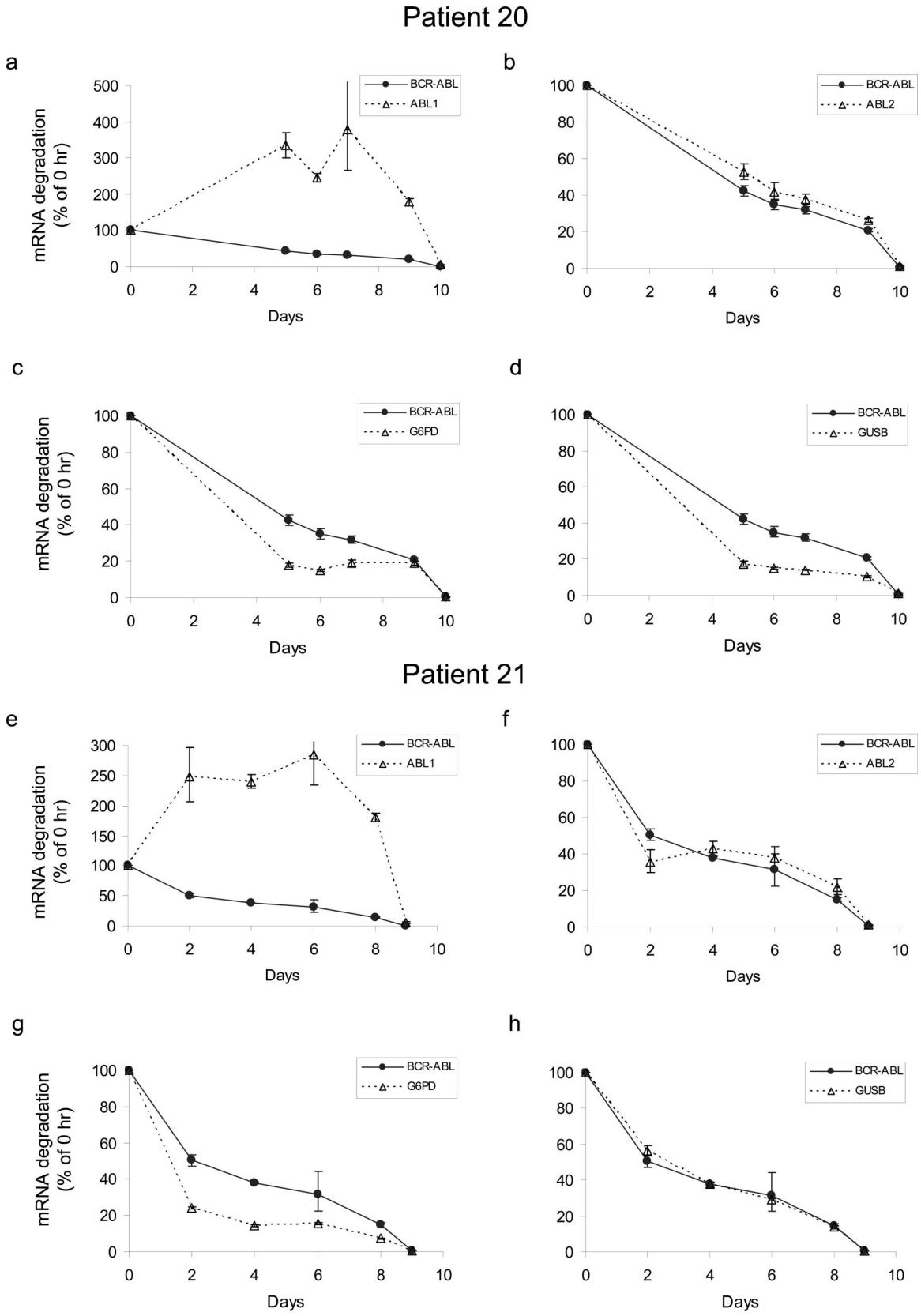


Figure 4. The degradation kinetics of four internal control transcripts in comparison with *BCR-ABL*. RNA was extracted from mononuclear cells from the bone marrow of two CML patients (Table 1, patients 20 and 21), placed at the bench for various periods of time. Real-time RT-PCR reactions were performed simultaneously for *BCR-ABL* and four different control genes as indicated in the legend boxes. Data at each time point after day 0 are expressed as a percentage of the initial time point calculated by the ΔCt method (for details, see Materials and Methods). Means ± SD are plotted. Note different *y*-axis scale is used in **a** and **e**.

