

Real-time quantification of different types of bcr-abl transcript in chronic myeloid leukemia

MARILINA AMABILE, BARBARA GIANNINI, NICOLETTA TESTONI, VITTORIO MONTEFUSCO, GIANANTONIO ROSTI, CLAUDIA ZARDINI, CAROLINA TERRAGNA, SILVIA BUONAMICI, EMANUELA OTTAVIANI, SIMONA SOVERINI, MAURO FIACCHINI, SIMONA BASSI, ANTONIO DE VIVO, ELENA TRABACCHI, GIUSEPPE SAGLIO,^o FABRIZIO PANE,[#] MICHELE BACCARANI,^{*} SANTE TURA, GIOVANNI MARTINELLI

Institute of Hematology and Medical Oncology "Seràgnoli", University of Bologna, ^{*}Institute of Hematology, University of Udine; ^oDepartment of Medical Science and Human Oncology, University of Turin; [#]Department of Biochemistry & Medical Biotechnologies, Federico II University, Naples, Italy

Background and Objectives. The most common translocation in chronic myeloid leukemia (CML) t(9;22) (q34;q22) produces the BCR/ABL fusion gene. We set up and evaluated a rapid and reliable real-time reverse-transcription-polymerase chain reaction (RT-PCR) approach using TaqMan technology for detection and quantification of *bcr-abl* transcripts in CML patients at diagnosis and during therapy.

Design and Methods. A pair of primers and probe complementary to ABL exon 2 were designed, enabling detection of the most frequent *bcr-abl* transcripts, and also of the normal ABL-1a transcript as an internal control. Conditions were established to amplify less than 1⁻¹⁰ target molecules/reaction and detect one CML cell in 10⁶ cells from healthy donors. To determine the utility of the assay, we quantified the *bcr-abl*/ABL-1a ratio in 59 bone marrow samples (45 samples with evidence of different Ph⁺ chromosome percentages and 14 samples in complete cytogenetic remission) from 48 CML patients, 34 of them at diagnosis and 14 in clinical remission (CR). In 14 cases, this ratio was compared with results obtained by a competitive-quantitative RT-PCR/capillary electrophoresis method from contemporary specimens.

Results. By real-time RT-PCR, the median value of *bcr-abl*/ABL-1a ratio at diagnosis was 15.334 (range 3.3-28.81) and fell to 0.9 (range 0.003-26.1) in CR. The median value of *bcr-abl*/ABL-1a ratio at cytogenetic remission was 0.7 (range 0.003-2.83). The real-time *bcr-abl*/ABL-1a ratios correlated with those obtained by competitive RT-PCR ($p < 0.0001$) and the percentage of Ph⁺ metaphases ($p < 0.0001$). The high sensitivity and specificity of the real-time RT-PCR procedure was confirmed in all 14 patients with minimal residual disease.

Interpretation and Conclusions. We conclude that this real-time RT-PCR procedure is a reliable and sensitive method of monitoring CML patients after therapy, and that the *bcr-abl*/ABL-1a ratio correlates strongly with cytogenetic analysis.

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Key words: chronic myelogenous leukemia, real-time RT-PCR, *bcr-abl* transcript, minimal residual disease, quantitative PCR

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Correspondence: Giovanni Martinelli, M. D., Institute of Hematology and Medical Oncology "Seràgnoli", University of Bologna, via Massarenti 9, 40138 Bologna, Italy.
Phone: international +39-051-6364075 - Fax: international +39-051-6364037 - E-mail: gmartino@kaiser.alma.unibo.it

