Leukemia (2010) 24, 1719–1724

www.nature.com/leu

ORIGINAL ARTICLE

Patients with chronic myeloid leukemia who maintain a complete molecular response after stopping imatinib treatment have evidence of persistent leukemia by DNA PCR

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Around 40–50% of patients with chronic myeloid leukemia (CML) who achieve a stable complete molecular response (CMR; undetectable breakpoint cluster region-Abelson leukemia gene human homolog 1 (*BCR–ABL1*) mRNA) on imatinib can stop therapy and remain in CMR, at least for several years. This raises the possibility that imatinib therapy may not need to be continued indefinitely in some CML patients. Two possible explanations for this observation are (1) CML has been eradicated or (2) residual leukemic cells fail to proliferate despite the absence of ongoing kinase inhibition. We used a highly sensitive patient-specific nested quantitative PCR to look for evidence of genomic *BCR–ABL1* DNA in patients who sustained CMR after stopping imatinib therapy. Seven of eight patients who sustained CMR off therapy had *BCR–ABL1* DNA detected at least once after stopping imatinib, but none has relapsed (follow-up 12–41 months). *BCR–ABL1* DNA levels increased in all of the 10 patients who lost CMR soon after imatinib cessation, whereas serial testing of patients in sustained CMR showed a stable level of *BCR–ABL1* DNA. This more sensitive assay for *BCR–ABL1* provides evidence that even patients who maintain a CMR after stopping imatinib may harbor residual leukemia. A search for intrinsic or extrinsic (for example, immunological) causes for this drug-free leukemic suppression is now indicated.

Leukemia (2010) **24,** 1719–1724; doi:10.1038/leu.2010.185; published online 2 September 2010
Keywords: chronic myeloid leukemia; minimal residual disease; PCR; imatinib mesylate

Introduction

After 5 years on imatinib (Glivec; Novartis Pharmaceuticals, Basel, Switzerland) treatment, an estimated 40% of chronic phase chronic myeloid leukemia (CML) patients will achieve a complete molecular response (CMR), as defined by sensitive real-time reverse transcriptase quantitative PCR (RQ-PCR).[1] Over 50% of patients who stop imatinib after a period of stable CMR will relapse within 6 months of stopping treatment, whereas nearly all of the remaining patients sustain a stable CMR with follow-up beyond 18 months.[2–4] There is an apparent dichotomy between early relapse and stable CMR after stopping imatinib. Early relapse is consistent with the unchecked proliferation of a pool of viable CML cells. A sustained CMR without ongoing pharmacological kinase inhibition might indicate eradication of the leukemic clone. We hypothesized that the detection of minimal residual disease (MRD) below the threshold of CMR would identify a group of patients with a higher risk of relapse when imatinib kinase inhibition was withdrawn.

Virtually all CML patients express either or both of the common breakpoint cluster region Abelson leukemia gene human homolog 1 (*BCR–ABL1*) transcripts, e13a2 and e14a2, and this enables molecular monitoring by reverse transcriptase PCR. Nested reverse transcriptase PCR with multiple replicates has been shown to achieve a lower detection limit than conventional RQ-PCR.[5,6] However, this highly sensitive method can also detect *BCR–ABL1* mRNA in up to 30% of normal individuals.[5] Consequently, a positive result in a patient might represent MRD, but might also represent a rare chimeric transcript in a non-leukemic cell.

In a laboratory that handles large numbers of CML patient samples, there must be a risk of cross-contamination between samples, complicating the interpretation of low-level positive results.