Table 4. Reasons for Exclusion

Genes	Main reasons for exclusion
ABL1	Much higher Ct than *BCR-ABL*, different levels in CML and non-CML cells, different degradation kinetics from *BCR-ABL*, and being a translocation partner of *BCR-ABL*
ABL2	Different levels in CML and non-CML cells, and ability to detect the *ABL* portion of *BCR-ABL*
ACTB	More abundant than *BCR-ABL* and unusual amplification curve
B2M	More abundant than *BCR-ABL* and different levels in CML and non-CML cells
GAPDH	More abundant than *BCR-ABL* and different levels in CML and non-CML cells
G6PD	Presence of molecular variants and X chromosome location
HPRT	Different levels in CML and non-CML cells
PGK	Different levels in CML and non-CML cells
TBP	Different levels in CML and non-CML cells

the degradation kinetics of *BCR-ABL* and control genes, mononuclear cells from the bone marrow of two CML patients were placed at room temperature to let cells die and RNA degrade. Levels of *BCR-ABL*, *ABL* by ABL1 and ABL2, *G6PD,* and *GUSB* were determined by real-time RT-PCR in aliquots of cells collected at different time points. Expression levels relative to the immediate sample (day 0) were analyzed and plotted in Figure 4. Apparently, patient-to-patient variation in the degradation of *BCR-ABL* and the four control genes existed. Nevertheless, of the four controls studied, *G6PD*, *GUSB*, and ABL2 showed similar degradation kinetics to *BCR-ABL* in both patients. Although degradation of *ABL* by ABL2 primer/probe followed almost perfectly with that of *BCR-ABL* (Figure 4, b and f), this finding is not unexpected, because ABL2 detects the *ABL* portion of the *BCR-ABL* fusion transcripts. As for *ABL* levels measured by ABL1, we observed unexpected upward changes of *ABL* after the initial time point (Figure 4, a and e). Interestingly, this upward change in *ABL* level was seen in both patient samples, suggesting that the finding was not incidental. It is possible that transcription of ABL kinase is up-regulated in response to cellular stress, likely nutrient/growth factor deficiency experienced by cells placed on the bench. ABL is implicated in various signaling pathways initiated by growth factor, DNA damage, oxidative stress, and integrin stimulation.[24–27] No matter what causes this unexpected change in the *ABL* level, these results demonstrate that *ABL* is regulated by cellular conditions and cannot serve as a reference for *BCR-ABL* quantification, although it is widely used in current clinical practice.

Discussion

Recommendation of the Internal Control Gene for BCR-ABL *Quantification*

After applying three criteria to select the most suitable control genes, we have found that among nine genes tested with 10 primer/probe sets, only *G6PD* and *GUSB* meet all three criteria. Reasons for exclusion of other genes are summarized in Table 4. *G6PD*, as part of a commercially available kit for *BCR-ABL* quantification, is one of the most commonly used internal control genes. However, we do not recommend using *G6PD* for the following reasons. First, *G6PD* is located on the X chromosome. X chromosome location is normally avoided because there might be sex difference in expression levels.[23] Second, *G6PD* deficiency is a fairly common genetic abnormality leading to anemia. Up to 5% of Chinese, 20% of Italians, 32% of Greeks, and 65% of Saudis are affected by *G6PD* deficiency.[28] Furthermore, in the Online Mendelian Inheritance in Man database of the National Center for Biotechnology Information, 56 molecularly characterized variants of *G6PD* are currently documented together with a very long list of mutants that have not been characterized. We are concerned that frequent sequence variations may affect the binding of PCR primers and/or probe, leading to false-negative results.

In contrast, *GUSB* is located on the long arm of chromosome 7. Mutations in *GUSB* cause mucopolysaccharidosis (MPS) VII, also known as Sly syndrome. MPS is currently known to consist of 13 subclasses.[29] These are rare genetic disorders with a combined frequency of approximately 1 in 20,000.[28] Moreover, among the 13 subclasses, MPS VII is the rarest of all forms of MPS.[30] We therefore recommend *GUSB* over *G6PD* as the most suitable internal control for *BCR-ABL* quantification.

