Accurate quantification of minimal residual disease (MRD) during treatment of chronic myeloid leukemia (CML) guides clinical decisions. The conventional MRD method, RQ-PCR for BCR-ABL1 mRNA, reflects a composite of the number of circulating leukemic cells and the BCR-ABL1 transcripts per cell. BCR-ABL1 genomic DNA only reflects leukemic cell number. We used both methods in parallel to determine the relative contribution of the leukemic cell number to molecular response. BCR-ABL1 DNA PCR and RQ-PCR were monitored up to 24 months in 516 paired samples from 59 newly-diagnosed patients treated with first-line imatinib in the TIDEL-II study. In the first three months of treatment, BCR-ABL1 mRNA values declined more rapidly than DNA. By six months, the two measures aligned closely. The expression of BCR-ABL1 mRNA was normalized to cell number to generate an expression ratio. The expression of e13a2 BCR-ABL1 was lower than that of e14a2 transcripts at multiple time points during treatment. BCR-ABL1 DNA was quantifiable in 48% of samples with undetectable BCR-ABL1 mRNA, resulting in MRD being quantifiable for an additional 5-18 months (median 12 months). These parallel studies show for the first time that the rapid decline in BCR-ABL1 mRNA over the first three months of treatment is due to a reduction in both cell number and transcript level per cell, whereas beyond three months, falling levels of BCR-ABL1 mRNA are proportional to the depletion of leukemic cells.

ABSTRACT

Introduction

Real-time reverse transcriptase quantitative PCR (RQ-PCR) for BCR-ABL1 mRNA is widely used for the routine monitoring of chronic myeloid leukemia (CML) patients receiving tyrosine kinase inhibitor (TKI) therapy. The achievement of molecularly-defined therapeutic targets during TKI treatment is associated with superior progression-free and overall survival.1 The BCR-ABL1 mRNA level is a composite measurement that reflects both the proportion of leukemic cells in the sample, and the expression of BCR-ABL1 relative to its control gene. Pre-analytical factors, such as the rate of degradation of the target mRNA, and methodological factors, such as the efficiency of reverse transcription or the choice of control gene, may have a significant influence on the final result of RQ-PCR.2,3 Substantial effort has been invested to minimize variation due to such factors through the development of an International Scale (IS) for BCR-ABL1.4

BCR-ABL1 genomic DNA PCR response kinetics during first-line imatinib treatment of chronic myeloid leukemia

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An alternative approach to overcome the variability in Q-PCR is to measure *BCR-ABL1* genomic DNA, since the overwhelming majority of chronic phase CML patients will have a single copy of *BCR-ABL1* and two copies of an autosomal control gene in each leukemic cell. In the past, this approach was not practical due to the complexity of sequencing individual genomic breakpoints. Almost all CML patients express one or both of the two common *BCR-ABL1* mRNA transcripts (e13a2, e14a2), whereas the genomic fusion sequences involve introns that are spliced out from the mRNA, and are essentially unique to each individual patient.6 Advances in sequencing technology have made it relatively simple to detect *BCR-ABL1* genomic breakpoints, and several methods have been published.5,7

It should be emphasized that DNA PCR and RQ-PCR are not expected to yield identical results. This is perhaps best exemplified by the comparison of RQ-PCR with metaphase karyotyping in CML, which shows that a partial cytogenetic response [≤55% Philadelphia-positive (Ph⁺) cells] is roughly equivalent to *BCR-ABL1* ≤10%.8 Whereas both techniques are clinically useful, measures of the size of the CML clone the end point of each assay is qualitatively different. *BCR-ABL1* DNA PCR is analogous to fluorescence in situ hybridization, in that both methods measure the simple proportion of cells in a sample that carry the Philadelphia rearrangement.

We used quantitative *BCR-ABL1* DNA techniques, Q-PCR and digital PCR (dTaq PCR), to monitor a cohort of patients in the Australasian Leukaemia and Lymphoma Group (ALLG) CML9 study (TIDEL-II).10 These results were compared with routine RQ-PCR monitoring. Since the number of copies of *BCR-ABL1* DNA is directly related to the number of leukemic cells in a sample, we used DNA and mRNA-based methods in order to determine the relative contribution of cell number and expression changes to molecular response in CML. Secondly, where there were differences between RQ-PCR and DNA PCR, we explored whether these differences might provide additional predictive information concerning treatment response.