In CML, the genomic breakpoints in *BCR* and *ABL1* are dispersed over intervals of 3.0 kb and ~150 kb, respectively. Each patient's fusion sequence is therefore virtually unique, unlike the mRNA transcript in which the highly variable intronic sequences containing the breakpoints are spliced out. *BCR–ABL1* DNA therefore provides a patient-specific marker of MRD, greatly reducing the risk of false positive results in replicate PCR assays, whether due to cross-contamination between samples from different patients or rare transcripts in non-leukemic cells.[5,6]

We developed a highly sensitive, patient-specific method for the detection of genomic *BCR–ABL1* DNA, and tested the method in a subset of 18 patients enrolled in the ongoing Australasian Leukaemia & Lymphoma Group CML8 study (ACTRN01260600 0118505). In this study, patients who had maintained a stable CMR for at least 2 years on imatinib treatment were asked to stop the therapy and were monitored monthly by RQ-PCR in a central laboratory. We found that 17 of the 18 patients had detectable MRD on one or more occasions, including 7 out of 8 patients who remained in a stable CMR without therapy.

Materials and methods

Patients were eligible for the study if they had achieved a CMR on imatinib, and sustained the CMR on continuing imatinib

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Received 19 April 2010; revised 1 July 2010; accepted 11 July 2010; published online 2 September 2010

therapy for at least 2 years, as defined by RQ-PCR. Patients who had previously received an allograft were excluded, but other previous pharmacological therapies (except for alternative kinase inhibitors) were permitted. The research was approved by the relevant ethics committees of the six participating hospitals. All participants gave written informed consent.

We report on a subset of 18 patients enrolled in the ongoing CML8 study with a known *BCR–ABL1* breakpoint and a minimum of 6 months of follow-up. The patients were 7 men and 11 women, with a median age of 57 years (range, 42–72 years). For the whole study, the median time from diagnosis of CML to study entry was >7 years (range, 3.5 to 19 years). Many patients had no suitable diagnostic sample available for breakpoint detection; hence, DNA PCR could not be performed.

RQ-PCR testing

Conventional RQ-PCR was performed following the standard procedures of our laboratory.[7] Briefly, total RNA was extracted from whole blood. RNA (2 μg) was reverse transcribed using random oligonucleotide primers. Real-time PCR was performed using 2.5 μl cDNA, with primers and hydrolysis probes specific for *BCR–ABL1* mRNA and for the *BCR* control gene. The entire assay (RNA extraction, cDNA synthesis and Q-PCR) was performed in duplicate in separate runs. Some centers routinely perform nested reverse transcriptase PCR to confirm CMR,[8] but we and others have found that the commonly used nested reverse transcriptase PCR method, first described by Cross *et al.*,[9] is no more sensitive than RQ-PCR in our hands,[10,11] and, therefore, nested reverse transcriptase PCR was not included in the definition of CMR that we used to determine study eligibility.

The lower limit of detection of each RQ-PCR sample was estimated using the Europe Against Cancer method (see also Supplementary Methods).[12] The number of copies of the control gene reflects both the quantity and quality of the RNA sample, and the efficiency of cDNA synthesis and Q-PCR. In our laboratory, a sample with 400 000 *BCR* transcripts has a calculated sensitivity of 4.5 log below the pretreatment baseline level of *BCR–ABL1*. A screening RQ-PCR at study entry was required to achieve a calculated sensitivity of 4.5 log with no detectable *BCR–ABL1* transcripts. A patient with detectable *BCR–ABL1* mRNA in any sample during the 2 years before screening was ineligible. Molecular relapse was defined as detectable *BCR–ABL1* mRNA at any level in two consecutive peripheral blood RQ-PCR assays.

BCR–ABL1 *DNA breakpoint detection and primer design*

Genomic DNA was extracted from patient cells stored at the time of diagnosis. The *BCR–ABL1* breakpoint region was amplified in 20 ng genomic DNA by long-range PCR using a single forward primer in *BCR* and a panel of 20 reverse primers spanning both alternative first exons of *ABL1*[13] (Supplementary Table 1). Patient-specific primers were designed to amplify approximately 100–200 bases on either side of the breakpoint. Nested primers were designed for Q-PCR, with the hydrolysis probe spanning (or adjacent to) the *BCR* breakpoint.

