

Quantification of bcr/abl mRNA expression by a rapid real-time reverse transcription-polymerase chain reaction assay in patients with chronic myeloid leukemia

A real-time reverse transcription-polymerase chain reaction (RT-PCR) assay was validated and used to quantify bcr/abl b3a2 and b2a2 mRNAs in clinical samples. bcr/abl mRNA was correlated to Philadelphia chromosome levels and differed significantly between cytogenetic response groups. A bcr/abl mRNA threshold value correctly identified 96% of samples without a major or complete cytogenetic response.

Sensitive and reproducible quantitative molecular assays might be useful for disease monitoring in patients with chronic myeloid leukemia (CML). We developed a real-time reverse transcription (RT)-polymerase chain reaction (PCR) assay and used it to quantify bcr/abl b3a2 and b2a2 mRNAs in clinical samples using the GeneAmp 5700 Sequence Detection System (PE Biosystems). The K562 cell line, carrying the bcr/abl rearrangement, was used as a positive control and as a standard for quantification, and the HeLa cell line as a negative control. The determination of bcr/abl rearrangement by qualitative RT-nested PCR was performed by a previously described method¹ with a lower sensitivity limit of 10^{-5} (10 pg of K562 RNA in 1 μ g of bcr/abl-negative cells). Real-time PCR was carried out with single set of PCR primers allowing the detection of both b3a2 and b2a2 transcripts.² The mRNA encoding for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference.

The performance of real-time quantification was evaluated in 10 independent assays. In each assay total RNA extracted from the K562 cell line was serially diluted in total RNA from HeLa cells. The range of quantification was linear until 10^{-4} dilution (from 250 ng to 25 pg of K562 RNA), corresponding to 1 K562 cell diluted in 10^4 HeLa cells (5 log dynamic range). Percent intra- and inter-assay coefficients of variation are shown in Table 1. The b3a2 and b2a2 variants could be amplified with comparable efficiency by the real-time RT-PCR method. GAPDH mRNA was readily detected in all samples and could be amplified from cDNA with equal efficiency compared to the bcr/abl target, confirming that GAPDH is a suitable reference for the quantification of bcr/abl mRNA.³⁻⁵ Therefore results were expressed as ng of K562 total RNA with the same level of bcr/abl mRNA expression and were normalized to GAPDH mRNA expression (bcr/abl normalized dose, nD).

Of the 80 peripheral blood or marrow samples from 29 CML patients analyzed, 7 could not be quantified by bcr/abl primers despite detectable GAPDH mRNA and were considered as negative (i.e., below the detection limit) for bcr/abl mRNA (nD = 0). bcr/abl mRNA was undetectable by qualitative RT-nested PCR in 3 of them, and Philadelphia chromosome (Ph)-positive metaphases were absent in the 5 samples with available matched cytogenetic data. Thus the different sensitivity thresholds between the qualitative and quantitative assays may be of little clinical relevance. Among the 73 samples that could be quantified by real-time RT-PCR, the b3a2 variant and the b2a2 variant had similar levels of expression (b3a2: median 22.22 nD, range 0.05-3446; b2a2: median 41.25 nD, range 0.2-138.9, $p=NS$). At diagnosis, the median level of bcr/abl expression in 23 patients was 44.3 nD in peripheral blood samples (range: 7.45-1880) and 53.7 nD in marrow samples (range: 7.55-3446) ($p=0.392$). bcr/abl mRNA levels did not correlate with the prognostic parameters of the Sokal index.

Matched cytogenetic and real-time RT-PCR data could be compared in 55 samples derived from 23 patients, 11 of whom were treated with interferon (rIFN) for a median of 24 months. Overall results showed a significant correlation between per-

