

Detection and Quantification of the Abelson Tyrosine Kinase Domains of the *bcr-abl* Gene Translocation in Chronic Myeloid Leukaemia Using Genomic Quantitative Real-time Polymerase Chain Reaction

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Abstract

Introduction: Since undetectable BCR-ABL mRNA transcription does not always indicate eradication of the Ph⁺ CML clone and since transcriptionally silent Ph⁺ CML cells exist, quantitation by genomic PCR of *bcr-abl* genes can be clinically useful. Furthermore, hotspot mutations in the Abelson tyrosine kinase (ABLK) domain of the *bcr-abl* gene translocation in Philadelphia chromosome-positive (Ph⁺) chronic myeloid leukaemia (CML) cells confer resistance on the specific kinase blocking agent, STI571. **Materials and Methods:** Genomic DNA from K562, CESS and patient CML cells were amplified using rapid cycle quantitative real-time polymerase chain reaction for the gene regions spanning the mutation hotspots. In assays for ABLK exons 4 or 6, exonic or intronic PCR primers were used. **Results:** We show that separation of cycle threshold (CT) values for log-fold amplicon quantification was 2.9 cycles for ABLK exon 4, and 3.8 cycles for exon 6 with rapid amplification times. K562 CML cells were found to have a ~2 log-fold ABLK gene amplification. In contrast, patient CML cells had CT differences of 2.2 for both exon, suggesting that there was no significant ABLK gene amplification. DNA sequencing confirmed that neither K562 nor patient CML cells contained ABLK hotspot mutations. Messenger RNA transcription analysis permitted the assessment of BCR-ABL transcription, which was qualitatively correlated to genomic amplification. **Conclusions:** This novel Q-PCR assay was found to have high fidelity and legitimacy, and potentially useful for monitoring minimal residual disease, transcriptionally silent Ph⁺ CML cells, and *bcr-abl* gene amplification.

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Introduction

Chronic myeloid leukaemia (CML) is a clonal stem cell malignancy characterised by massive proliferation of mature and immature granulocytes, basophils and spleen cells, but not cells of T cell lineage.¹ The molecular hallmark of CML is the reciprocal translocation between chromosomes 9 and 22, t(9;22), which produces the Philadelphia (Ph) chromosome, the *bcr-abl* hybrid gene, the BCR-ABL mRNA transcript and the BCR-ABL fusion protein.²⁻⁴ Even in certain Ph chromosome-negative (Ph⁻) CML patients, the presence of a legitimate BCR-ABL mRNA transcript is detectable by polymerase chain reaction (PCR),⁵ and this occurs because of relocation of the t(9;22) complex

containing the *bcr-abl* hybrid gene into another part of the genome that is permissive to transcription.

Philadelphia chromosome-positive (Ph⁺) CMLs constitute greater than 90% of cases,⁶ and are thought to arise directly as a result of the oncogenic activity⁷ of a 210 kiloDalton (kDa) tyrosine kinase (p210^{BCR-ABL} or BCR-ABL).⁸ Specifically, the Abelson kinase (ABLK) domain on the ABL portion of BCR-ABL fusion protein is a constitutively active protein kinase,⁹ which deregulates intracellular signal transduction pathways, and thereby mediates abnormal cell cycling, inhibition of apoptosis, and increased cell proliferation.¹⁰⁻¹² A major advancement in the treatment of CML came with the discovery that a specific inhibitor of the

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activation loop of the ABLK, STI571 or imatinib or Glivec (Novartis Pharma, Basel, Switzerland),^{13,14} and blockade of ABLK by STI571 leads to growth arrest and apoptosis of CML cells. As expected, this occurs only in Ph⁺ and not in Ph⁻ CML cells.¹⁵ Major clinical and cytogenetic responses have been reported in Ph⁺ CML patients treated with STI571, including patients in blast crises.¹⁶⁻¹⁸

The value of PCR, especially reverse transcription (RT)-PCR and more recently, quantitative real-time RT-PCR (RQ-PCR), as tools for monitoring BCR-ABL transcription and minimal residual disease (MRD) during treatment of CML is undisputed.¹⁹ However, it is not clear whether undetectable BCR-ABL mRNA transcription equates to eradication of the Ph⁺ CML clone^{20,21} since transcriptionally silent Ph⁺ CML cells clearly exist.^{22,23} In fact, DNA analysis becomes even more important in STI571-treated patients because of several reasons, including: the increased likelihood of transcriptionally silent Ph⁺ CML clones that contain ABLK mutations; as well as the emergence of clonal Ph⁻ chromosomal abnormalities,²⁴⁻²⁶ other Ph⁻ disorders, including myelodysplastic syndrome (MDS)²⁷ and acute lymphoblastic leukaemia.^{28,29}

