Reverse Transcription with Random Pentadecamer Primers Improves the Detection Limit of a Quantitative PCR Assay for BCR-ABL Transcripts in Chronic Myeloid Leukemia: Implications for Defining Sensitivity in Minimal Residual Disease

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BACKGROUND: Real-time quantitative reverse transcription PCR (RQ-PCR) assay for BCR-ABL is used to monitor treatment response in chronic myeloid leukemia (CML). BCR-ABL transcript levels decline over several years of imatinib treatment, and increasing numbers of patients have BCR-ABL transcripts at or below the limit of detection. More sensitive PCR methods are required to assess whether these patients have a long-term continuing decline in residual disease.

METHODS: We used random pentadecamer (R15) primers for reverse transcription in RQ-PCR and compared the results with our established method that uses random hexamers. An increase in assay sensitivity would be detected as an increase in the number of BCR-ABL transcripts.

RESULTS: BCR-ABL transcripts increased by 86% with R15 primers. We used R15 primers to retest 19 samples from selected CML patients who had no BCR-ABL transcripts recently detectable with hexamer primers and detected BCR-ABL transcripts in 68% of the samples. Use of R15 primers showed variable increases in the transcripts for control genes BCR (breakpoint cluster region), ABL1 (c-abl oncogene 1, receptor tyrosine kinase), and GUSB (glucuronidase, beta), depending on the gene examined. The reported BCR-ABL/control gene ratio was affected, and the estimated detection limit of the assay, which was based on increased control gene copy number, was different for each control gene.

CONCLUSIONS: This simple modification to the reverse transcription methodology improved the detection limit of the RQ-PCR assay for BCR-ABL transcripts. In the field of CML, these results have important implications for defining the detection limit of an assay when the BCR-ABL transcript is undetectable. Random pentadecamer primers may also be useful in other reverse transcription PCR assays for which the abundance of the target RNA is low.

Real-time quantitative reverse transcription PCR (RQ-PCR) assay for transcripts of the BCR-ABL fusion gene is used to monitor treatment response in chronic myeloid leukemia (CML). Serial monitoring shows that the depth of response to the kinase inhibitor, imatinib, increases progressively over time, with approximately half of patients in first-line treatment having no detectable BCR-ABL transcripts after 5 years. The ability to detect BCR-ABL transcripts is limited by the quality of the RNA sample and by the detection limit of the RQ-PCR assay. The ability to detect low concentrations is required to state with confidence that a result of no detectable BCR-ABL transcripts indicates a stable or declining amount of minimal residual disease (MRD). An estimated detection limit of 4.5 logs below the median pretreatment BCR-ABL value (standardized baseline) was used in the IRIS study to define samples as having no detectable BCR-ABL transcripts.

Our established RQ-PCR assay uses random hexamer (R6) oligonucleotide primers for reverse transcription. Stangeva et al. reported that the optimal length of a random primer was a 15-mer (pentadecamer). We compared the use of R6 and random pentadecamer (R15) primers for reverse transcription to evaluate whether the use of R15 primers improves the detection limit of the RQ-PCR assay for BCR-ABL transcripts.

The optimal concentration of R15 primers (Sigma–Aldrich) was 25 μmol/L, which is similar to that for R6 primers. The RQ-PCR assay for BCR-ABL transcripts (B3A2 and B2A2) and the BCR (breakpoint cluster region) control gene was performed as described previously. In brief, total RNA was isolated from Trizol (Invitrogen) preparations of blood or bone marrow leukocytes after erythrocyte lysis and was stored at −70°C until reverse transcription. We added 2 μg RNA and 400 U Superscript II reverse transcriptase (Invitrogen) to 2 reverse transcription reactions (20 μL each) containing either R6 or R15 primers at the same final concentration. Quantitative PCR was performed with the ABI Prism 7000 cycler and Sequence Detection software (Applied Biosystems). Transcripts for GUSB (glucuronidase, beta) and ABL1

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3 Nonstandard abbreviations: RQ-PCR, real-time quantitative reverse transcription PCR; CML, chronic myeloid leukemia; R6, random hexamer; R15, random pentadecamer; MRD, minimal residual disease.
4 Human genes: BCR, breakpoint cluster region; GUSB, glucuronidase, beta; ABL1, c-abl oncogene 1, receptor tyrosine kinase.
(c-abl oncogene 1, receptor tyrosine kinase) control genes were quantified in selected samples (12, 13). The estimated detection limit of the assay was determined according to the Europe Against Cancer collaborative group formula (8, 13). Nested PCR with standard primers (14) was performed on samples with sufficient cDNA.

