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BIO-TECHNICAL METHODS SECTION

BTS Leukemia

Transcription-mediated amplification and hybridisation protection assay to determine *BCR-ABL* transcript levels in patients with chronic myeloid leukaemia

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Detection of *BCR-ABL* transcripts in chronic myeloid leukaemia (CML) is used to confirm the diagnosis and to monitor residual disease. Quantitative techniques are required to predict response to therapy or early relapse. We have evaluated an assay in which transcription-mediated amplification (TMA) of *BCR-ABL* and *ABL* transcripts is achieved using reverse transcriptase and RNA polymerase. The products are quantified in the hybridisation protection assay (HPA) using acridinium ester-labelled DNA probes and chemiluminescence. The method is a single tube procedure which uses small amounts of RNA (<500 ng/triplicate analysis), is technically simple (requiring just two waterbaths and a luminometer), rapid (total assay time <4 h) and sensitive (capable of detecting one *BCR-ABL*-positive K562 cell in the presence of 10^4–10^5 *BCR-ABL*-negative cells). *BCR-ABL* signals from patient RNA samples were quantified relative to known amounts of K562 RNA and normalised to levels of *ABL*. *BCR-ABL/ABL* ratios ranged from 0.15 to 1.59 (median 0.65) in RNA from diagnostic blood or bone marrow of 18 CML patients and were ⩽0.0001 in 20 normal controls. Sequential samples analysed from six CML patients post-allogeneic bone marrow transplantation who relapsed and received donor lymphocyte infusions showed *BCR-ABL/ABL* ratios which reflected patient status or treatment. A *BCR-ABL/ABL* ratio of 0.01 served as a useful arbitrary indicator value, with results above and below this value generally correlating with relapse or remission, respectively.
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Introduction

The Philadelphia chromosome is the hallmark of chronic myeloid leukaemia (CML). At diagnosis, either a balanced t(9;22) translocation can be demonstrated by cytogenetics and/or the consequent mRNA fusion product, *BCR-ABL*, can be detected by RT-PCR in greater than 95% of patients.[1,2] As the prospects for effective therapy have improved, for example, with the introduction of allogeneic bone marrow transplantation (alloBMT) followed by donor lymphocyte infusions (DLI) in cases of relapse[3,4] or strategies to specifically inhibit the BCR-ABL tyrosine kinase activities,[5,6] so the t(9;22) translocation has been used increasingly as a marker of minimal residual disease. Cytogenetics and fluorescence *in situ* hybridisation (FISH) are insufficiently sensitive to detect t(9;22)-positive cells comprising less than 1% of the total population[7] and therefore most studies have relied on detection of *BCR-ABL* transcripts. However, the sensitivity of qualitative RT-PCR techniques varies and as many patients remain *BCR-ABL* PCR positive for some considerable time following alloBMT, the usefulness of these types of studies in predicting clinical outcome remains controversial.[8]

In order to address this issue, quantitative methods have been introduced. For example, in competitive PCR a synthetic competitor molecule is constructed which contains the sequences necessary for *BCR-ABL* PCR but which produces a fragment of slightly larger size than the *BCR-ABL* under investigation, ie b2a2 or b3a2 fusion products. Increasing amounts of competitor are added to a series of PCR reactions from patient cDNA and comparison of the resulting product intensities is used to estimate the level of transcript in the patient sample. This method has been shown to detect a fall in *BCR-ABL* levels after alloBMT[9–11] and an elevation prior to relapse[12,13] and has been used to determine the rate of response to DLI once relapse has occurred.[14] However, the method requires multiple PCR reactions for each sample, which is time-consuming, and reproducibility is not good. More recently, an alternative approach using 'real-time' PCR has been introduced whereby the number of *BCR-ABL* molecules is estimated on the basis of the number of amplification cycles required to reach a threshold level of detection. Studies using 'real-time' quantification have been used to demonstrate falls in *BCR-ABL* levels post alloBMT and after treatment with DLI for relapse.[15–17] The method can detect one *BCR-ABL*-positive K562 cell in the presence of 10^4–10^5 *BCR-ABL*-negative cells, but it requires sophisticated and expensive equipment.

We have therefore evaluated an RNA-based method of quantifying *BCR-ABL* transcripts in samples from peripheral blood or bone marrow of CML patients in which multiple RNA copies of the specified transcript are produced by transcription-mediated amplification (TMA). They are then hybridised to acridinium ester (AE)-labelled DNA probes and the resulting chemiluminescent signal quantified in the sensitive hybridisation protection assay (HPA).[18,19] We show that this single-tube method is simple, rapid, and capable of quantifying *BCR-ABL* transcripts at both diagnosis and following alloBMT and DLI.