**Methods**

**Patients’ characteristics and samples**

Fifty-nine newly diagnosed chronic phase CML patients from the TIDEL-II clinical trial9 were included in our study. Details of these patients and of the samples analyzed are presented in the Online Supplementary Tables S1 and S2. The overall clinical characteristics and treatment responses of the selected cohort were not significantly different from those of the overall study population. The subset of patients included here were selected in three categories: indetectable MRD (UMRD) achieved within the first 2 years (n=26); treatment failure (n=9); and 24 additional patients not falling into either of the first two categories. Treatment failure was defined following the European LeukemiaNet (ELN) criteria as loss of complete hematologic response, loss of complete cytogenetic response, loss of major molecular response (MMR; *BCR-ABL1* ≤0.1%), kinase domain mutations, or progression to accelerated phase/blast crisis.11

Peripheral blood samples for molecular analysis were collected prior to commencing TKI treatment (baseline); at one, two, and three months; and every three months thereafter up to 24 months. RQ-PCR was performed centrally in the diagnostic laboratory of SA Pathology, Adelaide, Australia, using the *BCR control gene.*11 The results were reported as *BCR-ABL1*/*BCR% applying an IS conversion factor (Online Supplementary Appendix).10

**Breakpoint detection**

The *BCR-ABL1* genomic DNA breakpoint was determined, as previously described, in blood samples collected at diagnosis using long range PCR with a single forward primer in *ABL1* and multiple reverse primers in *ABL1* to amplify the breakpoint (Online Supplementary Appendix).10

**Quantification of *BCR-ABL1* DNA**

Genomic DNA was extracted from peripheral blood leukocytes. The amount of amplifiable DNA in each sample was measured using the *GUSB* control gene. The earlier assays were performed using real-time Q-PCR with standard curves for both *BCR-ABL1* (patient’s diagnostic DNA assigned a value of 100%) and *GUSB* (plasmid) diluted in non-human DNA. Later assays used digital PCR (dPCR) for both *BCR-ABL1* and *GUSB* with the aim of improving precision. Results were reported as *BCR-ABL1*/*GUSB%* (corrected for the two copies of *GUSB* per cell) normalized against the individual patient’s diagnostic sample. Further details are provided in the Online Supplementary Appendix and Online Supplementary Figures S1-S3.

**Statistical analysis**

Statistical analysis was performed using the GraphPad Prism 7 statistical software (GraphPad Prism Inc., La Jolla, CA, USA). Agreement between assays was assessed using the method of Bland and Altman.11 Correlation between non-parametric values was assessed using Spearman rank coefficient. Differences between *BCR-ABL1* DNA and mRNA measurements were compared using a Mann-Whitney test. The cumulative incidence of MMR and MR4.5 was calculated using the Fine and Gray regression method in R. Any event leading to the permanent discontinuation of imatinib/nilotinib (including treatment failure, intolerance, and death) was treated as a competing risk. P<0.05 was considered statistically significant.

**Results**

**Comparison between DNA and mRNA before treatment**

Since DNA Q-PCR quantifies *BCR-ABL1* relative to the diagnostic DNA, we considered only the absolute dPCR values at diagnosis (n=29) and compared these values with the corresponding mRNA levels and the percentage of Ph⁺ bone marrow metaphase cells. The median value of *BCR-ABL1* DNA prior to TKI treatment was 100% by karyotyping (range, 85-100%) and 84% (range, 45-164%) by dPCR. The corresponding median *BCR-ABL1* mRNA value was 70%, with values ranging from 3.7% to 425% (Figure 1A). Two of the 3 patients (#1 and #3) with low *BCR-ABL1* mRNA had stored peripheral blood cells available for interphase fluorescence in situ hybridization, which showed excellent agreement with the *BCR-ABL1* DNA values obtained by dPCR (Table 1). Two of these 3 patients experienced treatment failure (blast crisis/secondary resistance with a kinase domain mutation) and the third patient had ELN warning features at baseline (high

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S2.
Sokal score and additional clonal chromosomal abnormalities in Ph+ cells) and failed to reach MMR by 12 months and MR4.5 by 24 months. All 3 of these patients expressed e13a2 BCR-ABL1 transcripts (one expressed both e13a2 and e14a2) and all had unusually low white blood cell counts at study entry (<20x10^9/L) (Online Supplementary Table S3). The post-treatment molecular responses of these 3 patients are shown in Figure 1B-D.

