β -Glucuronidase Is an Optimal Normalization Control Gene for Molecular Monitoring of Chronic Myelogenous Leukemia

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Quantitative monitoring of breakpoint cluster region (BCR)-Abelson kinase (ABL) transcripts has become indispensable in the clinical care of patients with chronic myelogenous leukemia. Because quantity and quality of RNA in clinical samples are highly variable, a suitable internal normalization control is required for accurate BCR-ABL quantification. However, few studies have examined suitability of the control genes using criteria relevant to residual disease testing. In this study, we evaluated a number of control genes with the application of several novel criteria, including control gene performance on serial patient sample testing and in a residual disease model. We also examined expression of the control genes in BCR-ABL-positive K562 cells in response to Gleevec treatment. We found that β -glucuronidase is the best control gene among those studied. Importantly, ABL, a widely used control gene, generates misleading BCR-ABL changes that potentially affect the clinical management of chronic myelogenous leukemia patients. (*J Mol Diagn* 2006, 8:385–389; DOI: 10.2353/jmoldx.2006.050150)

Quantitative determination of breakpoint cluster region (BCR)-Abelson kinase (ABL) transcripts using real-time polymerase chain reaction (PCR) technology is currently the method of choice for chronic myelogenous leukemia (CML) monitoring. It is used to assess patients' therapeutic response to Gleevec treatment.^{1–4} Rising BCR-ABL transcripts suggest acquired resistance and are used as an indicator for ABL mutation analysis.^{5–7} BCR-ABL monitoring will also be indispensable for clinical trials of emerging new drugs.^{8,9}

Application of an appropriate internal normalization control is essential for an accurate determination of BCR-ABL levels because RNA derived from clinical samples varies to a large degree in both quality and quantity. However, it is unclear what control genes are the most appropriate to serve for this purpose. Currently, many different genes are used in clinical practice,^{2,4,10,11} mak-

ing it hard to compare results from one laboratory to another. As an effort to unify the methodology, the Europe Against Cancer (EAC) program conducted a comprehensive study evaluating 14 commonly used control genes, including ABL, β -glucuronidase (GUSB), and β 2-microglobulin.^{12,13} Major criteria applied in the study included absence of pseudogenes, high or medium expression level, and similar control gene expression between normal and leukemic peripheral blood (PB) samples and between PB and bone marrow (BM) samples. In a follow-up investigation by EAC, the stability of several selected control genes was also studied.¹⁴ Based on these evaluations, it was concluded that ABL is the most suitable control gene for normalization of leukemic fusion genes. However, this study raised some concerns. First, the primers for ABL control amplify not only normal ABL but also the BCR-ABL fusion transcripts. Consequently, the ratio of BCR-ABL/control becomes BCR-ABL/(BCR-ABL + ABL). Because BCR-ABL changes with therapy, the denominator used as a normalization control changes along with it. Second, diagnostic specimens were used to evaluate the control genes that may be of limited relevance to residual disease monitoring.

In a previous study, we compared nine control genes for three criteria, including their mRNA expression levels in CML diagnostic specimens, their expression levels in CML versus non-CML cells, and their degradation kinetics. We found that GUSB is the most suitable control for BCR-ABL quantification.¹⁵ In the current study, we applied several novel criteria, including 1) how control genes perform on serial sample testing, 2) how control genes perform in a residual disease model, and 3) whether control gene expression changes significantly with Gleevec treatment. Unlike previous reports that studied one time point during the disease course,^{12,15} the dynamics of BCR-ABL and control genes at different times in the treatment course were evaluated. Residual disease specimens or mimics of such were used whenever possible, making it highly relevant to CML monitoring.

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Materials and Methods

Specimens

PB or BM aspirates were obtained from 12 CML patients under an institutional review board protocol. For serial testing, pairs of samples were of the same specimen type, either PB or BM aspirates. Leukocytes from a healthy donor were purchased from the New York Blood Center (New York, NY). Mononuclear cells were isolated using Ficoll-Paque PLUS density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions.