ABL *as the Internal Control Gene*

ABL is probably the most widely used normalization control for *BCR-ABL* quantification in Europe and North America. The EAC study has evaluated several commonly used control genes and concluded that *ABL* is the most suitable one.[23] However, the primer/probe set used to assay *ABL* level (designated as ABL2 in this study) also detects the *ABL* portion of the *BCR-ABL* transcript. The ratio of *BCR-ABL* to control then becomes *BCR-ABL*/(*BCR-ABL*+*ABL*) with a changing denominator. We question whether a translocation partner is qualified to serve as an internal control gene for normalization because different gene structures in the malignant and nonmalignant cells may lead to different gene expression levels. It is also of our concern that *ABL* assayed this way would change along with the *BCR-ABL* during the leukemia/treatment course, giving rise to an inaccurate *BCR-ABL*-to-control ratio. We therefore designed our study using different criteria for control gene selection. We mimicked diagnostic and residual disease specimens using pure samples and samples mixed with non-CML

hematopoietic cells. We selected control genes to ensure that for the same amount of RNA input, the level of a particular control gene does not change significantly between the pure and mixed samples. We found that *ABL* assayed by two differently designed primer/probe sets failed to meet this criterion. Lower Ct by ABL1 and higher Ct by ABL2 was observed in K562 cells mixed with HL60 cells (Table 3). Because a lower Ct value represents a higher level of mRNA, the lower Ct by ABL1 in the cell mixture (K562/HL60 = 1:9) suggests that a higher amount of *ABL* transcript is present in HL60 cells. This is not unexpected because HL60 cells contain two wild-type alleles of *ABL,* whereas K562 cells contains only one, and the other allele is disrupted by the translocation. Alternatively, expression of *BCR-ABL* may suppress the transcription of the wild-type *ABL* in K562, making it lower than HL60 cells. In contrast, ABL2 detects both wild-type *ABL* and translocated *ABL* in the *BCR-ABL* fusion transcript. The lower Ct value by ABL2 in K562 may simply reflect that the *BCR-ABL* plus *ABL* transcripts in K562 are much more abundant than *ABL* in HL60 cells. More importantly, in the mixing study performed with patient samples, *ABL* levels by ABL1 and ABL2 are different between pure and mixed samples, suggesting that the amounts of *ABL* message are different in CML and non-CML cells.

We have also applied another criterion that was not applied by the EAC study. In our opinion, degradation of the control gene should be proportional to degradation of the *BCR-ABL* transcripts. A control gene that is not degraded in the same fashion as *BCR-ABL* may lead to under- or overestimation of the *BCR-ABL*. This criterion is particularly important to diagnostic laboratories because varying degrees of degradation exist in clinical samples. We examined the degradation kinetics of several control genes and found that wild-type *ABL* assayed by ABL1 does not meet this criterion. Although degradation of *ABL* assayed by ABL2 paralleled that of the *BCR-ABL*, it is expected because ABL2 detects the *ABL* portion of the *BCR-ABL*.

An additional concern for use of *ABL* as an internal control is that the EAC study found that ABL2 amplifies genomic DNA in 7% of 150 samples tested. Ct values resulting from genomic amplification ranged from 35 to 45 cycles. As stated in the article, these high Ct values were far away from the Ct values obtained from good-quality RNA samples. However, RNA samples of variable quality are seen in routine clinical practice. In a partially degraded clinical specimen with high *ABL* Ct, contribution from amplification of *ABL* genomic locus would lead to a falsely low *BCR-ABL* result.

GUSB *as the Internal Control Gene*

By our three criteria plus the rare presence of sequence variations, we recommend *GUSB* as the most suitable control gene for *BCR-ABL* quantification. However, the EAC study found that *GUSB* levels are different between normal and leukemia samples at diagnosis. The leukemia samples used combined CML with acute lymphocytic leukemia and acute myelogenous leukemia samples in the analysis. Separate comparison of normal and CML samples was not provided. In addition, the primer/probe set of *GUSB* used in the EAC study was designed by the group and differs in nucleotide sequences from the commercially available set that we used in the current analysis, potentially accounting for the different results in the two studies. Of note, our studies were conducted using ABI primer/probe sets on ABI Prism 7000 instrument only. Whether similar results and conclusion can be obtained using other primer/probe sets for the same control genes or using other real-time instruments remains to be determined.