**Agreement of Q-PCR and dPCR for BCR-ABL1 DNA**

Forty-six serial samples from 9 patients on TKI treatment were quantified by both Q-PCR and dPCR for BCR-ABL1 DNA. The results were highly correlated ($r=0.94$, $P<0.0001$). Agreement between the two methods was further assessed using a Bland-Altman plot (Online Supplementary Figure S4). The mean bias was -0.11-log with the 95% limits of agreement ranging from -1.02-log to 0.80-log (±8.1 fold) indicating that there was no systematic difference between the results obtained by the two DNA PCR methods after diagnosis. In subsequent analyses of BCR-ABL1 DNA during treatment the two sets of data were combined.

**Faster reduction in BCR-ABL1 mRNA than DNA early in treatment**

In our cohort of 59 patients, the quantified BCR-ABL1 mRNA and DNA results (undetectable values excluded) were highly correlated across the range of values during TKI treatment ($r=0.88$; $P<0.0001$) (Figure 2A). However, during the first three months of therapy BCR-ABL1 DNA values were significantly higher than mRNA, whereas from six months onwards there was good agreement between methods (Figure 2B). The median reduction in BCR-ABL1 from baseline to three months was 2.05-log versus 1.75-log for BCR-ABL1 DNA (Online Supplementary Figure S5). This bias was independent of the BCR-ABL1 DNA quantification method (seen with both dPCR and Q-PCR; see Online Supplementary Figure S6).

**Early molecular response assessment by BCR-ABL1 mRNA and DNA**

A reduction in BCR-ABL1 to ≤10% at three months [early molecular response (EMR)] has emerged as an early treatment milestone that is strongly associated with later achievement of optimal response and progression-free survival.15,16 The predictive effect of EMR was confirmed in the overall TIDEL-II study population of 210 patients.9 In this smaller subgroup, no patient with BCR-ABL1 levels >10% at three months went on to achieve MMR or MR4.5. We tested the predictive value of BCR-ABL1 levels by both mRNA and DNA at the 3-month landmark using

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**Figure 1. DNA and mRNA prior to treatment.** (A) Proportion of leukemic cells and BCR-ABL1 expression before treatment assessed by conventional cytogenetic analysis (green), DNA dPCR (red) and RQ-PCR (blue). Three patients with discrepant DNA and mRNA values are highlighted (red square). (B-D) Molecular response of patients (pts) with BCR-ABL1 <10% despite DNA values close to 100%. Absolute DNA dPCR values are represented at diagnosis. TKI: tyrosine kinase inhibitor; Ph+: Philadelphia positive; Und: undetectable.

**Table 1. BCR-ABL1 values in patients with low mRNA values relative to DNA values.**

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>mRNA, IS%</th>
<th>DNA dPCR, %</th>
<th>iFISH, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>6.96</td>
<td>99.4</td>
<td>88.0</td>
</tr>
<tr>
<td>#2</td>
<td>5.10</td>
<td>81.2</td>
<td>N/A</td>
</tr>
<tr>
<td>#3</td>
<td>3.70</td>
<td>101.8</td>
<td>87.8</td>
</tr>
</tbody>
</table>

IS: International Scale; dPCR: digital PCR; iFISH: interphase fluorescence in situ hybridization; N/A: not available.
the established BCR-ABL1 transcript IS cut-offs of 10% and 1%. Both mRNA and DNA levels were predictive of later MMR and MR4.5, and the BCR-ABL1 DNA level did not improve the predictive value of conventional RQ-PCR (Online Supplementary Figure S7). The optimal BCR-ABL1 DNA cut-off for prediction of later molecular response could not be determined in this study due to the small number of patients and the potential bias due to the selection of patients on the basis of response.