In this study, we report the development of a rapid and sensitive method of detecting and quantifying exons 4 and 6 of the ABLK domain using gDNA and rapid cycle quantitative real-time PCR (Q-PCR) on the LightCycler System (Roche Diagnostics, Mannheim, Germany), where over 18 mutations of the ABLK were found. This method potentially facilitates diagnosis and monitoring of all CML patients regardless of Ph status, and is especially useful in characterising MRD, transcriptionally silent Ph⁺ CML patients and patients who attain Ph-negativity following successful treatment, especially if the prior treatment was STI571. This method is useful for analysis of archival tissue [e.g., frozen peripheral blood (PB) samples], and for determining ABLK gene amplification. It is also simple and rapid, and hence, highly applicable for use in a high turnover haematology clinic.

Materials and Methods

Cells and Cell Culture

Fresh CML cells were obtained (with informed consent) from the PB of a patient with Ph⁺ CML in the accelerated phase. Patient CML cells were tested to be Ph⁺ using routine conventional nested PCR. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque density gradient sedimentation (Amersham, Pharmacia, Uppsala, Sweden), and used for subsequent experiments. K562 CML (CCL-243) and CESS Epstein-Barr virus (EBV)-immortalised (TIB-190) cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI 1640 with

L-glutamine medium supplemented with 10% fetal bovine serum (FBS), 25 IU/mL penicillin, 25 mg/mL streptomycin, 5 mmol/L L-glutamine (all from Life Technologies, Invitrogen, Carlsbad, CA), at 37°C, in 5% CO₂ and a humidified atmosphere.

Extraction of gDNA and Total RNA

Genomic DNA was extracted from purified PB MNCs of a patient with Ph⁺ CML in accelerated phase, as well as a K562 CML and CESS EBV-immortalised normal B cell (control) cell lines, using the Tissue DNeasy Kits (Qiagen GmbH, Hilden, Germany). Total mRNA was extracted from both cell lines using the RNeasy Mini kit from the same manufacturer. One nanogram of DNA was used in each Q-PCR reaction, and 250 ng of total RNA was used in each RQ-PCR reaction.

Q-PCR

Rapid cycle quantitative real-time PCR was performed on 10.0 µL final volume using FastStart DNA Master SYBR Green I Kit (Roche Diagnostics), in accordance with the manufacturer’s protocol and the Roche Light Cycler instrument. The CESS EBV-immortalised normal B (control) cell line was used for optimisation reactions. For Q-PCR optimisation, varying MgCl₂ concentrations, forward/reverse primer concentrations, amounts of gDNA, or annealing temperatures were used. Primers used in the experiments for Q-PCR and RQ-PCR (Alpha DNA, Montreal, Quebec) were designed using the Roche Probe Design Software.

ABL exon 4 F	5'-TCCAGCCCCAAAGCGCAAC-3'	58,683 to 58,701
ABL exon 4 R	5'-CACGGCCACCGTCAGG-3'	58,848 to 58,833
ABL exon 6 F	5'-CAGTTGGGAGCGGAGCC-3'	68,636 to 68,652
ABL exon 6 R	5'-CCACGCCCTGTGACTCCAT-3'	68,909 to 68,891

The fluorescence signal was continuously measured during both the amplification as well as the melting phases of Q-PCR; i.e., both quantification crossing point (CP) or cycle threshold,^{30,31} as well as melting curve data were analysed (Roche LightCycler Data Analysis Software, Roche Diagnostics). CP/CT here is defined as cycle number where fluorescence levels of all samples are the same or just above the background level. Optimised Q-PCR conditions were then used to amplify gDNA from K562 (known to have amplification of ABLK gene, but no ABLK mutations),^{32,33} and CML patient (unknown ABLK gene status) PBMCs.