Conclusions

In conclusion, we recommend using *GUSB* assayed by the primer/probe set from ABI as the control gene for determination of *BCR-ABL* level in CML patients. Using a commercially available source of primer/probe facilitates standardization of reagents among different laboratories. It remains to be determined whether *GUSB* can be used as the control gene for quantification of fusion genes found in other types of leukemia. Because the degradation kinetics of each fusion gene is likely different, the control gene may need to be evaluated and selected on a target-by-target basis.

Note Added in Proof

After acceptance of the current article, a related manuscript[31] was accepted for publication and will be appearing in an upcoming issue of *The Journal of Molecular Diagnostics.* This study applied additional clinically relevant criteria for further evaluation of the control genes.

Acknowledgments

We thank Wayne Tam, M.D., Ph.D., and Botond Timar, M.D., for critical reading of the manuscript.

References

1. Nowell PC, Hungerford DA: A minute chromosome in human chronic granulocytic leukemia. Science 1960, 132:1497–1499
2. Rowley JD: Letter: a new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 1973, 243:290–293
3. Hochhaus A, Weisser A, La Rosee P, Emig M, Muller MC, Saussele S, Reiter A, Kuhn C, Berger U, Hehlmann R, Cross NC: Detection and quantification of residual disease in chronic myelogenous leukemia. Leukemia 2000, 14:998–1005
4. Kantarjian H, Talpaz M, O'Brien S, Garcia-Manero G, Verstovsek S, Giles F, Rios MB, Shan J, Letvak L, Thomas D, Faderl S, Ferrajoli A, Cortes J: High-dose imatinib mesylate therapy in newly diagnosed Philadelphia chromosome-positive chronic phase chronic myeloid leukemia. Blood 2004, 103:2873–2878
5. Hughes TP, Kaeda J, Branford S, Rudzki Z, Hochhaus A, Hensley ML, Gathmann I, Bolton AE, van Hoomissen IC, Goldman JM, Radich JP: Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. N Engl J Med 2003, 349:1423–1432