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Principles of TMA and HPA

Transcription-mediated amplification

In TMA a sequence-specific primer, which also contains the promoter sequence for RNA polymerase at its 5′ end, is hybridised to the target RNA to initiate reverse transcription and create a DNA copy (Figure 1). The RNA in the resulting RNA:DNA heteroduplex is degraded by the RNAse H activities of the reverse transcriptase. This enables another primer to bind to the DNA copy and a second strand of DNA is synthesised. Both strands of the created double-stranded DNA molecule now contain promoter sequences for RNA polymerase, and this enzyme can therefore use it as a template and initiate transcription. Each of the newly synthesised RNA amplicons serves as the template for a new round of replication, leading to an exponential expansion of the RNA target.

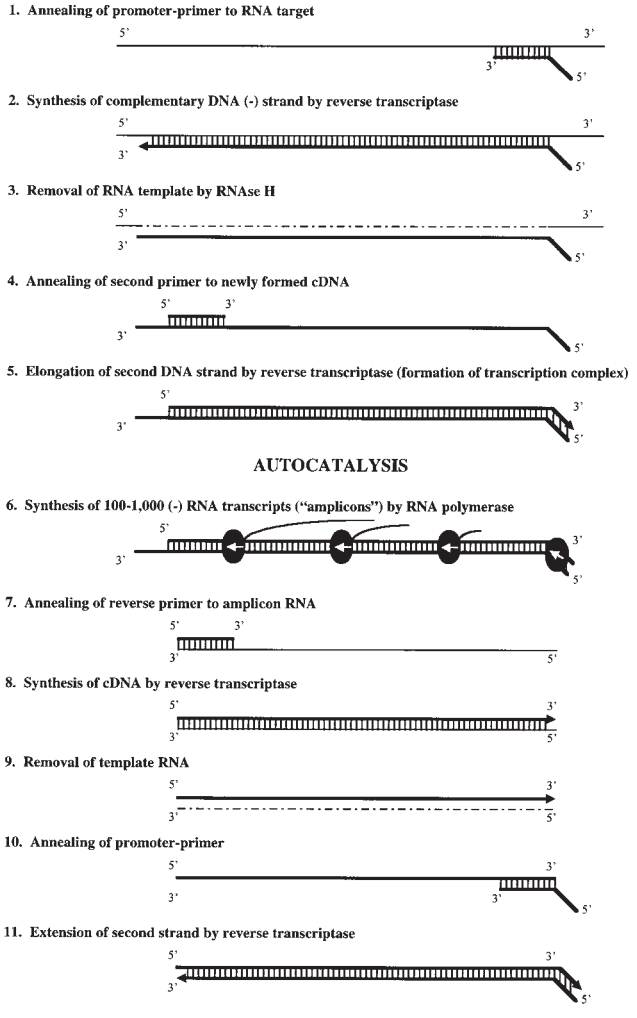

Figure 1 Transcription-mediated amplification (TMA) cycle. Step 1: *ABL* promoter-primer binds to 3′ end of RNA target; step 2: reverse transcriptase (RT) creates DNA copy of RNA target; step 3: RNAse H activities of RT degrades the RNA; step 4: 5′ *ABL* or *BCR* primer binds to the DNA; step 5: RT completes the double-stranded DNA template including the RNA polymerase promoter sequence; step 6: RNA polymerase initiates transcription of RNA from DNA template, producing 100–1000 copies of either *BCR-ABL* or *ABL* amplicon; step 7: 5′ *ABL* or *BCR* primer binds to the RNA amplicon; step 8: RT creates an RNA:DNA heteroduplex; step 9: RNAse H degrades the RNA; step 10: *ABL* promoter-primer binds to the newly synthesised DNA; step 11: RT creates double-stranded DNA. Products from this step are then available as templates for step 6 in an autocatalytic cycle.

Hybridisation protection assay

In the HPA, sequence-specific oligonucleotide probes labelled with an acridinium ester (AE) are allowed to hybridise to the amplicons produced in the TMA reaction (Figure 2). Unhybridised probe is removed by rapid hydrolysis and the chemiluminescent signal from the remaining, more slowly hydrolysed, probe-amplicon hybrids is measured in a luminometer. The number of RNA amplicons produced is directly proportional to the number of target molecules in the starting sample, and only one molecule of AE-labelled probe can bind to an RNA amplicon. The chemiluminescent signal obtained is therefore a measure of starting template concentration.