RQ-PCR

Similarly, rapid-cycle quantitative real-time PCR was performed on 20.0 µL final volume using FastStart RNA Master SYBR Green I Kit (Roche Diagnostics), in accordance with the manufacturer’s protocol. Following reverse transcription, optimised Q-PCR conditions were

applied to complete the RQ-PCR [61°C for 20 min for reverse transcriptase; 95°C for 30 sec for initial denaturing 35 cycles of 4 sec (95°C), 5 sec (61°C), and 7 sec (72°C) for amplification of cDNA]. As in above, the fluorescence signal was continuously measured during both the amplification as well as the melting phases of Q-PCR; i.e., both quantification cycle threshold,^{30,31} as well as melting curve data were analysed. Only ABLK exon 4 was analysed because the primers used were entirely exonic and expected to anneal to cDNA obtained following reverse transcription. In contrast, ABLK exon 6 primers were intronic and not expected to anneal to cDNA. The fluorescence signal was continuously measured during both the amplification as well as the melting phases of Q-PCR.

Purification and DNA Sequencing of PCR Products

The Q-PCR products were resolved in a 2% agarose gel and ethidium bromide staining to determine the size of the products. Thereafter, PCR products were purified using the MinElute PCR Purification Kit (Qiagen), followed by DNA sequencing using the Big-Dye terminator method (PE Biosystems, Foster City CA 94404, USA), in 10.0 mL final volume, according to the manufacturer's recommendation. The same forward and reverse PCR primers were used for DNA sequencing on the ABI PRISM 3100 genetic analyser (Applied Biosystems Asia Pte Ltd).

Results

Optimisation of Q-PCR MgCl₂ and Primer Concentration for Amplification of ABLK Exons 4 and 6 in the CESS Cell Line

In this study, we first determined optimal conditions for Q-PCR for both exons 4 and 6 of the kinase domain of ABLK of the *bcr-abl* gene. The first parameter analysed was the Mg²⁺ concentration. We used the CESS EBV-immortalised normal B cell line, which has normal ABLK genes, for the optimisation studies. Optimal MgCl₂ concentrations for exons 4 and 6 were 4.0 mM and 3.0 mM (Figs. 1a and 1b), respectively. Moreover, the best CT values for exons 4 and 6 were 22.5 cycles and 19.8 cycles, respectively. Melt curve analyses demonstrated low primer-primer dimer signals even among bracketed reactions, suggesting high fidelity of the Q-PCR reactions (Figs. 1c and 1d).

Having determined optimal Mg²⁺ concentrations for Q-PCR, we next tested primer concentrations for detecting exon 4 and exon 6 ABLK mutations. Figures 1e and 1f show that the optimum primer concentration was 0.7 mM for both ABLK exons 4 and 6. Again, the best CT values for exons 4 and 6 were 24.8 cycles and 20.8 cycles, respectively. Melt curve analyses showed low primer-dimer signals even among reactions. Moreover, fidelity of

Q-PCR was good and appeared to be more efficient for exon 6 (Fig. 1h) than exon 4 (Fig. 1g).

Optimisation of Genomic DNA and Annealing Temperature for Q-PCR Amplification of ABLK Exons 4 and 6 in the CESS Cell Line

We next optimised Q-PCR reactions for the amount of gDNA, using optimal concentrations of MgCl₂ and primer concentrations of 0.7 μM. Figures 2a and 2b show good (~3 cycles per log-fold difference) separation of CT values for gDNA between 0.1 and 10.0 ng (i.e., over 2 log-fold) differences. Specifically, ABLK exon 4 log-fold signal separation between CTs were 26.8, 24.0 and 21.0; and exon 6 CTs were 28.7, 25.6 and 21.2, respectively. These data suggest that the assays were sensitive and quantitative. Separation of CT values for log-fold amplicon quantification was 2.9 (2.8 to 3.0) cycles for ABLK exon 4, and 3.8 (3.1 to 4.4) cycles for exon 6. Again, melt curve analyses demonstrated good fidelity of Q-PCR (Figs. 2c and 2d).

To complete optimisation of Q-PCR, the annealing temperature was varied, whilst keeping the above optimised conditions for Mg²⁺, primer concentrations and amount of DNA. The optimum annealing temperature was found to be 67°C for both exon 4 and exon 6 (Figs. 2e and 2f). The final best CT values between exons 4 and 6 were very similar, 21.7 cycles and 23.8 cycles, respectively. And again, melt curve analyses showed good fidelity of the Q-PCR reaction (Figs. 2g and 2h). The final optimised conditions for Q-PCR of ABLK exon 4 or exon 6 are summarised in Table 1. PCR amplification of the *actin* housekeeping gene indicated good quality of the genomic DNA in all samples; e.g., the CT values were equal and the melting curves produced similar peaks) (data not shown).