Molecular analysis of chromosomal translocations associated with hematopoietic tumors has provided important tools for the diagnosis and monitoring of patients' response to therapy. A consistent translocation, t(9;22), is present in the cells of most chronic myeloid leukemia (CML) patients.¹ Reciprocal translocation of the two genes involved – ABL and BCR on chromosomes 9 and 22, respectively – gives rise to a fusion gene which produces the neoplastic BCR-ABL fusion protein.² In 99% of CML patients, two types of *bcr-abl* junction (namely, "b2-a2" and "b3-a2") can be detected by reverse transcription (RT) polymerase chain reaction (PCR), due to the exclusion (b2-a2) or inclusion (b3-a2) of 75 base pairs (bp) from BCR exon 14 (or b3 of a major-breakpoint cluster region, M-BCR).³ RT-PCR detection of the *bcr-abl* transcript can be used for diagnosis of CML and monitoring of the Ph clone.⁴ It has allowed more precise definitions of disease subsets, and provided potentially valuable prognostic information for the management of patients.^{4,5} Qualitative RT-PCR has already been widely used to reveal the presence of detectable levels of minimal residual disease (MRD) in patients in complete cytogenetic remission (CR) after α -interferon therapy (α -IFN).^{4,6} However, the recent introduction of 'real-time' quantitative RT-PCR⁷ should eventually allow actual quantification of the target gene to be routinely performed at diagnosis and during follow-up. In real-time RT-PCR, the 5'-3' nuclease activity of the Taq polymerase cleaves an internal fluorogenic probe specific for the target sequence, causing the emission of a fluorescent signal that can be detected during amplification. Several groups have used real-time RT-PCR with TaqMan technology to quantify MRD with leukemia-specific chromosome aberrations.⁸ Complete suppression of the Ph⁺ clone has been observed in the majority of allotransplanted patients⁹ and a few of those treated with α -IFN.^{4,10-11} These considerations suggest that routine quantitative analysis using real-time RT-PCR could be of clinical value in CML patients submitted to these treatments.

Table 1. Qualitative molecular characterization of chronic myeloid leukemia patients.

CML patients studied/samples	CML patients studied at diagnosis/after BMT	Male/female	Type e1/a2 bcr-abl	Type e13/a2 bcr-abl	Type e14/a2 bcr-abl
48/59	34/14	26M/22F	0 (0%)	20 (41%)	28 (59%)

In the present study, we have developed *bcr-abl* quantification by real-time RT-PCR using the ABI PRISM 7700 (Perkin Elmer), a new technique which allows simple and rapid quantification of a target sequence during the extension phase of PCR amplifications. A fluorogenic probe labeled with both a reporter-dye at the 5' end and a quencher-dye at the 3' end hybridizes to the target sequence on the second exon of the ABL gene. The exonuclease activity of the Taq DNA polymerase cleaves the probe and releases the reporter-dye, resulting in an increase in the fluorescence signal. This allows identification and quantification of specific RT-PCR products as the reaction proceeds.¹² In an unknown sample, the absolute copy numbers of the *bcr-abl* target sequence and of a control gene, such as ABL, can be calculated at the end of the reaction using a calibration curve prepared from a set of *bcr-abl* RNA standards. The results are then expressed as a *bcr-abl*/ABL ratio. Quantitative data can be rapidly produced with a very wide dynamic detection range of over five orders of magnitude. We set up probes for *bcr-abl* and tested the method on bone marrow (BM) samples previously obtained from a group of CML patients, some of whom had already been studied by an established competitive RT-PCR method.^{13,14} In the latter cases, we compared the reliability and sensitivity of the two methods.

Design and Methods

Patients and therapy

A total of 59 BM samples (45 samples with cytogenetic evidence of disease and 14 samples from patients in complete cytogenetic remission) (Table 1) from 48 CML patients (26 males, 22 females), thirty-four at diagnosis and 14 in clinical remission (CR) after allotransplantation were analyzed. Informed consent was always obtained, as required by the Declaration of Helsinki. At diagnosis, all patients were Ph⁺. In 14 cases, this ratio was compared with results obtained by a competitive-quantitative RT-PCR/capillary electrophoresis method from contemporary specimens.

Cytogenetic analysis

Cytogenetic analysis was routinely performed according to a standard technique.¹⁵ At least 20 mitoses were analyzed for each sample.¹⁶

Sample and RNA isolation

Mononuclear cells from BM (3-5 mL) samples were obtained by Ficoll-Hypaque density gradient centrifugation and stored at -80°C in GITC as reported else-

where.¹⁷ Total cellular RNA was extracted as previously described,¹⁸ and 1 µg of total RNA was used in the RT-reaction.¹³

Qualitative RT-PCR analysis

Qualitative RT-PCR was routinely performed at diagnosis and during follow-up, as previously described.^{13,15,17} To assess the quality and quantity of the amplifiable RNA isolated from samples, RT-PCR of ABL control gene transcripts was performed as previously described.^{13,15,17} The following stringent criteria for negativity were applied: no amplification of the *bcr-abl* transcript in three independent assays at a sensitivity of 1:10⁵ (see below) always accompanied by successful amplification of the ABL transcript. To determine the level of sensitivity of breakpoint sequence amplification, experiments using *bcr-abl* positive RNA (types e13/a2 and e14/a2) were conducted by serially diluting *bcr-abl* positive RNA in HL60 cell line RNA, as reported elsewhere;^{13,15,17} TOM1 cell line was used as an e1/a2 positive control. Positive and negative controls were performed in all assays. Positive controls were *bcr-abl* positive RNA extracted from a BM sample from a CML patient. Negative controls consisted in reactions with either RNA from a *bcr-abl* negative CML patient or HL60 cell line RNA. Precautions taken to avoid contamination included use of a specifically designed UV-flow cabinet and PCR-designated pipettes with filter tips.^{15,17} All tests were done twice to confirm the results.