Specificity of BCR–ABL1 *DNA PCR*

Owing to the high frequency of genomic repeat elements in either *BCR* or *ABL1*, it is possible that a single primer pair will result in a low level of nonspecific amplification involving repeat sequences. DNA from three patients and from two CML cell lines (K562 and MOLM-1) was used to test the specificity of the corresponding patient-specific primer sets of the three patients. Each patient-specific PCR amplified only the patient's own *BCR–ABL1* DNA. The primer sets for every patient were tested using DNA pooled from five normal individuals. Three of 18 non-nested DNA Q-PCR assays showed nonspecific amplification in normal DNA. In two cases, this was eliminated by redesigning the primers. Using a nested PCR method, it was possible to eliminate nonspecificity in all cases.

Sensitivity of BCR–ABL1 *DNA PCR*

The lower limit of detection of any assay is dependent on the amount of material assayed as well as the efficiency of the assay. We measured the amount of amplifiable genomic DNA in each patient sample using Q-PCR for a control gene, glucuronidase β (*GUSB*). *GUSB* was selected because it is on chromosome 7 and is rarely involved in the *BCR–ABL1* rearrangement. This enabled us to express the MRD result relative to the number of amplifiable genomes. This is analogous to the use of a control gene in RQ-PCR, instead of relying on an amount of RNA (determined by spectrophotometry) to ensure a consistent amount of patient material in the assay. Spectrophotometry may be inaccurate if there is partial degradation of the DNA or if the solution contains protein or RNA. The concentration of amplifiable DNA in each sample was determined by Q-PCR for a region spanning an intron–exon boundary in *GUSB* (Supplementary Methods). A *GUSB* DNA standard was prepared using DNA extracted from 2×10^6 normal peripheral blood leukocytes. The concentration of amplifiable DNA in patient samples was determined relative to the standard curve, which was a dilution series of the *GUSB* DNA standard. Using this method, the estimated DNA yield was 5.7 pg per diploid human cell.

Genomic DNA was extracted from patient samples (peripheral blood $n=64$; marrow $n=3$) collected at study entry, and at various follow-up time points. Having first determined the concentration of amplifiable DNA in each sample using the *GUSB* value, the volume of DNA in each assay was adjusted to ensure that there was 500 ng DNA in each PCR replicate. The total amount of DNA tested was 2–10 μg (4–20 replicates), according to the frequency of detection of *BCR–ABL1*. If few or no replicates were positive, a larger number of replicates was needed to increase the accuracy of the estimate of MRD. Each assay included negative controls (at least 10 μg normal human DNA in 20 replicates amplified using patient-specific primers) to detect nonspecificity or contamination. The number of cells assayed in 10 μg genomic DNA was $\sim 1.75 \times 10^6$, giving a lower detection limit of $10^{-6.2}$.

Calculation of semiquantitative BCR–ABL1 *DNA results*

Serial dilutions of patient-specific *BCR–ABL1* DNA (DNA from the sample used for breakpoint detection) were amplified in parallel with the follow-up samples to generate a *BCR–ABL1* standard curve. The number of amplifiable leukemic genomes in each replicate was calculated from the patient-specific standard curve. The final MRD result was expressed as the total number of amplifiable leukemic genomes divided by the total number of amplifiable genomes in all replicates (Supplementary Methods).

Results

BCR–ABL1 DNA was detected in 17 of 18 patients on at least one occasion when in CMR at baseline and/or during

follow-up. This confirms the higher sensitivity of DNA PCR in patient samples (Supplementary Table 2). The estimated level of MRD in these 17 patients ranged from 4.5 to 6.0 log below the pretreatment baseline. These semiquantitative *BCR–ABL1* DNA results indicate that a substantial proportion of CML patients with a sustained CMR on imatinib have a level of MRD near the estimated detection limit of RQ-PCR, or at most only 1.5 log below this threshold.

Of the 18 patients, 8 remained in a stable CMR at the last follow-up after a median of 2 years (range, 12–41 months). *BCR–ABL1* DNA was detected on at least one occasion in 7 of these patients (Figure 1; patients no. 1–7). Only one patient (no. 8) had no detectable *BCR–ABL1* DNA on every occasion. In contrast to the increase seen in most patients who relapsed, serial testing of patients in ongoing CMR showed a stable level of *BCR–ABL1* DNA (patient no. 5; Figure 2a). In one case, there was possibly a reduction in *BCR–ABL1* DNA over time, as it was not detectable at the last two time points (patient no. 3; Figure 1).