ABLK Exons 4 and 6 Gene Amplification in K562 and Patient CML Cells

Having completed optimisation of the Q-PCR reaction for ABLK exons 4 and 6, we next studied ABLK gene

Table 1. Optimal Q-PCR Conditions Used for ABLK Exons 4 and 6 Hotspot Mutation Detection

Variables	Exon 4	Exon 6
DNA concentration (ng)	1.0	1.0
MgCl ₂ (mM)	4.0	3.0
Forward primer (μM)	0.7	0.7
Reverse primer (μM)	0.7	0.7
Denaturation (°C/s)	94.0/5.0	94.0/5.0
Annealing, T _m (°C/s)	67.0/5.0	67.0/5.0
Extension (°C/s)	72.0/7.0	72.0/11.0
Cycle time (s)	17.0	21.0
Number of cycles	30	30

The final reaction volume was 10.0 μL/sample.

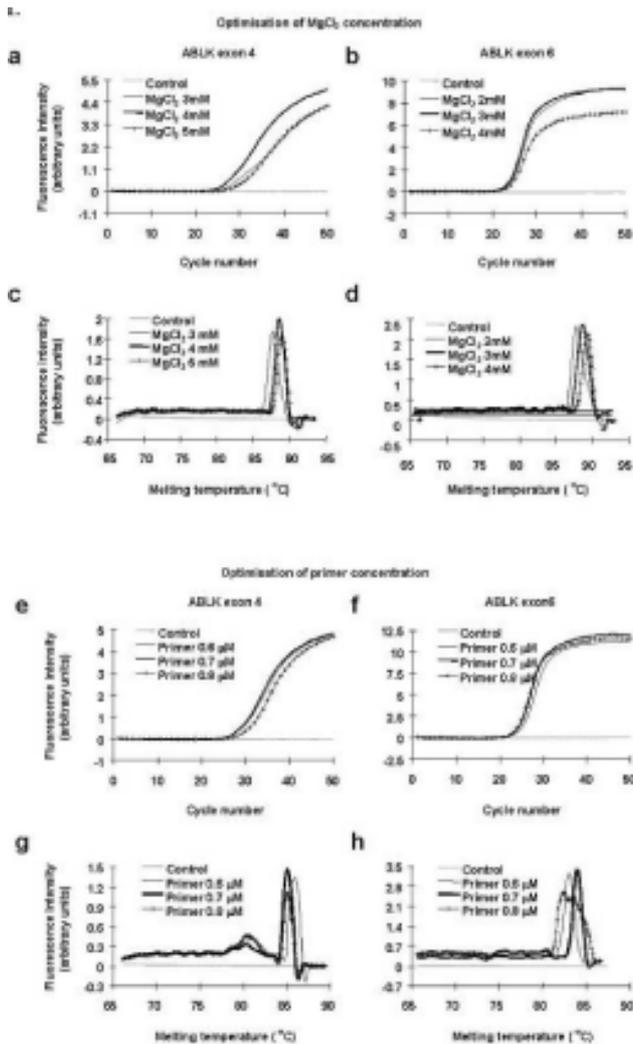


Fig. 1. Optimisation of Q-PCR MgCl₂ and primer concentration for amplification of ABLK exons 4 and 6 in the CESS cell line. Genomic DNA (1.0 ng/sample) was extracted from the CESS EBV-immortalised normal B cell line in log phase growth, and amplified separately using Q-PCR (10.0 μL reaction volume) for exons 4 (a and c) or 6 (b and d) of the ABLK gene. MgCl₂ concentrations (1.0, 2.0, 3.0, 4.0 or 5.0 mM) were varied for each reaction and exon. One micromolar of each primer and an annealing temperature of 67°C were arbitrarily used as initial conditions for Q-PCR. Experiments were performed in triplicate, control reactions contained no gDNA, and bracketed data is presented both as quantification curves (a and b), as well as melting curves (c and d). Analysis was performed on the Roche LightCycler Data Analysis Software (Roche Diagnostics). Optimal MgCl₂ (4 mM for exon 4 and 3.0 mM for exon 6) concentrations were used to further optimise Q-PCR reactions for exons 4 (e and g) or 6 (f and h) of the ABLK gene. Specifically, primer concentrations (0.5, 0.6, 0.7, 0.8, 0.9 or 1.0 μM) were varied for each reaction and exon. Genomic DNA (1.0 ng/sample) and an annealing temperature of 67°C were arbitrarily used. Experiments were performed in triplicate, control reactions contained no gDNA, and bracketed data are presented both as quantification curves (e and f), as well as melting curves (g and h).