We obtained archived RNA samples from 105 CML patients and 10 individuals with BCR-ABL–negative hematologic disorders (negative controls). In addition, we used 91 routine duplicate analyses (independent RNA extraction, reverse transcription, and quantitative PCR) performed in our laboratory with the standard R6 method to estimate the measurement error inherent in the assay. We used Bland–Altman analysis to assess the agreement or difference in results between methods (15). The Research Ethics Committee of our institution permits the use of anonymized patient samples. We selected 19 CML patients likely to have residual BCR-ABL transcript numbers close to the threshold of detection (undetectable BCR-ABL transcripts in the index sample, but ≥1 positive RQ-PCR result within 6 months). Eighteen of 19 patients had been treated with ABL kinase inhibitors, and 1 patient had been treated with interferon α. We carried out RQ-PCR assays on archived RNA samples with R6 primers and with R15 primers. Duplicates (2 independent reverse transcription reactions, 2 quantitative PCRs) were performed for each primer length. In the case of discordant results, we performed a third reverse transcription reaction with the same sample and produced a consensus result via our usual procedure. BCR-ABL was not explainable by inherent assay imprecision: the difference between the first and second samples of 91 duplicates assayed with R6 primers was 0.9-fold for both BCR-ABL transcripts.

We measured transcript copy number for the BCR control gene in 105 CML patient samples in both R6 and R15 primer–based assays. BCR transcript copy numbers obtained with R15 primers were higher in 103 of the 105 samples. BCR transcript copy number was >1.5-fold higher in 88 samples (84%) and >2-fold higher in 64 samples (61%). The assay with R15 primers yielded a mean 2.3-fold higher copy number than the assay with R6 primers (Fig. 1). The observed increase was substantially greater than the 0.9-fold difference observed for 91 duplicate measurements of BCR transcripts with the R6 assay. In our assay, a BCR transcript copy number of 400 000 indicates an estimated detection limit 4.5 logs below the standardized baseline number. The median BCR copy number was 1 120 000 with R15 primers, vs 494 000 with R6 primers, and the use of R15 primers increased the median estimated detection limit from 4.6 logs to 5.0 logs. With R6 primers, 42 (40%) of 105 samples had control gene values below the detection limit recommended for MRD analysis (<4.5 logs), whereas 15 (14%) of 105 samples were deemed suboptimal in assays with R15 primers. A comparison of the reported BCR-ABL/BCR transcript ratio obtained with R15 primers yielded a mean difference of 0.8, indicating that the R15 ratio is lower than the R6 ratio by approximately 20%. This finding is consistent with the observation that the increase in BCR transcript copy number is greater than for BCR-ABL (see Fig. 1 in the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol54/issue9). In a subset of 30 samples, the fold increase with R15 primers for GUSB and ABL1 was 1.5 and 1.3, respectively (see Fig. 2 in the online Data Supplement).

To confirm that an increased BCR-ABL transcript number produced a clinically useful difference in patient samples, we selected 19 CML patients likely to have undetectable BCR-ABL transcript numbers in the index sample, with ≥1 positive RQ-PCR result within 6 months. Eighteen of 19 patients had been treated with ABL kinase inhibitors, and 1 patient had been treated with interferon α. We carried out RQ-PCR assays on archived RNA samples with R6 primers and with R15 primers. Duplicates (2 independent reverse transcription reactions, 2 quantitative PCRs) were performed for each primer length. In the case of discordant results, we performed a third reverse transcription reaction with the same sample and produced a consensus result via our usual procedure. BCR-ABL was not explainable by inherent assay imprecision: the difference between the first and second samples of 91 duplicates assayed with R6 primers was 0.9-fold for both BCR-ABL transcripts.