The 10 molecular relapses occurred at a median interval of 4 months after stopping imatinib (range, 2–8 months). Relapse occurred in six of nine patients treated with imatinib *de novo* in early chronic phase and in four of nine patients treated with imatinib after interferon-α treatment. All 10 relapsed patients had detectable *BCR–ABL1* DNA at least 2 months before molecular relapse, and in most cases before stopping imatinib (Figure 1; patients no. 9–18). The median rate of increase in *BCR–ABL1* DNA in the 10 patients who relapsed was around 0.02 log per day, equivalent to a leukemia doubling time of 13 days. This is comparable with the rate determined using RQ-PCR kinetic data.[14] In most relapses, *BCR–ABL1* DNA increased exponentially (Figure 2b), but in the patient with the latest relapse, there was only a modest increase in *BCR–ABL1* DNA (Figure 2c). This patient chose not to recommence imatinib, despite meeting the study definition of 'relapse', and at last follow-up had no detectable *BCR–ABL1* mRNA in a sample of adequate quality.

Our hypothesis was that the detection of *BCR–ABL1* DNA at study entry would identify patients with a higher risk of relapse. The positive predictive value for relapse of a positive DNA PCR result at baseline was 62%. The negative predictive value was 75%. The finding of *BCR–ABL1* DNA in most of the patients, even in stable CMR, limited the utility of this test as a predictor of relapse risk.

The higher sensitivity of the *BCR–ABL1* DNA PCR method is dependent on the large number of replicates performed, and it is possible that a similar sensitivity could have been achieved by performing multiple RQ-PCR assays for *BCR–ABL1* mRNA. We prepared RNA from the blood of three normal individuals and two CML patients in CMR, and performed multiple replicate RQ-PCR assays following the routine laboratory procedure. A total of 12 μg RNA from each sample was tested in 18 replicates (Supplementary Table 3). *BCR–ABL1* mRNA was detected in the blood of both CML patients (in 1 of 18 replicates each). However, 5 of 53 replicates from the three normal individuals also contained detectable *BCR–ABL1* transcripts.