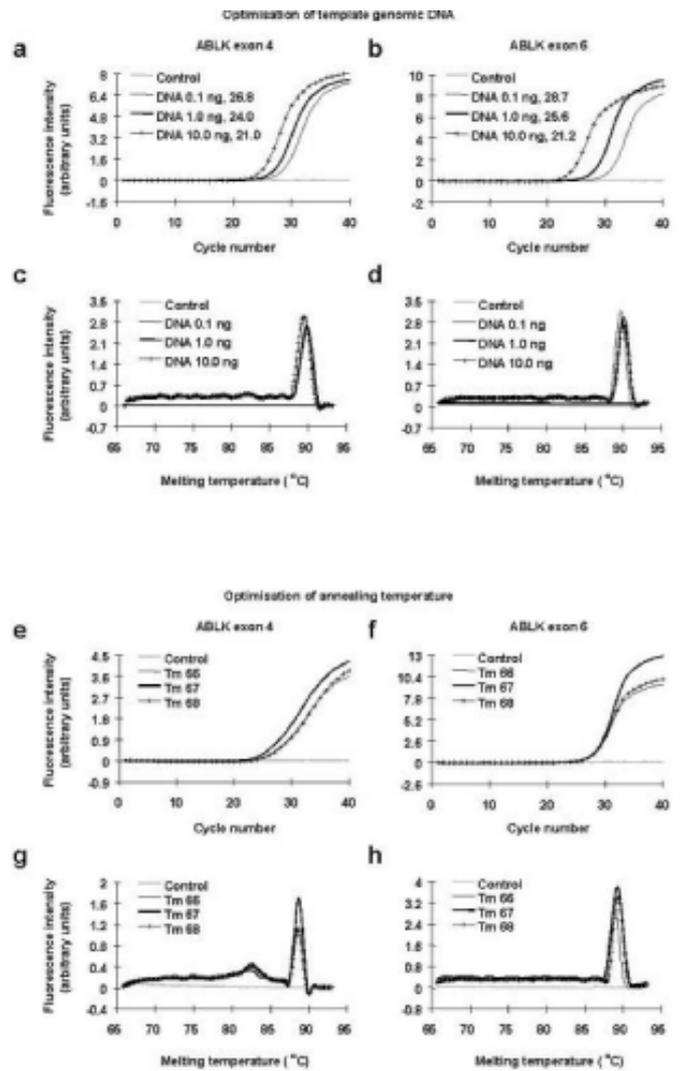


Fig. 2. Optimisation of amount of gDNA and annealing temperature for Q-PCR amplification of ABLK exons 4 and 6 in the CESS cell line. Optimal MgCl₂ (4.0 mM for ABLK exon 4 and 3.0 mM for ABLK exon 6) were combined with optimal primer concentrations (0.7 μM for each primer) for the optimisation of amount of gDNA (0.0, 0.1, 1.0 or 10.0 ng) for Q-PCR. Again, the CESS EBV-immortalised normal B cell line and an arbitrary annealing temperature of 67°C were used for Q-PCR (10.0 μL reaction volume). Experiments were performed in triplicate and bracketed data are presented both as quantification curves (a and b), as well as melting curves (c and d). Genomic DNA (1.0 ng/sample) was extracted from CESS EBV-immortalised normal B cells and amplified using Q-PCR (10.0 μL reaction volume; 4 mM MgCl₂ for ABLK exon 4 or 3 mM MgCl₂ for ABLK exon 6; and 0.7 μM primer concentration) for exons 4 (e and g) or 6 (f and h) of the ABLK gene. Optimisation of the Q-PCR was first performed for the following annealing temperatures, 66°C, 67°C or 68°C. Control reactions contain no gDNA. Experiments were performed in triplicate and bracketed data are presented both as quantification curves (e and f), as well as melting curves (g and h). Control reactions contained no gDNA.