Methodology

TMA

Test RNA (100 ng unless otherwise stated) in a total volume of 25 μl was mixed with 50 μl core amplification reagent (Gen-Probe, San Diego, CA, USA; US patent 5 888 779) containing 2 × amplification buffer (2 mM each dNTP, 8 mM each rNTP, 80 mM Tris-HCl pH 7.5 at 25°C, 50 mM $MgCl_2$, 35 mM KCl, 10% (w/v) polyvinylpyrrolidone) and 15 pmol promoter-primer and each reverse primer (Table 1) in a 12 × 75 mm polypropylene tube and incubated at 60°C for 10 min under oil to allow denaturation of the RNA. The mixture was then cooled to 42°C for 5 min before adding 25 μl of enzyme mix containing MMLV reverse transcriptase (2000 units/assay) and T7 RNA polymerase (2000 units/assay) in 8 mM Hepes pH 7.5, 50 mM N-acetyl-L-cysteine, 0.04 mM zinc acetate, 80 mM trehalose, 140 mM Tris-HCl pH 8.0 at 25° C, 70mM KCl, 1 mM

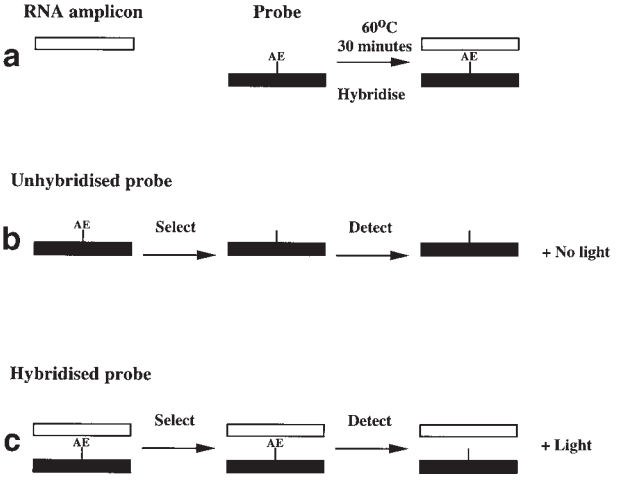

Figure 2 Detection of amplicon with DNA probes and the hybridisation protection assay (HPA). (a) An excess of acridinium ester (AE)-labelled probe is added and allowed to hybridise to *BCR-ABL* or *ABL* target sequences within the amplicon produced in the TMA reaction. Separation of hybridised from unhybridised probe is achieved by the addition of a selection reagent which hydrolyses the AE on the unhybridised probe. (b) No light is emitted in the luminometer from the hydrolysed unhybridised probe. (c) The AE on the hybridised probe is protected within the double helix and is not hydrolysed by the selection reagent. Light is emitted and detected by the luminometer.

Table 1 TMA primer and HPA probe sequences for the amplification and detection of *BCR-ABL* and *ABL* transcripts.

Primer/probe	*Exon location*	*Sequence (5′→3′)*
ABL promoter primer	a2	TAAATTAATACGACTCACTATAGGGAGACTCAGACCCTGAGGCTCAAAGTCAGA
BCR reverse primer	b2	GACCAACTCGTGTGTGAAACTCCA
ABL reverse primer	Ib	CAAAGGAAGCAGGGAAGAAGG
BCR-ABL AE-probe	b2	GACTGTCCACAGCATTCCGCTGACC
ABL AE-probe	Ib	GTGGAACATGAAGCCCTTCAGCGG

EDTA, 0.01% (w/v) phenol red, 10% (v/v) Triton X-100 and 20% (v/v) glycerol) and incubation continued for a further 60 min at 42°C.

HPA

One hundred microlitres of probe mix containing 7.5 nM AE-labelled oligonucleotide probe complementary either to the *BCR* exon b2 (Table 1) for *BCR-ABL* products or to an *ABL* exon 1b sequence (Table 1) specific for *ABL* products in hybridisation buffer (100 mM lithium succinate pH 4.7, 2% (w/v) lithium lauryl sulphate, 15 mM aldrathiol-2, 1.2 M lithium chloride, 20 mM EDTA, 20 mM ethylene glycol-bis-(-amino ethyl ether) N, N, N′, N′-tetracetic acid (EGTA), 3% ethanol; Gen-Probe), was then added to the TMA reaction, the mixture vortexed and incubated for 30 min at 60°C. To remove unhybridised probe, 300 μl hydrolysis buffer (600 mM sodium tetraborate pH 8.5, 1% (v/v) Triton X-100) was then added and the sample incubated at 60°C for a further 10 min. After cooling the tubes were placed in a luminometer with an automated reagent-injection system (Leader 450i; Gen-Probe) and 200 μl Reagent 1 (1 ml/l H_2O_2, 1 mM nitric acid) and 200 μl Reagent 2 (1 M NaOH) were added. The resulting chemiluminescence was integrated for 2 s and recorded as relative light units (RLU). Each sample was tested in triplicate and the mean result after subtraction of the background (ie no RNA) level calculated.