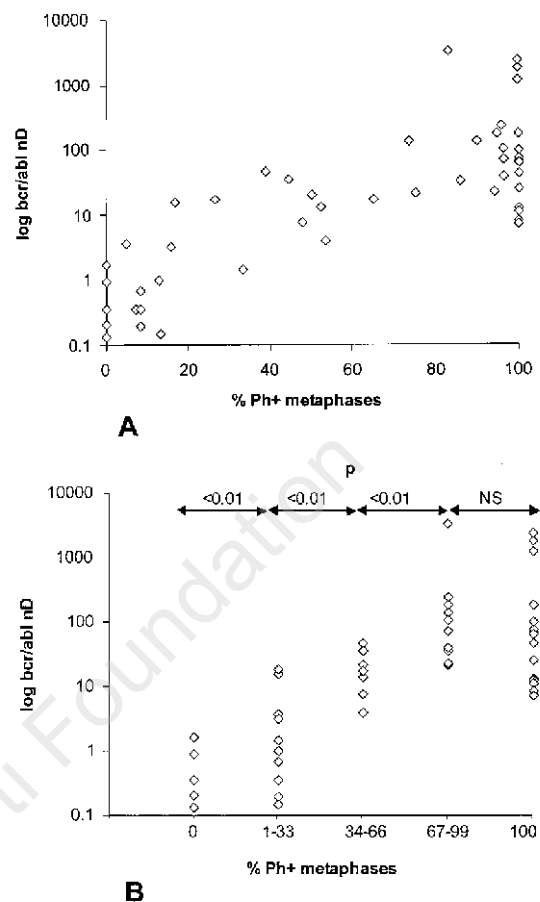


Figure 1. Relationship between bcr/abl normalized mRNA levels and cytogenetic data. A: Correlation between the percentage of Ph chromosome-positive metaphases and bcr/abl normalized dose (nD) in matched peripheral blood or bone marrow samples ($p < 0.001$, Spearman coefficient). B: Bcr/abl nD according to cytogenetic response groups. The significance levels of the differences between groups (Mann-Whitney test) are reported.

Table 1. Variability of the real-time quantitative PCR assay.

Variability	N. of assays	Input K562 RNA	Coefficient of variation (CV)
Intra-assay	10 replicates	2.5 ng	0.54%
Inter-assay	10 RT-PCR assays	250 ng	0.98%
		25 ng	1.45%
		2.5 ng	0.7%
		250 pg	0.72%
		25 pg	2.44%

centage of Ph-positive metaphases and bcr/abl nD ($p < 0.001$; Figure 1A). Five of 11 samples with complete cytogenetic response (KR) had no detectable bcr/abl mRNA expression by real-time RT-PCR. The bcr/abl nD at disease onset (median 143.5 nD, range 13.2-2,479) did not appear to predict the subsequent achievement of Ph negativity with rIFN therapy by 5 patients. Significant differences were observed between samples with complete and major KR ($p < 0.01$), major and minor KR ($p < 0.01$), minor and minimal KR ($p < 0.01$) (Figure 1B). Similar observations have been reported previously in patients after allogeneic bone marrow transplantation^{3,4,6,7} and in patients treated with rIFN,⁸ using a different real-time PCR method. A bcr/abl mRNA nD cut-off of 4 could reliably discriminate samples with major-complete response (#22, 20 of which with nD < 4) from those with minor-no response (# 33, all with nD > 4), reaching 100% sensitivity, 90.9% specificity, 100% negative predictive value, 94.3% positive predictive value and 96.4% accuracy for the identification of minor-no cytogenetic response.

These results show that this real-time RT-PCR method can be used for the rapid and reliable quantification of bcr/abl mRNA in clinical samples and may become an alternative to serial cytogenetic analyses in patients treated with rIFN. Its role in the monitoring of CML during treatment with new therapeutic agents such as tyrosine kinase inhibitors will be the subject of further studies.

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References

1. Martiat P, Maisin D, Philippe M, et al. Detection of residual bcr-abl transcripts in chronic myeloid leukemia patients in complete remission using the polymerase chain reaction and nested primers. *Br J Haematol* 1990; 75:355-8.
2. Mensink E, van de Locht A, Schattenberg A, et al. Quantitation of minimal residual disease in Philadelphia chromosome positive chronic myeloid leukemia patients using real-time quantitative RT-PCR. *Br J Haematol* 1998; 102:768-74.
3. Emig M, Saussele S, Wittor H, et al. Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real time quantitative RT-PCR. *Leukemia* 1999; 13:1825-32.
4. Barbany G, Hagberg A, Olsson-Strömberg U, Simonsson B, Syvanen AC, Landegren U. Manifold-assisted reverse transcription-PCR with real-time detection for measurement of the BCR-ABL fusion transcript in chronic myeloid leukemia patients. *Clin Chem* 2000; 46:913-20.
5. Elmaagacli AH, Beelen DW, Opalka B, Seeber S, Schaefer UW. The amount of BCR-ABL fusion transcripts detected by the real-time quantitative polymerase chain reaction method in patients with Philadelphia chromosome positive chronic myeloid leukemia correlates with the disease stage. *Ann Hematol* 2000; 79:424-31.
6. Kreuzer KA, Lass U, Nagel S, et al. Applicability of an absolute quantitative procedure to monitor intra-individual bcr/abl transcript kinetics in clinical samples from chronic myelogenous leukemia patients. *Int J Cancer* 2000; 86:741-6.
7. Bolufer P, Sanz GF, Barragan E, et al. Rapid quantitative detection of BCR-ABL transcripts in chronic myeloid leukemia patients by real-time reverse transcriptase polymerase-chain reaction using fluorescently labeled probes. *Haematologica* 2000; 85:1248-54.
8. Branford S, Hughes TP, Rudzki Z. Monitoring chronic myeloid leukaemia therapy by real-time quantitative PCR in blood is a reliable alternative to bone marrow cytogenetics. *Br J Haematol* 1999; 107:587-99.