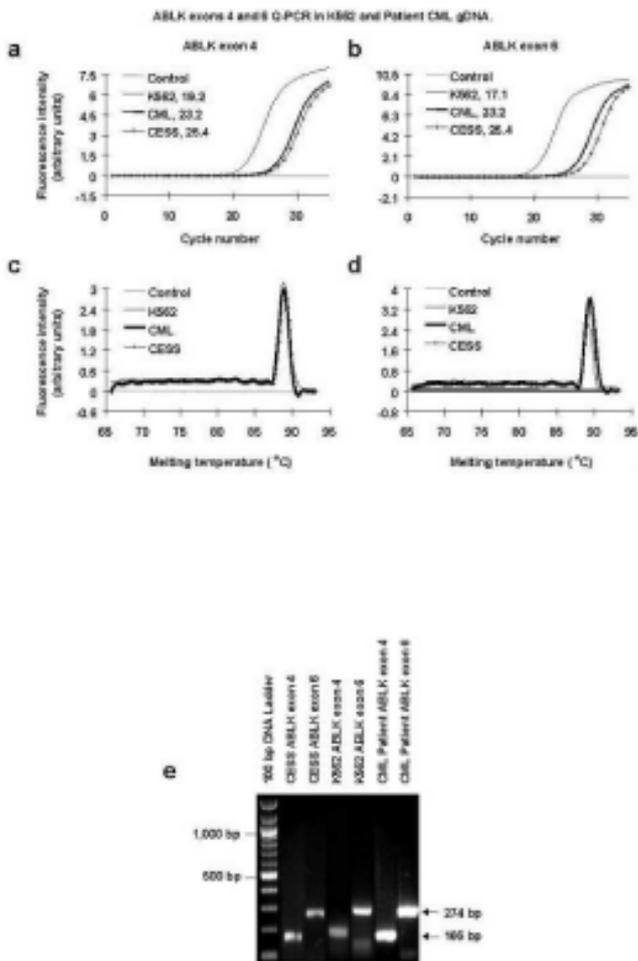


Fig. 3. ABLK exons 4 and 6 gene amplification in K562 and patient CML cells.

Genomic DNA (1.0 ng/sample) was extracted from K562 and patient CML cells, and the CESS EBV-immortalised normal B cell line in log phase growth. Optimal Q-PCR conditions: 4.0 mM MgCl₂ for ABLK exon 4 or 3.0 mM MgCl₂ for ABLK exon 6; 0.7 μM primer concentration, and annealing temperature 67°C, were used to amplify both exon 4 (a and c) or exon 6 (b and d) of the ABLK gene (10.0 μL reaction volume). Experiments were performed in triplicate and bracketed data are presented both as quantification curves (a and b), as well as melting curves (c and d). Thereafter, Q-PCR products were resolved in a 2% agarose gel and detected using ethidium bromide staining (e). The expected sizes of the ABLK exons 4 and 6 amplicons were 166 bp and 274 bp, respectively. A 100-bp DNA ladder was loaded in lane 1. CESS ABLK exons 4 and 6 (lanes 2 and 3), K562 ABLK exons 4 and 6 (lanes 4 and 5), and CML patient ABLK exons 4 and 6 (lanes 6 and 7) were respectively analysed.

amplification in K562 and patient CML cells using our novel Q-PCR assay. Cycle durations were 17.0 s for ABLK exon 4 and 21.0 s for exon 6. Moreover, because of the differences in annealing temperature, ABLK exon 4 or exon 6 Q-PCR reactions had to be performed separately. A total of 30 cycles of Q-PCR were recommended for template gDNA of 1.0 ng/sample. Hence, once gDNA had been