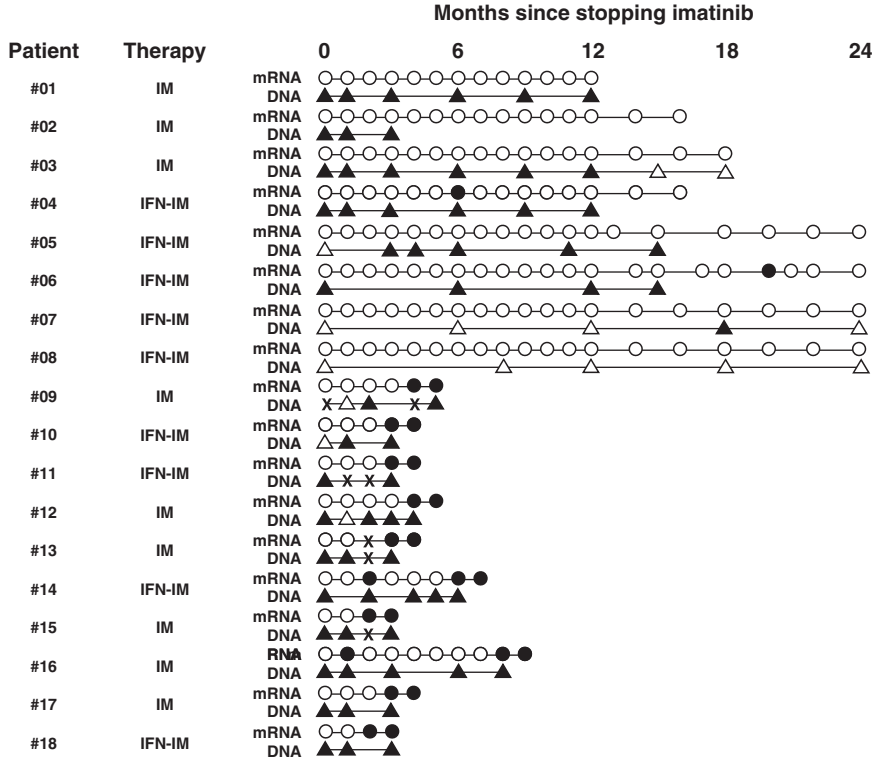

Figure 1 *BCR–ABL1* DNA and mRNA detection in follow-up samples. Patients no. 1–8 remained in CMR at last follow-up: seven of these eight patients had detectable *BCR–ABL1* DNA without loss of CMR. Patients no. 9–18 had a molecular relapse, indicated by two consecutive positive results in RQ-PCR. In all of these 10 patients, *BCR–ABL1* DNA was detectable before *BCR–ABL1* mRNA. *BCR–ABL1* mRNA results (circles) are shown on the upper line and *BCR–ABL1* DNA results (triangles) are shown on the lower line. Open symbols indicate that *BCR–ABL1* was not detected; solid symbols indicate that *BCR–ABL1* was detected; crosses indicate that no sample was available for testing at that time point.