Competitive RT-PCR and capillary electrophoresis (CE) assays

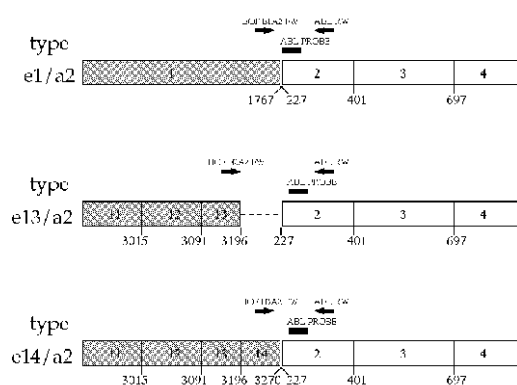
Competitive RT-PCR and CE assays were performed as described previously.¹³

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was retrospectively performed on stored samples. The principles and procedure of real-time RT-PCR quantification using the TaqMan probe (or a similar approach)¹⁹ have already been described.²⁰

Primers and probe for real-time RT-PCR of bcr-abl

In order to define an amplicon no longer than 200 bp, the TaqMan probe (BOP ABL probe) and reverse primer (BOR ABL primer) were located in exon 2 of the ABL gene for all types of transcript (Figure 1 and Table 2). The forward primers were located on exon 1 (BOF E1A2 primer), on exon 13 (BOF B2A2 primer) and exon 14 (BOF B3A2 primer) of the BCR gene for types e1/a2, e13/a2 and e14/a2 respectively. Probes were labeled by



BOF E1A2 FW : 5' CGC AAG ACC GGG CAG AT 3'
 BOF B2A2 FW : 5' GCA TTC CGC TGA CCA TCA AT 3'
 BOF B3A2 FW : 5' TCC ACT CAG CCA CTG GAT TTA A 3'
 BOF ABL : 5' TCC TCC AGC TGT TAT CTG GAA GA 3'
 BOR ABL : 5' TGG GTC CAG CGA GAA GGT T 3'
 BOP ABL : 5' CCA GTA GCA TCT GAC TTT GAG CCT CAG GG 3'

Figure 1. Figure shows a schematic representation (not in scale) of the set of primers and probes used for real-time RT-PCR assay for amplification of either e1/a2 and e13/a2 or e14/a2 *bcr-abl* transcripts. Open boxes and numbers represent BCR exons. Dotted boxes and numbers represent ABL exons. Arrows indicate primers used for RT-PCR with their orientations. Filled black rectangles represent the TaqMan probe. Numbers below the boxes represent nucleotides at exon boundaries. Sequences of primers and probes as in Table 2 are also named under the boxes.

a 5' FAM reporter and 3' TAMRA quencher. One set of primers and probe was also defined for the ABL control gene (exon 1a) (BOF ABL primer in Table 2).² Primers and TaqMan probes were all designed using Primer Express software (Perkin Elmer, Foster City, CA, USA).

PCR conditions for real-time RT-PCR of *bcr-abl*

Reaction mixtures of 25 μ L contained 12.5 μ L of TaqMan buffer A with the ROX dye as the passive reference, 5 mM MgCl₂, 200 μ M dATP, dCTP, dGTP, 400 μ M dUTP, 1.25 U AmpliTaq Gold DNA polymerase, 0.5 U AmpErase uracil N-glycosylase (UNG), 300 nM forward and reverse primers, 200 nM specific TaqMan probe and 6 μ L of plasmid or cDNA (diluted 1:3). All the reagents were from Perkin Elmer/Applied Biosystem.

All real-time RT-PCR experiments were performed at least in triplicate. Before determining the sensitivity of the target, the real-time RT-PCR set up was optimized. In particular, the amounts of forward and reverse primer producing the highest Δ Rn and lowest C_T were determined. In the primer-matrix experiment, nine combinations of 50, 300 and 900 mM for each primer were tested in triplicate: i.e. 50/50, 50/300, 50/900, 300/50,

Table 2. Oligonucleotides used in the real-time RT-PCR assay for quantification of BCR-ABL transcripts.