Statistical analysis

Simple regression analysis was used to verify the linearity of calibration curves. Student's *t*-test was employed to analyse the difference in both *BCR-ABL* and *ABL* levels of CML and control patient samples: a *P* value of less than 0.05 was deemed to be significant. Coefficients of variation (CV) were calculated to assess technical variation in multiple sample analysis.

Results

Technical evaluation of TMA/HPA

Sensitivity and linearity: In order to evaluate the sensitivity of the technique and linearity of the increase in RLUs obtained with increasing template concentration, the *BCR-ABL* and *ABL* signals from varying amounts (0–500 ng) of total RNA from the *BCR-ABL*-positive cell line K562 were measured as described above. In addition, RNA was extracted from mixtures containing 1 K562 cell in 10, 10^2, 10^3, 10^4, 10^5 or 10^6 HL60 cells (*BCR-ABL* negative) and 100 ng used to assay the *BCR-ABL* and *ABL* signals. In the *BCR-ABL* assay, levels above background were detected using greater than 0.005 ng K562 RNA (Figure 3a) and this correlated to the detection of 1 K562 cell in 10^4–10^5 HL60 cells (Figure 3c). Regression analysis of the values obtained for 0.05, 0.5, 5 and 50 ng starting RNA gave r^2 values of 0.906–0.996 (mean 0.960, median 0.968) in 12 analyses. The *ABL* assay required greater than 0.5 ng K562 RNA to detect levels above background (Figure 3b), probably reflecting that there are fewer *ABL* than *BCR-ABL* transcripts per K562 cell.[20] Regression analysis of the *ABL* values obtained for 0.5, 5, 50 and 500 ng starting RNA gave r^2 values of 0.887–0.992 (mean 0.960, median 0.961) in 12 analyses.

Reaction kinetics and product stability: In order to evaluate kinetics of the reaction and stability of the products created during TMA, samples from three patients with high, medium and low *BCR-ABL* values, respectively, were incubated at 42°C for times varying between 30 and 150 min, then HPA performed as described. Maximum levels were achieved after approximately 60 min of incubation in all three samples and remained stable for a period of at least a further 90 min (Figure 4a).

Reproducibility: Sample reproducibility was assessed using RNA from three patients with high, medium and low *BCR-ABL* levels and three with high, medium and low *ABL* levels, respectively. Eight separate aliquots were prepared from each sample, each of 300 ng, and assayed in triplicate (100 ng/test) using the same batch of reagents. The results obtained are shown in Figure 4b. For the *BCR-ABL* assays, the CVs of the mean values from the eight aliquots were 11%, 26% and 27% for the three samples, respectively, and for the *ABL* assays they were 14%, 16% and 26%, respectively. Day to day technical variation was assessed using the data from 12 calibration curves created using four different dilutions of K562 RNA (Figure 4c). The CVs were 41%, 18%, 21% and 28% for 50, 5, 0.5 and 0.05 ng RNA, respectively, in the *BCR-ABL* assay, and 35%, 17%, 19% and 24% for 500, 50, 5 and 0.5 ng RNA in the *ABL* assay.

Analysis of CML patient samples

For each series of patient or control samples analysed, calibration curves using samples of 0.05, 0.5, 5 and 50 ng starting K562 RNA for *BCR-ABL* analysis and 0.5, 5, 50 and 500 ng for *ABL* analysis were constructed as described above and the slopes used to convert RLUs obtained into post-amplification quantities of RNA. One hundred nanograms template RNA was selected as the optimal amount to use per test as this gave post-amplification *BCR-ABL* and *ABL* levels within the linear