6. Rosti G, Martinelli G, Bassi S, Amabile M, Trabacchi E, Giannini B, Cilloni D, Izzo B, De Vivo A, Testoni N, Cambrin GR, Bonifazi F, Soverini S, Luatti S, Gottardi E, Alberti D, Pane F, Salvatore F, Saglio G, Baccarani M: Molecular response to imatinib in late chronic-phase chronic myeloid leukemia. Blood 2004, 103:2284–2290
7. Mauro MJ, Druker BJ, Maziarz RT: Divergent clinical outcome in two CML patients who discontinued imatinib therapy after achieving a molecular remission. Leuk Res 2004, 28(Suppl 1):S71–S73
8. Cortes J, O'Brien S, Kantarjian H: Discontinuation of imatinib therapy after achieving a molecular response. Blood 2004, 104:2204–2205
9. Hochhaus A, La Rosee P: Imatinib therapy in chronic myelogenous leukemia: strategies to avoid and overcome resistance. Leukemia 2004, 18:1321–1331
10. Druker BJ: Overcoming resistance to imatinib by combining targeted agents. Mol Cancer Ther 2003, 2:225–226
11. Lee M, Khouri I, Champlin R, Kantarjian H, Talpaz M, Trujillo J, Freireich E, Deisseroth A, Stass S: Detection of minimal residual disease by polymerase chain reaction of bcr/abl transcripts in chronic myelogenous leukaemia following allogeneic bone marrow transplantation. Br J Haematol 1992, 82:708–714
12. Hochhaus A, Reiter A, Saussele S, Reichert A, Emig M, Kaeda J, Schultheis B, Berger U, Shepherd PC, Allan NC, Hehlmann R, Goldman JM, Cross NC: Molecular heterogeneity in complete cytogenetic responders after interferon-alpha therapy for chronic myelogenous leukemia: low levels of minimal residual disease are associated with continuing remission. German CML Study Group and the UK MRC CML Study Group. Blood 2000, 95:62–66
13. Paschka P, Muller MC, Merx K, Kreil S, Schoch C, Lahaye T, Weisser A, Petzold A, Konig H, Berger U, Gschaidmeier H, Hehlmann R, Hochhaus A: Molecular monitoring of response to imatinib (Glivec) in CML patients pretreated with interferon alpha. Low levels of residual disease are associated with continuous remission. Leukemia 2003, 17:1687–1694
14. Emig M, Saussele S, Wittor H, Weisser A, Reiter A, Willer A, Berger U, Hehlmann R, Cross NC, Hochhaus A: Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real time quantitative RT-PCR. Leukemia 1999, 13:1825–1832
15. Schoch C, Schnittger S, Bursch S, Gerstner D, Hochhaus A, Berger U, Hehlmann R, Hiddemann W, Haferlach T: Comparison of chromosome banding analysis, interphase- and hypermetaphase-FISH, qualitative and quantitative PCR for diagnosis and for follow-up in chronic myeloid leukemia: a study on 350 cases. Leukemia 2002, 16:53–59
16. Preudhomme C, Revillion 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
17. Branford S, Hughes TP, Rudzki Z: Monitoring chronic myeloid leukaemia therapy by real-time quantitative PCR in blood is a reliable alternative to bone marrow cytogenetics. Br J Haematol 1999, 107:587–599
18. Neumann F, Herold C, Hildebrandt B, Kobbe G, Aivado M, Rong A, Free M, Rossig R, Fenk R, Schneider P, Gattermann N, Royer-Pokora B, Haas R, Kronenwett R: Quantitative real-time reverse-transcription polymerase chain reaction for diagnosis of BCR-ABL positive leukemias and molecular monitoring following allogeneic stem cell transplantation. Eur J Haematol 2003, 70:1–10
19. Jones CD, Yeung C, Zehnder JL: Comprehensive validation of a real-time quantitative bcr-abl assay for clinical laboratory use. Am J Clin Pathol 2003, 120:42–48
20. Eder M, Battmer K, Kafert S, Stucki A, Ganser A, Hertenstein B: Monitoring of BCR-ABL expression using real-time RT-PCR in CML after bone marrow or peripheral blood stem cell transplantation. Leukemia 1999, 13:1383–1389
21. Gabert J, Beillard E, van der Velden VH, Bi W, Grimwade D, Pallisgaard N, Barbany G, Cazzaniga G, Cayuela JM, Cave H, Pane F, Aerts JL, De Micheli D, Thirion X, Pradel V, Gonzalez M, Viehmann S, Malec M, Saglio G, van Dongen JJ: Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia: a Europe Against Cancer program. Leukemia 2003, 17:2318–2357
22. Zanella I, Rossi G, Finazzi D, Capucci A, Albertini A, Cariani E: Quantification of bcr/abl mRNA expression by a rapid real-time reverse transcription-polymerase chain reaction assay in patients with chronic myeloid leukemia. Haematologica 2001, 86:318–319
23. Beillard E, Pallisgaard N, van der Velden VH, Bi W, Dee R, van der Schoot E, Delabesse E, Macintyre E, Gottardi E, Saglio G, Watzinger F, Lion T, van Dongen JJ, Hokland P, Gabert J: Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR): a Europe against cancer program. Leukemia 2003, 17:2474–2486
24. Van Etten RA: c-Abl regulation: a tail of two lipids. Curr Biol 2003, 13:R608–R610
25. Hernandez SE, Krishnaswami M, Miller AL, Koleske AJ: How do Abl family kinases regulate cell shape and movement? Trends Cell Biol 2004, 14:36–44
26. Wange RL: TCR signaling: another Abl-bodied kinase joins the cascade. Curr Biol 2004, 14:R562–R564
27. Shaul Y, Ben-Yehoyada M: Role of c-Abl in the DNA damage stress response. Cell Res 2005, 15:33–35
28. Connor M, Ferguson-Smith M: Medical Genetics, ed 5. Oxford, Blackwell Science Ltd, 1997
29. Goetz CG: Textbook of Clinical Neurology, ed 2. St. Louis, MO, W.B. Saunders Company, 2003
30. Behrman RE, Kliegman RM, Jenson HB: Nelson Textbook of Pediatrics, ed 17. St. Louis, MO, W.B. Saunders Company, 2004
31. Lee JW, Chen Q, Knowles DM, Cesarman E, Wang YL: β-Glucoronidase is an optimal normalization control gene for molecular monitoring of chronic myelogenous leukemian. J Mol Diagn 2006, (in press)