Primers and detection probes	Sequences (5' - 3')	Mapping
BOF E1A2 primer	CGAAGACCGGGCAGAT	BCR exon 1
BOF B2A2 primer	GCATTCGCTGACCATCAAT	BCR exon 13
BOF B3A2 primer	TCCACTCAGCCACTGGATTTAA	BCR exon 14
BOF ABL primer	TCCTCCAGCTGTATCTGGAAGA	ABL exon 1a
BOR ABL primer	TGGGTCAGCGAGAAGGTT	ABL exon 2
BOP ABL probe	FAM-CCAGTAGCATCTGACTTTGAGCCTCAGGG-TAMRA	ABL exon 2

Table shows primers and probes used for *bcr-abl* and for ABL-1a real-time RT-PCR quantification. FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine.

300/300, 300/900, 900/50, 900/300 and 900/900. RT-PCR reactions were set in MicroAmp optical 96-well reaction plates closed with MicroAmp optical caps (Perkin Elmer/Applied Biosystem). After 2 minutes at 50°C to allow UNG to destroy potential contaminant RT-PCR products, and 10 minutes at 95°C to denature UNG and activate AmpliTaq Gold, the amplification was carried out by 50 cycles at 95°C for 15 seconds and 65°C for 60 seconds in the ABI/Prism 7700 Sequence Detector System (ABI/Perkin Elmer, Foster City, CA, USA).

Construction of "standard curves" for BCR/ABL fusion gene and ABL control gene

We cloned the RT-PCR products obtained by amplification with the primers listed in Table 2, derived at diagnosis from TOM1 cell line, from patients #1 and 2 corresponding to *bcr-abl* types e1/a2, e13/a2 and e14/a2, respectively, and the ABL-1a amplification product. The four products were cloned into the pCR II-TOPO vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). We called the resulting plasmids pTA-e1/a2, pTA-e13/a2 and pTA-e14/a2, pTA-a1/a2, respectively. In order to construct the standard curve for the quantification of each type of *bcr-abl* and ABL-1a transcript, serial dilutions of the corresponding plasmid were used. For each assay, 10-fold dilutions, starting at 10⁴ plasmid copies,²¹ were analyzed in triplicate using Sequence Detector System software V1.6 (Perkin Elmer, Applied Biosystem). A standard curve was established by plotting the CT and the known copy number on a logarithmic scale.

Statistical analysis

Comparison between qualitative and real-time RT-PCR positivity was performed as previously reported.³ All analyses were carried out using the SPSS software package (SPSS Inc., Chicago, IL, USA).²²

Results

Cytogenetic analysis

Cytogenetic results are summarized in Table 3 (a, b, and c). A total of 59 BM analyses were performed. At diagnosis, all 34 patients showed Ph⁺ chromosome (Table 3a). Furthermore, all the 14 CML patients who

Table 3 (a), (b), (c). Molecular and cytogenetic evaluation of clinical samples of CML patients.

<i>CML</i> Cases studied	Phase of Disease	Ph+%	Quantitative real time RT-PCR analysis for <i>bcr-abl/ABL-1a</i> ratios	Qualitative RT-PCR analysis for <i>bcr-abl</i>
(a) Cases from 1 to 34	Chronic phase and diagnosis	100	median 15.334 (range 3.36-28.81)	+ all (positive)
<i>CML</i> Cases studied	Phase of Disease	Ph+%	<i>bcr-abl/ABL-1a</i> ratios	Qualitative RT-PCR analysis for <i>bcr-abl</i>
(b) Cases from 35 to 41 (UPN of patients transplanted in chronic phase)	allograft transplantation (months from BMT)	median 0.013 (range 0.003-0.7720)		
164	+13	0	0.0120	- (negative)
194	+35	0	0.0700	-
249	+2	0	0.3180	-
262	+6	0	0.0010	-
274	+15	0	0.0170	-
292	+3	0	0.7720	-
	+6	0	0.0003	-
301	+6	0	0.0008	-
<i>CML</i> Cases studied	Phase of disease	Ph+ %	Quantitative real-time RT-PCR analysis for <i>bcr-abl/ABL-1a</i> ratios	Qualitative RT-PCR analysis for <i>bcr-abl</i>
(c) Cases from 42 to 48 (UPN of patients transplanted in accelerated or blastic phase)	allo-transplantation (months from BMT)	median 2.5 (range 0.04-20.16)		
141	+62	0	2.8340	+ (positive)
	+64	9.3	1.0760	+
	+72	0	0.1040	-
	+77	0	2.5000	-
190	+15	3.9	2.5910	+
	+25	8.5	15.9690	+
	+36	16.6	17.4040	+
	+41	2	8.9610	+
	+51	4	1.8650	+
	+56	28	21.4310	+
	+61	58	26.1130	+
199	+2	40.5	20.1610	+
214	+2	3.3	0.7720	+
267	+3	0	0.0880	-
	+4	0	0.2050	-
285	1	0	0.04	-
288	+3	25	20.014	+