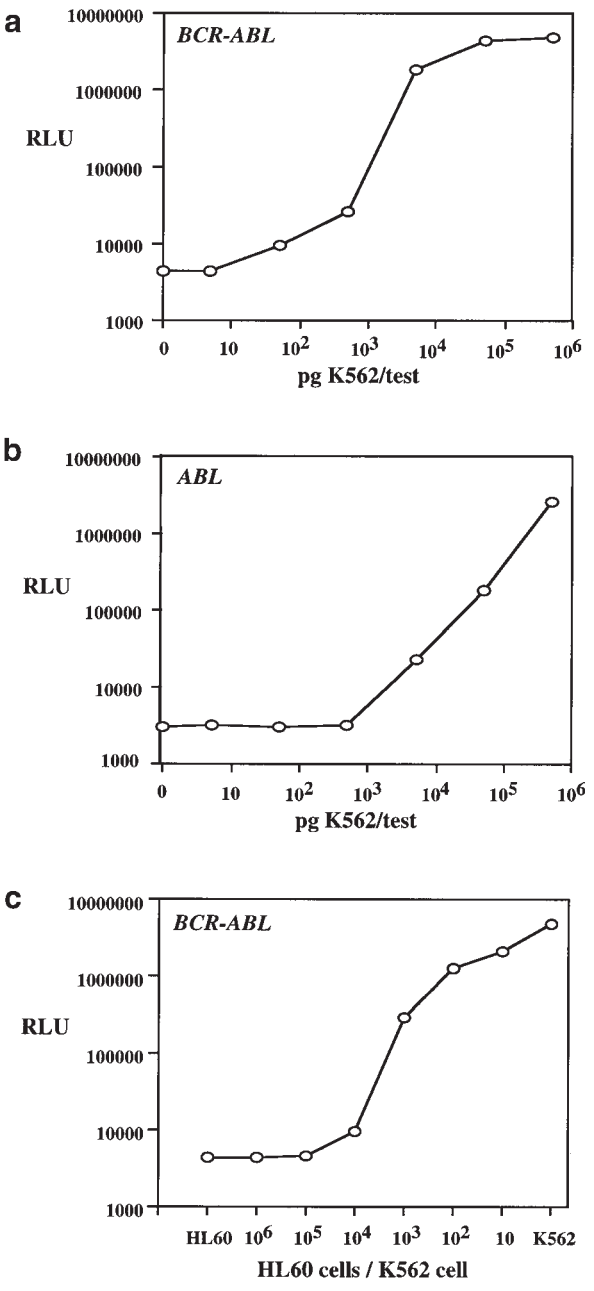

Figure 3 Linearity and sensitivity of assays. Increasing concentrations of K562 RNA were assayed for levels of (a) *BCR-ABL* and (b) *ABL* transcripts. (c) *BCR-ABL* levels were assayed in RNA from 1 K562 cell in the presence of increasing numbers of HL60 cells.

range of the calibration curve for samples with varying levels of target (data not shown).

Diagnosis: Levels of *BCR-ABL* and *ABL* transcripts were quantified in 100 ng total RNA from bone marrow (BM, n = 10) or peripheral blood (PB, n = 10) of 20 CML patients at presentation with clinical and cytogenetic t(9;22) disease in chronic phase, and from BM (n = 10) or PB (n = 10) of 20 haematologically normal controls (HNC) who all had a normal karyotype and were *BCR-ABL* negative by conventional RT-PCR analysis.[21] In order to accommodate variations in RNA quality, *BCR-ABL* transcript levels were normalised by expressing the results as *BCR-ABL/ABL* ratios. The *BCR-ABL* level was not above background in 19 of the control samples and was at the background level at 0.005 ng in one BM sample (Figure 5a) whereas the *ABL* levels varied between 1.9 and 49.2 ng (median 14.1 ng) (Figure 5b). All 20 controls had *BCR-ABL/ABL* ratios $\leq$0.0001 (Figure 5c). In 18 of the CML patients (8 BM, 10 PB), *BCR-ABL* levels ranged between 0.2 and 43.0 ng (median 8.5 ng), *ABL* levels between 0.6 and 82.6 ng (median 14.9 ng) and the *BCR-ABL/ABL* ratios between 0.15 and 1.59 (median 0.65). There was no significant difference between the *ABL* levels of the CML patients and normal controls, but *BCR-ABL* levels and *BCR-ABL/ABL* ratios were both significantly higher in the 18 CML patients than the controls (P = 0.0001). In the two remaining CML samples, both from BM, the *BCR-ABL* levels were $<$0.005 ng (Figure 5a), the *ABL* levels were normal (7.1 and 14.0 ng, respectively) and the *BCR-ABL/ABL* ratios were 0.0001 and 0.0004 (Figure 5c). These two samples were also *BCR-ABL* negative by conventional RT-PCR.