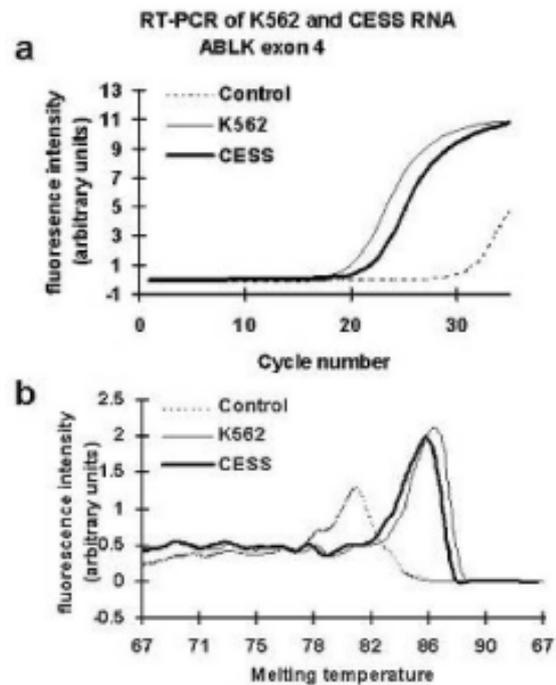


Fig. 4. Relative expression of the ABLK exon 4 mRNA transcript in K562 and CESS cells.

Total RNA (250.0 ng/sample) was first extracted from K562, and the CESS EBV-immortalised normal B cell line in log phase growth. Following reverse transcription, RQ-PCR was completed using optimal Q-PCR conditions: i.e., 4 mM MgCl₂; 0.7 μM primer concentration (20.0 μL reaction volume). Experiments were performed in duplicate and bracketed data are presented as quantification curves (a), as well as melting curves (b).

extracted, the time required for the completion of Q-PCR was only 30 minutes. To study ABLK gene amplification in CML patient cells, K562 CML cells, which are known to have ABLK gene amplifications,³³ but not mutations, were used as a positive control. As can be seen in Figures 3a and 3b, the CT values for CESS EBV-immortalised normal B cells (which are presumed to contain 2 normal alleles), were 25.4 cycles for both ABLK exons 4 and 6, respectively. Cycle threshold values of the K562 CML cell line for ABLK exons 4 and 6 were 19.2 cycles and 17.1 cycles, respectively. This translated into a ~2 log-fold (CT differences of 5.8 for exon 4 and 7.7 for exon 6) ABLK gene amplification for K572 CML cells. In contrast, patient CML cells were found to have CT values of 23.2 cycles or CT differences of 2.2 for both exons; suggesting that there was no significant ABLK gene amplification. Melt curve analyses confirmed equal fidelity of the Q-PCR reactions for all samples (Figs. 3c and 3d).

In order to confirm that the Q-PCR products were legitimately amplified, we first resolved the Q-PCR products in an agarose gel (Fig. 3e) and confirmed the expected Q-

PCR products of sizes 166 bp for ABLK exon 4 (lanes 2, 4 and 6) and 274 bp for ABLK exon 6 (lanes 3, 5 and 7). Next, we purified DNA from the respective bands in the agarose gel and performed forward and reverse DNA sequencing for each of the 6 Q-PCR products, using the same primers. DNA sequencing confirmed legitimate ABLK exon 4 or exon 6 DNA sequences for all 6 samples (data not shown). However, neither K562 nor patient CML cells contained a detectable ABLK mutation. These data confirm the potential utility of this assay for analysing both gene amplification of ABLK exons 4 or 6, as well as the presence of hotspot ABLK mutations.

Relative Expression of the ABLK Exon 4 mRNA Transcript in K562 and Patient CML Cells

Having determined the relative level of gene amplification in K562 and patient CML cells, we next determined the relative transcription level of the ABLK gene. This would permit us to assess the level of possible transcriptional silencing in a semi-quantitative manner and to compare the genomic and messenger RNA levels. To do this, we performed RQ-PCR for ABLK exon 4 using equal amounts of total RNA from K562 and the CESS EBV-immortalised normal B cell line. Exon 4 (and not exon 6) of the *abl* gene was selected because we designed exonic primers for exon 4, whereas intronic primers were used for exon 6. As can be seen in Figures 4a and 4b, the curves for the amplification of the K562 *abl* gene appears sooner than that of the CESS *abl* gene, indicating that it is present in larger amounts. The calculated CT value for the K562 was 19.93 while the values for CESS were 21.7 and 22 in two independent experiments. The products were highly specific and identical for both cell lines, as can be seen by the melting curve analysis. Thus, these data confirm that the Q-PCR data are consistent with the RQ-PCR data.