Cell Culture and Treatment

BCR-ABL-positive K562 cells were cultured in Iscove's modified Dulbecco's medium (American Type Culture Collection, Manassas, VA) supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin. Cells were incubated at 37°C under 5% CO₂ and subcultured 1:4 every 96 hours. Cells (1×10^6 /ml) were treated with 1 μ mol/L Gleevec that was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland). Fresh medium containing 1 μ mol/L Gleevec was added to the culture every 48 hours to maintain the cell density and nutrition balance. A dose of 1 μ mol/L was chosen because it is the concentration of Gleevec that is required to inhibit *ABL* tyrosine kinase activity *in vitro*, and the dose is below the steady-state plasma concentrations of Gleevec (2.0 to 4.4 μ mol/L) in patients taking a daily dose of 400 mg.¹⁶ Cells were collected for reverse transcriptase-PCR (RT-PCR) analysis before and at various times after treatment.

RNA Extraction, 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 μ L 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- μ L reaction volume using Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's protocol.

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 μ L of $1 \times$ TaqMan Universal PCR master mix. The reaction contains 300 nmol/L of primers and 200 nmol/L probe. PCR was conducted using 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.

TaqMan primer and probe sets for detection of glucose 6-phosphate dehydrogenase (*G6PD*) (hs00166169_m1), *GUSB* (4333767F), and TATA-box binding protein (*TBP*) (4333769F) were purchased from ABI. Sequences of these primers and probes are not provided by the manufacturer. Two *ABL* primer/probe sets were custom-made by ABI, and their sequences and relative positions to *ABL* and *BCR-ABL* genes are illustrated in Figure 1.¹⁵ Real-time PCR results were analyzed with ABI Prism 7000 SDS software, and auto-thresholds and auto-baselines determined by the software for each individual gene target were applied to generate values of corresponding threshold cycles.

Results and Discussion

Based on the previous study, four control genes detected by five primer-probe sets (referred to as five control genes hereafter for simplicity) were selected for the current investigation: *G6PD*, *GUSB*, *TBP*, *ABL1* (which detects normal *ABL* transcripts), and *ABL2* (which detects both normal *ABL* and the *BCR-ABL* fusion transcripts; same as the EAC primer/probe, illustrated in Figure 1). We first tested how the control genes performed in serial sample testing. Pairs of samples were obtained at different times from six patients treated with Gleevec. Fold reduction in *BCR-ABL* between the first and second samples (R) were determined using five different control genes as the normalizer. Thus, five Rs were generated for each pair of samples. Theoretically, these Rs should be the same for the same patient, no matter which normalizer is used. However, as shown in Figure 2, big differences existed among *BCR-ABL* levels normalized by different control genes. Specifically, in patients 1, 2, and 3, fold reductions between two samples generated using *ABL2* as the normalizer appeared much larger than those generated using other control genes. For example, in patient 1, Rs by *ABL1*, *G6PD*, *GUSB*, and *TBP* were all less than fivefold (3.4, 0.8, 0.7, and 3.3, respectively), suggesting that the patient did not respond to treatment during this period, whereas R by *ABL2* was 16.1, suggesting that the patient responded well. The fivefold cutoff was used to distinguish a responder from a nonresponder because the interassay variability of our real-time RT-PCR assay may generate up to fivefold difference with a single specimen (see Materials and Methods). Similarly, in patients 4, 5, and 6, using *ABL1* to normalize *BCR-ABL* led to results that appeared much larger than values by other control genes. Notably, fold reductions in *BCR-ABL* normalized with *G6PD* and *GUSB* are very close for all six individuals.

We next evaluated the control genes using a clearly defined residual disease model. RNA samples from six CML patients were diluted 1:16 with RNA from a normal individual to mimic residual disease. Ratio of *BCR-ABL* transcripts in the pure sample to that in the diluted samples (R') was determined. Five R's were generated for each pair of samples using five normalizers, respectively,

Figure 1. Schematic diagram of the two different sets of primers/probes for *ABL* quantification (left, ABL1; right, ABL2). Light shaded cylinders represent *ABL* cDNA, and dark shaded cylinders represent *BCR-ABL* 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. **Left:** The forward primer of ABL1 set hybridizes to the exon 1, and the reverse 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. No PCR products are generated once *ABL* is fused to *BCR*. **Right:** In comparison, the forward primer of ABL2 set hybridizes to exon 2, and the reverse primer and probe hybridize to exon 3 of the *ABL* gene. It therefore detects both the wild-type *ABL* and translocated *BCR-ABL* messages. (Reprinted from J Mol Diagn 2006, 8:231–239 with permission from the American Society for Investigative Pathology and the Association for Molecular Pathology.)

and they are expected to be 16 by design. As shown in Figure 3, for all six patients, R's generated using *G6PD* and *GUSB* as normalizers clustered around 16 as expected. In contrast, normalization using ABL1 or ABL2 generated obviously wrong ratios in some cases. Taking patient 5 for example, normalization to ABL1 led to a result of ~160, which is nine times higher than the true

value. Taken together, these data provide strong evidence that *ABL* does not perform well as a normalization control.