Post alloBMT: Sequential levels of *BCR-ABL* and *ABL* transcripts were quantified using 100 ng total RNA from PB (Figure 6a–c) or BM (Figure 6d–f) of six CML patients following alloBMT who all received DLI for haematological or molecular relapse. The 'absolute' level of *BCR-ABL* transcripts varied from patient to patient according to status and treatment, with a range of 0.013–53.3 ng, but the range of *ABL* levels was 0.7–123.8 ng (median 24.2 ng) which was similar to the control and diagnostic samples. *BCR-ABL/ABL* ratios ranged from 0.0002 to 9.74. In five of the six patients it was possible to observe a trend in the *BCR-ABL/ABL* ratio which corresponded with clinical course and treatment (Figure 6a, b, d–f). In four patients where samples were available prior to relapse, high *BCR-ABL/ABL* ratios ($>$0.01) and/or an apparent increase of the *BCR-ABL/ABL* ratio were seen several months before relapse, although the extent of the increase inevitably depended on the timing of the samples (Figure 6a, b, d, e). Five patients achieved clinical remission after DLI and this was reflected in a subsequent decline of the *BCR-ABL/ABL* ratio in samples available from four of these patients (Figure 6a, b, d, f). One patient remained in chronic phase post-relapse, and no decrease in the *BCR-ABL/ABL* ratio in this patient had been observed by the time of the last available sample at 4 months after the third course of DLI (Figure 6e). In one patient, the only sample of the five analyzed which had a *BCR-ABL/ABL* ratio $>$0.001 was when the patient was in clinical remission, although the patient had relapsed about 3 months earlier (Figure 6c). *ABL* levels for all samples from this patient were within the accepted range (8.5–73.3 ng), indicating that these results were not due RNA degradation.

When the TMA/HPA results were compared with conventional RT-PCR analysis, there was no consistent *BCR-ABL/ABL* ratio which separated results which were PCR positive or negative. However, 35 out of 39 samples analysed with a TMA/HPA *BCR-ABL/ABL* ratio $>$0.01 were positive by PCR analysis; conversely, 15 out of 27 PCR-negative samples had ratios $<$0.01.

Discussion

Although there were hopes that qualitative RT-PCR of the *BCR-ABL* fusion gene in CML might be useful as an indicator

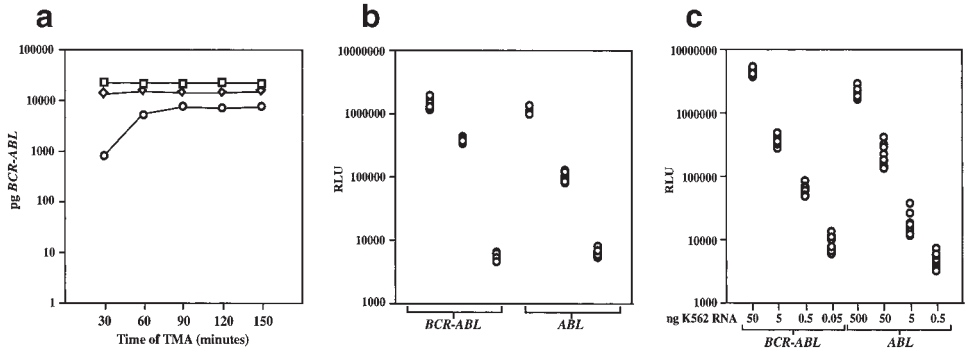

Figure 4 Assay kinetics and reproducibility. (a) Time course of TMA incubation using three patient samples with differing *BCR-ABL* levels. (b) Repeated analysis of samples with varying *BCR-ABL* or *ABL* levels. Each sample was assayed eight times in triplicate. (c) Day to day variation of four different concentrations of K562 RNA assayed on 12 separate occasions.