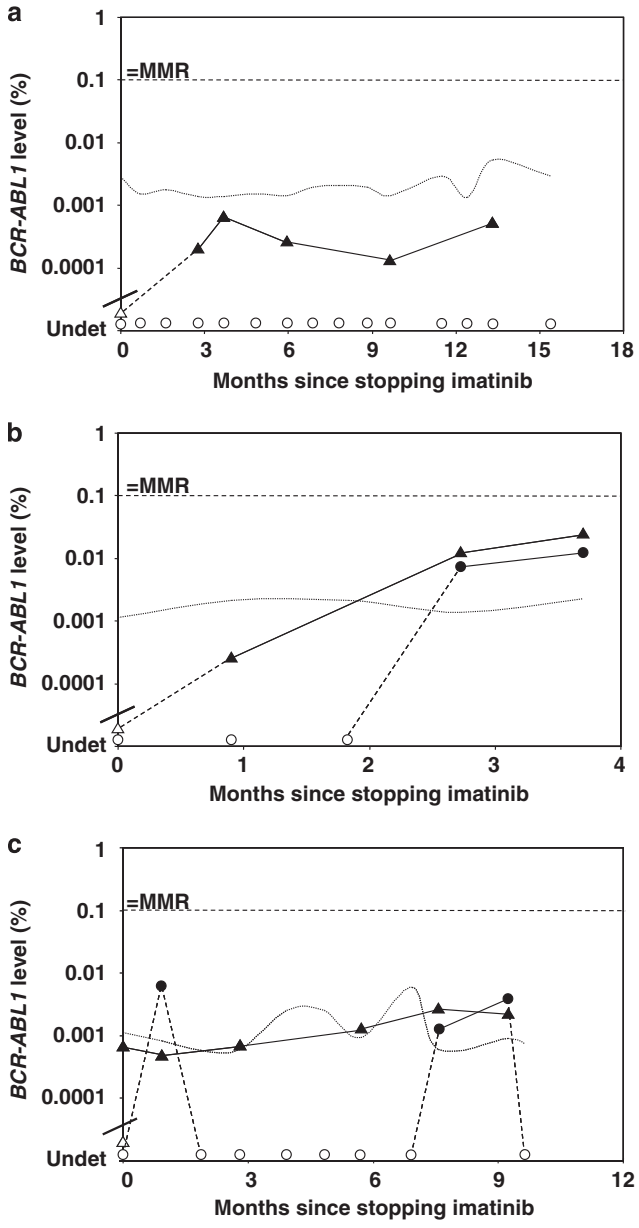

Figure 2 Examples of semiquantitative results for *BCR–ABL1* DNA in three patients with divergent outcomes after stopping imatinib. (**a**) Patient no. 5 remained in CMR 2 years after stopping imatinib with no detectable *BCR–ABL1* mRNA on any occasion, whereas *BCR–ABL1* DNA was detected on multiple occasions at a stable low level. (**b**) Patient no. 10 developed a molecular relapse 3 months after stopping imatinib. *BCR–ABL1* DNA was not detectable at study entry, but was detected 1 month after stopping imatinib and rose exponentially. (**c**) Patient no. 16 developed a molecular relapse 8 months after stopping imatinib. *BCR–ABL1* DNA was detected at study entry and on each subsequent occasion, but the estimated increase in *BCR–ABL1* DNA was less than in other relapses. The patient did not recommence imatinib treatment, but regained CMR spontaneously. *BCR–ABL1* DNA results are shown as triangles. *BCR–ABL1* mRNA results are shown as circles. Open symbols indicate that *BCR–ABL1* was not detected; solid symbols indicate that *BCR–ABL1* was detected. The broken line between detectable and undetectable *BCR–ABL1* results indicates that the slope of this line is arbitrary, because the true level from which *BCR–ABL1* has risen is not known. The estimated lower limit of detection of the patient's RQ-PCR samples is indicated by the curved dotted line, averaging about 4.5 log below the standardized baseline pretreatment level. Undet = undetectable; MMR = major molecular response.

Hence, it was not possible to distinguish between MRD in CML patients and rare transcripts in the samples of normal individuals using this approach, as previously described.[5]

Discussion

We have demonstrated, by the detection of *BCR–ABL1* DNA, that leukemic cells can persist in patients who maintain a CMR after imatinib cessation. A total of 7 of 17 patients in our study with MRD detectable at the genomic level on at least one occasion after imatinib cessation have not relapsed with up to 41 months of sensitive RQ-PCR monitoring. In the allograft setting the observation of very late relapses after a period of molecular remission provides strong evidence that CML MRD may be suppressed by an ongoing graft versus leukemia effect.[15] Cytotoxic T lymphocytes specific for leukemia-associated antigens have been demonstrated in patients treated with both interferon-α[16,17] and imatinib.[18] Further investigation is warranted to determine whether immunological reactivity against CML modifies the risk or timing of relapse when imatinib treatment is withdrawn.

Kinetic data using RQ-PCR have shown that the average rate of decline in MRD is <0.5 log per annum beyond the second year of imatinib treatment.[1,14] Most of the patients in this study had been in CMR for 2–3 years at study entry; hence, by extrapolation, one could predict that the level of MRD would average 1–1.5 log below the detection limit of RQ-PCR, that is, around 5–6 log below the pretreatment level of *BCR–ABL1*. At study entry, we detected *BCR–ABL1* DNA in 13 of 17 patients (one sample was lost) at a level that was consistent with the kinetic data, and in the remaining 4 patients, the level of MRD was below the limit of detection. If there is a continuing slow depletion of MRD at the same rate during prolonged imatinib treatment, it would be predicted that a decade or more of treatment after achieving a stable CMR would be required for complete eradication of the leukemic clone. The important finding of this study is that complete eradication of the leukemic clone appears not to be a prerequisite for sustained remission after imatinib withdrawal.

A study of *BCR–ABL1* DNA PCR in patients in long-term remission post-allograft was recently published.[19] The methodological approach was very similar to our own, except that nesting was not used. Nesting was found not to confer higher sensitivity than an efficient and specific real-time PCR. We used nesting to overcome nonspecificity in one or two patients, and this methodological finding of the Hammersmith group is not inconsistent with our own experience. It may be that there are rare patients in whom the specific *BCR–ABL1* fusion sequence complicates the design of real-time PCR with oligonucleotides of appropriate melting temperature and specificity.