By definition, genes used as normalization controls should not change significantly with cellular conditions. However, the literature suggests that *ABL* is involved in various signaling pathways initiated by growth factors,

Figure 2. Performance of the control gene in serial sample testing. *BCR-ABL* was first normalized to each control gene, as indicated in the legend box, to obtain R_{first} and R_{second} for the first and second serial samples. Fold reduction from the first to the second samples were then calculated to obtain $R = R_{first}/R_{second}$.

Figure 3. Performance of the control genes in a residual disease model. *BCR-ABL* was first normalized to each control gene, as indicated on the x axis, for pure and diluted samples to generate R_{pure} and R_{diluted} . The ratio of the amount of *BCR-ABL* transcript in pure patient samples and the corresponding 1:16 diluted samples were then calculated as $R' = R_{\text{pure}}/R_{\text{diluted}}$. The horizontal line intercepts with the y axis at 16.

DNA damage, and oxidative stress.^{17–19} In a previous study, we observed an unexpected rise of *ABL* mRNA after patient CML cells were left to die on the bench, whereas messages of other genes decrease with time as expected for RNA degradation.¹⁵ This finding suggests the possibility that transcription of *ABL* kinase is up-regulated in response to cellular stress. To further test this notion, we treated *BCR-ABL*-positive K562 cells with Gleevec and followed the mRNA of *BCR-ABL* and the control genes through the treatment course. As shown in Figure 4, most of the control genes decreased along with *BCR-ABL* during treatment. However, normal *ABL* mRNA detected by ABL1 was elevated during the first 2 days of

Gleevec treatment. *ABL* messages detected by ABL2 followed that of *BCR-ABL*. This was not unexpected because ABL2 also detects the *BCR-ABL* fusion transcripts.

In summary, using novel criteria that are emphasized in residual disease testing, we demonstrated that among the control genes studied, *G6PD* and *GUSB* meet all criteria as suitable control genes for *BCR-ABL* quantification. *G6PD* is not chosen because frequent mutations in the gene may impair the binding of PCR primers/probe, leading to false negative amplification. *G6PD* deficiency is a common genetic lesion that affects hundreds of millions of people, and more than 300 molecular variants throughout the gene have been described.²⁰ In contrast, mutations in the *GUSB* gene are rare events. Disease caused by *GUSB* mutations affects less than 1 in 20,000 of the population.²¹ For this reason, *GUSB* is preferred to *G6PD* as the control gene for *BCR-ABL* quantification.

As to ABL2, one may argue that it may be used when leukemic burden is reduced by therapy, because at that time, *BCR-ABL* in the denominator of the formula $BCR-ABL/(BCR-ABL + ABL)$ may be dropped, reducing the formula to $BCR-ABL/ABL$. However, normal *ABL* that is left in the denominator is regulated under different cellular conditions, as shown by both the current and previous studies. We conclude that no matter how primers are designed, they generate *BCR-ABL* levels that may mislead clinical decisions in many cases.

In conclusion, we found that *GUSB* is the control gene of choice for accurate quantitative determination of *BCR-ABL*, and we recommend its usage in standardized practice. *GUSB* is likely to be suitable for quantification of

Figure 4. Performance of the control genes in response to Gleevec treatment. K562 cells ($1 \times 10^6/\text{ml}$) were treated with $1 \mu\text{mol/L}$ Gleevec. Fresh medium containing $1 \mu\text{mol/L}$ Gleevec was added to the culture every 48 hours to maintain the cell density and nutrition balance. Cells were collected for RT-PCR analysis before and at indicated times after treatment. Data plotted are mean $\pm$ SD of the three independent experiments.

other leukemic fusion genes, but this remains to be determined.

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