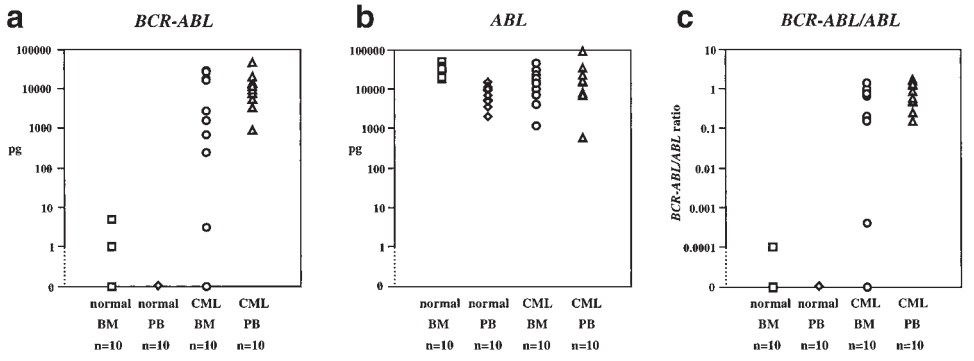

Figure 5 Post-amplification levels of (a) *BCR-ABL* and (b) *ABL* using RNA from 20 CML patients at diagnosis and 20 normal controls. (c) The corresponding *BCR-ABL/ABL* ratios.

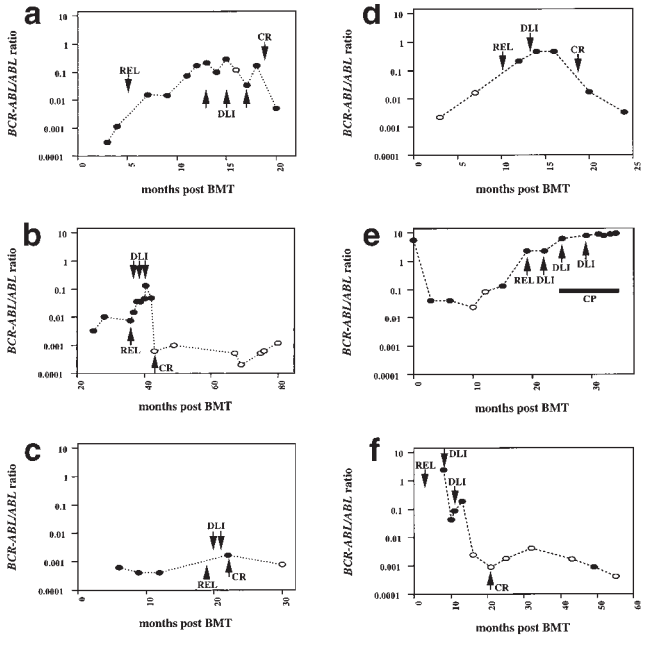

Figure 6 Sequential analysis of six patients who underwent alloBMT and subsequently relapsed at the molecular (a–c) or cytogenetic/haematological level (d–f). CR, complete remission; REL, relapse; DLI, donor lymphocyte infusion; CP, chronic phase; •, RT-PCR positive; O, RT-PCR negative.

of minimal residual disease, studies have shown that PCR positivity may persist after alloBMT, and in general this method is an unreliable predictor of impending relapse. As a consequence, a number of quantitative PCR methods have been introduced, but they either require multiple analyses of a single sample or expensive, sophisticated equipment. We have therefore evaluated an alternative method, TMA/HPA, for quantifying the level of *BCR-ABL* and *ABL* transcripts in RNA samples from patients and controls. It differs in many aspects from PCR. Whereas the latter involves one enzyme activity and a maximum of a two-fold increase in product at every discrete cycle, there are two very separate activities simultaneously taking place in TMA: (1) an RNA polymerase activity (comparable to a typical *in vitro* transcription reaction) and (2) a DNA polymerase function performed by the reverse transcriptase (comparable to the initial RT step in RT-PCR). Many transcripts or amplicons are made by RNA polymerase from each molecule of starting template, and each of these amplicons is immediately converted into a new template molecule for RNA polymerase by the RT activity. Since each of the created double stranded DNA template molecules can lead to the production of 100–1000 RNA amplicons, this expansion can result in billions of amplicons produced within 1 h and the fold increase is therefore considerably greater than in PCR. The amplicons are then quantified in the HPA by hybridising to chemiluminescently labelled sequence-specific probes.

Both component parts of this assay have been described previously but separately. The technique nucleic acid sequence-based amplification (NASBA) is similar to TMA but

uses AMV-RT instead of MMLV-RT.[22] These enzymes have different specificities and efficiencies of incorporation, and NASBA also requires a supplementary addition of RNAse H. It has been successfully used to amplify *BCR-ABL* transcripts in a small number of diagnostic CML samples where the products were detected using ethidium bromide.[23] HPA has been reported as a rapid, sensitive, non-radioactive alternative to hybridisation of radioactively labelled probes to semi-quantify *BCR-ABL* transcripts following conventional RT-PCR.[24]

In the present study TMA and HPA have been combined to amplify, detect and quantify *BCR-ABL* and *ABL* transcripts. The results have been converted into an 'absolute' value in nanograms of post-amplification product by equating the chemiluminescent signals from the amplicons produced using 100 ng patient RNA with the signals from known amounts of K562 RNA assayed at the same time. Levels of *ABL* transcripts were used as an indicator of RNA integrity and only samples with an *ABL* result >0.5 ng, the background level for *ABL* detection, were accepted. In order to account for variations in RNA quality, results were normalised by expressing them as *BCR-ABL/ABL* ratios.