Transcript type and molecular response

It has previously been reported that the BCR-ABL1 transcript type may influence treatment outcomes (reviewed by Marum and Branford17). Consequently, we compared molecular responses in patients having only e13a2 transcripts (n=32) or only e14a2 transcripts (n=17). There was no significant difference between BCR-ABL1 IS levels according to transcript type at any individual time point (Figure 3A). However, BCR-ABL1 DNA was significantly higher in e13a2 patients at multiple time points during treatment (Figure 3B). The median BCR-ABL1 expression ratio (mRNA%:DNA%) was 0.5 for e13a2 versus 1.09 for e14a2 (P=0.0005) (Figure 3C). This analysis was repeated using BCR-ABL1 DNA values from dPCR and Q-PCR separately and a similar pattern was observed (Online Supplementary Figure S8).

Sensitivity of RQ-PCR and DNA PCR

The median limit of detection achieved by RQ-PCR was MR4.6 (range, 3.2-5.1 log) in comparison with MR5.2 (range, 4.6-5.7 log) for DNA PCR. BCR-ABL1 DNA was detected in 42 of 86 samples with undetectable mRNA (49%) with a median value of 0.002% (range, 0.0002-0.07%). Two samples were mRNA-positive, DNA-negative with BCR-ABL1 levels of 0.003 and 0.02% (Figure 2A). The remaining 44 samples had undetectable BCR-ABL1 by both methods. The higher degree of sensitivity using BCR-ABL1 DNA led to MRD being quantifiable for an additional 5-18 months (median 12 months) of follow up. Samples collected after 24 months were not analyzed, so in some patients the duration of quantifiable MRD may have been longer than this estimate.

Discussion

BCR-ABL1 molecular monitoring by RQ-PCR is relied upon to ensure that TKI-treated patients are on track to achieve an optimal response, to define the end points of clinical trials, and to determine criteria for a safe trial of cessation of TKI therapy after having sustained a deep molecular response.10-20 Molecular responses defined by RQ-PCR have been shown to be robust indicators of clini-
ical outcome, yet the biology of BCR-ABL1 molecular response is relatively complex. Key to this complexity is the composite nature of the response: a reduction in the ratio of BCR-ABL1 mRNA to a control gene could be due to a reduction in the proportion of CML cells in the sample, a reduction in the expression of BCR-ABL1, an increase in the expression of the control gene, or even a change in the relative stability of these mRNA transcripts. Since the number of copies of genomic BCR-ABL1 is directly proportional to the number of leukemic cells, we reasoned that measuring both BCR-ABL1 DNA and mRNA would lead to a better understanding of the main determinants of variation in molecular response.

During the first three months of treatment, the

![Figure 3. BCR-ABL1 transcript type and molecular response. (A) Comparison of the BCR-ABL1 IS values during the first two years of tyrosine kinase inhibitor (TKI) treatment (e13a2 shown in green and e14a2 shown in black). (B) Comparison of e13a2 and e14a2 BCR-ABL1 DNA values in the same patients. Diagnostic values were assigned a value of 100%. Note that at later time points the proportion of e14a2 patients with undetectable BCR-ABL1 DNA was higher than for e13a2, which may result in an underestimation of the difference between the two transcript types. (C) Box and whiskers plot comparing BCR-ABL1 expression ratio (mRNA:DNA) for e13a2 and e14a2 transcripts. *P<0.05; ***P<0.001.](image-url)
BCR-ABL1 DNA values were significantly higher than the corresponding BCR-ABL1 DNA values. After three months, the reduction in BCR-ABL1 levels (2.05-log) was primarily due to depletion of CML cells (1.75-log), with only a small contribution from expression changes (0.3-log reduction; 2-fold decrease). A proportionally greater decline in expression than in cell number is likely due to the early depletion of higher expressing cells. From six months of treatment onwards, there was generally excellent agreement between the level of MRD measured by depletion of CML cells (1.75-log), with only a small 2-fold decrease). A proportionally greater decline in BCR-ABL1 IS levels (2.05-log) was primarily due to depletion of murine cells selected for low BCR-ABL1 expression. The median limit of detection of BCR-ABL1 DNA was MR5.2 versus MR4.6 for conventional RQ-PCR. This improvement in sensitivity led to around half of the samples with undetectable BCR-ABL1 mRNA having measurable MRD and extended the period of time in which there was detectable BCR-ABL1 by around a year. The median limit of detection for dPCR was MR5.2. These results are similar to those obtained by Alikian et al., who used dPCR for both BCR-ABL1 DNA and mRNA, and found higher sensitivity with the DNA-based assay. Whil...