Sobrinho-Simoes *et al*. tested a total of 36 replicates, containing 10.8 µg genomic DNA for each sample. Only five imatinib-treated patients were examined, many of whom had a relatively short duration of CMR. In most of these patients, *BCR–ABL1* DNA was detected, consistent with our findings in a larger number of patients, most of whom had a longer duration of CMR. Interestingly, their patients in long-term remission post-allograft appeared to have deeper molecular responses than our patients in stable CMR after stopping imatinib. An earlier study comparing MRD after imatinib or allografting used intermittent RQ-PCR positivity as a marker for a shallow CMR, and reached a similar conclusion.[20] Persistent RQ-PCR negativity could be a surrogate for a lower level of MRD in CMR. In this clinical trial, we deliberately excluded any patient with any detectable

BCR–ABL1 mRNA in the preceding 2 years, in order to select a group of patients with a stringent definition of CMR.

In those patients who relapsed, the rate of increase in *BCR–ABL1* DNA (0.02 log/day) was similar to RQ-PCR kinetic data, in which the cessation of imatinib is followed by an exponential increase in the level of *BCR–ABL1* mRNA.[14,21] Michor *et al*. predicted that unchecked proliferation of a single residual CML cell would lead to molecular relapse within a period of 6 months. In fact, we found that most patients relapsed within 4 months of imatinib withdrawal. The latest relapse (at 8 months) was associated with a much smaller rise in *BCR–ABL1* DNA, raising the possibility that the biology of late relapse might be different from the typical relapses in the first few months. To understand such observations, it would be necessary to define the measurement error of the DNA PCR assay so that a true increase in the level of MRD can be distinguished from the imprecision of the assay at low levels.

The quantification of MRD by genomic DNA enables an estimation of cell number, unaffected by mRNA expression levels and mRNA stability. Further assay development would be needed to standardize this method and to determine the precision of quantitative *BCR–ABL1* DNA results, as has been undertaken for DNA PCR monitoring of acute lymphoblastic leukemia.[22] Despite the added complexity of a patient-specific method, this assay has the potential to provide useful information about MRD in selected patients in a stable CMR that could not be provided by conventional monitoring strategies. It remains possible that a novel RNA-based assay might have enabled us to reach the same conclusion. However, in multiple replicate RQ-PCR assays, it was not possible to distinguish between MRD and the background low level of 'false-positive' *BCR–ABL1* mRNA in normal controls. Similar to Sobrinho-Simoes *et al*., we felt that a patient-specific assay was the best way to ensure that a positive result truly represents MRD in the patient being tested. There are now several published methods for the detection of the patient-specific *BCR–ABL1* genomic breakpoint,[23–25] and once the breakpoint is known the quantification of the patient-specific sequence is relatively simple. This approach has been used in childhood acute lymphoblastic leukemia for some years and, in the future, *BCR–ABL1* DNA PCR might have a role in the assessment of response to novel treatments, such as BCR–ABL1 peptide vaccination, with the aim of eradicating MRD.

Using a novel, patient-specific method for the detection of *BCR–ABL1* DNA, we were able to detect MRD in the majority of patients in a stable CMR on imatinib. Contrary to our hypothesis, the presence of *BCR–ABL1* DNA before stopping imatinib was not predictive of relapse, suggesting that factors other than the number of residual CML cells must contribute to the probability of stable CMR after withdrawal of imatinib.

Conflict of interest

DMR was a PhD Scholar of the Leukaemia Foundation of Australia. DMR, SB, JFS, APS, CA, AKM, APG and TPH have received honoraria from Novartis Oncology. CS is an employee of Novartis Oncology. PAB, CF, PD, RJF and JVM have no conflict of interest to declare.

Acknowledgements

Professor Nicholas Cross and Dr Joannah Score (Wessex Regional Genetics Laboratory, Salisbury, United Kingdom) developed the original long-range PCR method for the detection of *BCR–ABL1* genomic breakpoints. Dr John Reynolds, Ms Rachel Koelmeyer and Dr Ruth Columbus (Australasian Leukaemia & Lymphoma Group Trial Centre) contributed to the design of the clinical trial. Emeritus Professor Alexander Morley (Flinders University) provided advice on laboratory studies. This study was supported by research funding from Novartis Oncology to the Australasian Leukaemia & Lymphoma Group (clinical trial), and to the Institute of Medical & Veterinary Science, SA Pathology (laboratory studies).

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)