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transcripts were detected with R15 primers only in 9 of 19 samples, with both R6 and R15 primers in 4 samples, and with R6 primers only in 1 sample. Thus, 13 (68%) of 19 samples that had previously been reported as testing negative with our standard RQ-PCR method had measurable BCR-ABL transcripts when we used R15 primers instead. Five (26%) of 19 samples also tested positive with R6 primers, indicating that the detection limit improves with repeated analysis. In duplicate tests of 10 negative-control samples with R15 primers, BCR-ABL transcripts were not detected. Nested PCR did not improve the detection limit for BCR-ABL transcripts in our laboratory (see Table 1 in the online Data Supplement).

The use of R15 primers in our established RQ-PCR assay for BCR-ABL transcripts produced an increase in copy number for both BCR-ABL and the BCR control gene. Importantly, this change in primers permitted the detection of BCR-ABL transcripts in 68% of selected samples that had previously been reported as having no detectable transcript. This result indicates an improvement in the detection limit for BCR-ABL transcripts of approximately 2-fold. This finding should be confirmed with a larger number of samples, but it may be useful for laboratories that have large numbers of samples from CML patients with MRD close to the limit of detection. The incremental improvement in the detection limit obtained with R15 primers may be sufficient to extend the period of measurable disease in imatinib-treated patients who are experiencing a slow decline in BCR-ABL transcript number (8). This improvement is comparable to the improvement in assay sensitivity obtained with an increased concentration of reverse transcriptase (12), but the cost is considerably less.

We examined the effect of substituting R15 primers for R6 primers on alternative control genes ABL1 and GUSB. The use of R15 primers produced an increase in copy number for ABL1 and GUSB transcripts, as for the BCR-ABL and BCR transcripts. The increases were not proportional, however, with lesser increases noted for ABL1 and GUSB than for BCR. This finding should be validated in laboratories that have established ABL1 and GUSB assays. The increase in GUSB, ABL1, and BCR-ABL transcripts was independent of the increase in BCR transcripts. One would have anticipated that the use of a longer random primer would produce a proportional change in all genes measured, but this was not the case, either in our study or in the original report of Stangegaard and colleagues (10), who noted that the differences in the expression of 3 genes on changing from the R6 primer–based assay to the R15 primer–based assay differed by >2-fold.

For the longitudinal follow-up of CML patients, it is important that the reported BCR-ABL/control gene ratio not be affected by changes in assay methodology. We found a small difference in the overall BCR-ABL/BCR transcript ratio with R15 primers compared with R6 primers, indicating that BCR-ABL levels will be underestimated relative to those obtained with our established method. An acceptable difference is one that would not affect clinical decision making. For example, we use an increase in the BCR-ABL transcript level of >2-fold as a trigger to screen for resistance to treatment (16). The difference due to the use of R15 primers instead of R6 primers is well within this limit. Our findings demonstrate than any change in RQ-PCR assay conditions might affect the results we observe, a conclusion that underscores the importance of assay validation in each individual laboratory. A systematic difference introduced by the use of R15 primers could be overcome by a mathematical correction factor, similar to the process for standardizing the reporting of the BCR-ABL ratio (17).

The increasing numbers of patients treated with ABL kinase inhibitors who have undetectable levels of BCR-ABL transcripts indicates a growing need to establish a consensus method for interlaboratory comparison of assay detection limits. The Europe Against Cancer formula for estimating the limit of detection is a useful means of assessing the quality of individual RQ-PCR results produced within a single laboratory with a single uniformly practiced method. The calculated detection limit reflects variation in the quality of the samples from which RNA was extracted, in extraction efficiency, and in the efficiency of reverse transcription and real-time PCR (13). Our data demonstrate that this formula cannot be used accurately to compare the detection limits of RQ-PCR performed with different control genes in different assay systems. The estimated detection limit of an assay for BCR-ABL transcripts ideally should be based on the measured change in BCR-ABL transcripts.

This study has demonstrated that the use of R15 primers improves the detection limit of RQ-PCR assays for BCR-ABL transcripts in CML patients. Transcript copy numbers increased for all 5 mRNA species examined, indicating that these findings may have broader applicability in other assays for RNA transcripts in low abundance, such as in the monitoring of viral load and in antenatal diagnostics.

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