Calibration curves using varying amounts of K562 RNA demonstrated that linear results could be obtained over a range of 0.05–50 ng starting K562 RNA for *BCR-ABL* transcripts (Figure 3a) and 0.5–500 ng for *ABL* transcripts (Figure 3b) whereas the linear detection range of real-time quantitative RT-PCR is over 5 logs.[16,17] It is likely that linearity was lost above a certain copy number, 50 ng K562 RNA in the case of *BCR-ABL*, because the amplification machinery became saturated. A time course of the TMA incubation at 42°C indicated that most/all of the reaction occurred within the first 60 min and then slowed down, creating a stable plateau value (Figure 4a). We therefore chose 1 h as a suitable time to measure the amount of product. The results obtained were reproducible, as shown by repeated simultaneous analysis of three different RNA samples from patients with varying levels of either *BCR-ABL* or *ABL* transcripts (Figure 4b), and by separate analysis of four different dilutions of K562 RNA (Figure 4c).

BCR-ABL levels were undetectable or at background level only (0.005 ng) in RNA from all normal controls. They were high (0.2–43.0 ng) in RNA from PB or BM of 18 patients at diagnosis who had evidence of *BCR-ABL* positivity by conventional RT-PCR. The normalised *BCR-ABL/ABL* ratios for these 18 patients were all >0.1. Only one patient had a *BCR-ABL* value which was slightly greater than that obtained using 50 ng of K562 RNA, the highest template concentration using in the regression analysis. Although dilution of the RNA and re-analysis would have provided a more accurate *BCR-ABL* value for this patient, we did not feel it was warranted as it would not have altered interpretation of the result. Samples from two t(9;22) CML patients with very low or undetectable levels of *BCR-ABL* were also *BCR-ABL* negative by conventional RT-PCR and it is probable that these patients had *BCR* breakpoints outside the M-BCR.[25]

In five out of six patients studied who relapsed following alloBMT and received DLI treatment, results demonstrated changes in *BCR-ABL* levels and *BCR-ABL/ABL* ratios which reflected patient status or treatment. Apparent increases in levels were detected several months before relapse and were more discriminatory than conventional RT-PCR, which was consistently positive throughout this period in some cases (Figure 6a, b). Similarly, levels decreased following DLI treatment, despite persistent PCR positivity in some patients (Figure 6a, d, f). Furthermore, although sequential analysis of individual patients was the most informative means of assessing their clinical status, a *BCR-ABL/ABL* ratio of 0.01 served as a useful cut-off point. Of the 18 remission samples post-DLI treatment analysed from these five patients, 17 had values below this level, and the remaining sample had a ratio of 0.017 (Figure 6d). Conversely, all samples with values $\geqslant 0.01$ were associated with relapse. Results from the sixth patient were considered to be uninterpretable as they were all <0.01 (Figure 6c). The reason for this failure is not clear as *ABL* levels on all samples were within the accepted range. However, it is possible that the crucial events have been missed as no samples were available for analysis between 12 and 22 months post alloBMT, including at the time of relapse (19 months) and DLI treatment (approximately 20 months).

TMA/HPA offers several advantages over conventional qualitative and quantitative RT-PCR techniques. It is rapid, with a total assay time of less than 4 h, and requires only small amounts of RNA, less than 500 ng per triplicate analysis. The sensitivity is approximately equivalent to that of 'real-time' PCR, ie detecting one K562 cell in 10^4–10^5 HL60 cells, but it does not require expensive thermocycling equipment, simply two waterbaths to provide the different temperatures used for the amplification and hybridisation steps, and a basic luminometer. The whole procedure is carried out in one tube which minimises the risk of cross-contamination, and double-stranded DNA will not interfere with the assay as it will not act as a template for the initial reverse transcription step. Furthermore, the RNA amplicons produced in the TMA reaction are considerably more labile than DNA PCR products, substantially reducing the risk of carryover contamination and false positives. With appropriate primers and probes, TMA/HPA can be readily adapted to other translocations using the same core reagents.

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