Discussion

Preclinical and clinical studies suggest that resistance to anti-CML drugs may be the result of resistance resulting from BCR-ABL reactivation and/or resistance resulting from the activation of alternative signalling pathways.^{16,34} When drug resistance is due to reactivation of BCR-ABL activity, the possible mechanisms include *bcr-abl* gene amplification, and/or mutations in the ATP-binding pocket or activation loop of the ABLK domain that prevent ABLK blockade.¹⁶ Specifically, both patients still receiving STI571, as well as those relapsing after successful treatment with STI571, frequently exhibit *bcr-abl* gene amplification, and/or ABLK mutation(s). Prior studies have demonstrated that ABLK mutations leading to ABLK reactivation were more frequent than *bcr-abl* gene amplification.³⁵ Moreover, ABLK mutations were generally not present prior to therapy but developed as newly acquired mutational events during

the course of STI571 treatment. Hence, serial studies of ABLK mutations could assist decision making during therapy.³⁶ These data suggest that analysis of the status of the *bcr-abl* gene at both genomic as well as transcription levels might be important for the monitoring of patients treated with STI571.^{37,38}

Analysis of ABLK mRNA transcription provides a context-dependent assessment of kinase function, and is highly valuable for monitoring patients on STI571. Rapid methods for mRNA detection have already been devised.¹⁹ However, transcriptionally silent Ph⁺ CML cells clearly exist and these cells do not express the BCR-ABL mRNA transcript, do not respond to STI571 and evade detection by RQ-PCR and Western immunoblotting.²¹ Recently, 16 similarly transcriptionally silent Ph⁺ CML patient samples were analysed for the status of the *bcr-abl* gene using FISH and were found to harbour partial or total gene deletions in der(9)t(9;22) in 9 of 16 (56%) samples; as well as abnormal localisation of the 3' portion of *bcr* and the 5' portion of *abl* gene loci [i.e., variant t(9;22) translocation] in 5 of 16 (31%) patients.²⁵ By contrast, transcriptionally competent Ph⁺ CML cells (n = 50 samples) all (100%) contained non-variant t(9;22) translocations. The authors concluded that genomic aberrations were therefore the basic and most frequent cause of transcriptional silencing in Ph⁺ CML. Since chromosomal translocations and profound genomic instability³⁹ were the result of homologous DNA recombination deficiency, these data further suggest that transcriptionally silent Ph⁺ CML patients may not only have more advanced disease,^{28,40} but are also likely to be more resistant to STI571.

These transcriptionally silent mutations are associated with advanced CML and detectable only by analysis of gDNA. Hence, patients should also be followed up using both complementary assays (i.e., RQ-PCR and Q-PCR). Since these rapid gDNA-based assays that detect ABLK mutations are currently not available, we undertook this study to bridge this knowledge gap. We hypothesised that Q-PCR will more thoroughly enable the quantitative analysis of MRD and render such analysis more probable, not only of patients with CML.

In designing primers for our assays, we considered the position of the hotspot mutations carefully. Specifically, we were interested in assaying the kinase loop. Moreover, we were keen on developing high-stringency primer sets, both in terms of ease of primer-DNA binding, as well as (high) melting temperature. For ABLK exon 4, 11 known mutations are clustered in the middle of the exon, within a 12 nucleotide-long region. This is in contrast to ABLK exon 6, which has the same number of known mutations spread across a 4-fold longer span of DNA. This fortuitous situation permitted us to design exonic primers with high

stringency for the ABLK exon 4 hotspot. In contrast, intronic primers had to be designed for longer-spanning ABLK exon 6 mutations. Coincidentally, the exonic primers of ABLK exon 4 could be used for both Q-PCR and RQ-PCR, thus permitting easy comparison of data for mRNA transcription, gene mutation and gene amplification of ABLK exon 4.

Using optimised conditions for both ABLK exons, each cycle of Q-PCR in our assay required only 17.0 seconds for exon 4 amplification, and 21.0 seconds for exon 6 amplification. Hence, in a 30-cycle Q-PCR reaction, 25 mins to 30 mins are the typical durations required for Q-PCR. We are currently developing a quantitative and specific method using highly specific WT and mutant probes and the primers developed here to detect the presence of several of the most common mutations in both exons. These fluorescent multiprobe assays will allow us to determine the identification of a specific nucleotide (as either wild type or mutant) and the genomic levels of both in patients with CML. Whether these assays will eventually replace more cumbersome assays, including FISH, will require further evaluation.

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