This table shows clinical and molecular follow-up of three groups of CML patients: at diagnosis (a); in allografted patients in chronic phase (b); in allografted patients in accelerated or blastic phase (c). UPN = unidentified progressive number of transplanted CML patients. *Bcr-abl/1a* ratios are expressed as described in Design and Methods; qualitative PCR analysis was considered positive (+) or negative (-) as described in ref. #15.

were studied after BMT had been Ph⁺ before transplantation. Eleven samples from 5 allografted patients (all transplanted in blastic phase: UPN141, UPN190, UPN199, UPN214, and UPN288) showed different degrees of Ph positivity. All the 8 samples from seven patients allografted in chronic phase (UPN164, UPN194, UPN249, UPN262, UPN274, UPN292 and UPN301 in Table 3b) were in karyotypic remission (KR).

Qualitative RT-PCR analysis

Assays of all 59 samples from the patients were studied. All 48 patients were *bcr-abl* positive by conventional PCR at diagnosis. Twenty out of 48 (41%) patients expressed e13/a2 transcript,¹³ and 28 (58%) displayed type e14/a2; no patient expressed e1/a2. All samples that turned out to be ABL-1a negative were excluded from subsequent analyses.²³ Log sensitivities of the tran-

script primer sets were 10^{-6} for all *bcr-abl* types, as reported.^{3,24-27} Qualitative RT-PCR analysis for *bcr-abl* on samples is reported in Table 3 a, b, and c.

Application of real-time quantitative RT-PCR for *bcr-abl* analysis

For all types of *bcr-abl* transcript studied involving ABL exon 2 (e1/a2, e13/a2 and e14/a2), it was possible to develop primer/probe combinations that permitted amplification and real-time RT-PCR analysis. For all types of transcript, the TaqMan probe and forward primer were located in exon 2 of the ABL-1a gene. The reverse primers were located in exons 1, 13 and 14 of the BCR gene for transcripts e1/a2, e13/a2 and e14/a2, respectively (Figure 1 and Table 2). The number of target molecules of transcript in each sample was expressed as a percentage ratio between *bcr-abl* and ABL-1a in 6 μ L of cDNA.

Sensitivity of real-time PCR

In repeated tests, we reliably amplified ten e1/a2, e13/a2 or e14/a2 *bcr-abl* and ten ABL-1a transcripts per reaction. To calculate the maximum sensitivity, serial dilutions of 10, 100, 1,000, and 10,000 myeloid PB cells taken from e13/a2 and e14/a2 *bcr-abl* positive CML patients at diagnosis or from the TOM1 cell line were processed and analyzed in 10×10^7 leukocytes from a healthy donor. We routinely achieved a sensitivity of 10^{-6} (i.e. we could detect one Ph⁺ cell in 10^6 normal white blood cells). The same approach was performed with a dilution of TOM1 (e1/a2 *bcr-abl* positive cell line), of BV173 (e13/a2 *bcr-abl* positive cell line) and K562 (e14/a2 *bcr-abl* positive cell line) cells in HL60 (*bcr-abl* negative cell line) cells. Reproducible sensitivities of 10^{-7} were reached for all cell lines.

Reliability of real-time RT-PCR

Quantitative analysis of *bcr-abl* in 20 identical samples of 500 molecules of plasmids pTA-e13/a2 (similar results also being obtained with pTA-e1/a2 and with pTA-e14/a2) in one run (intra-assay comparison) resulted in a coefficient of variation (CV, calculated for the determined concentrations) of 0.18; the respective cycle threshold crossing points resulted in a CV of $r=0.02$. Analysis of 20 identical samples in 20 runs on 20 days

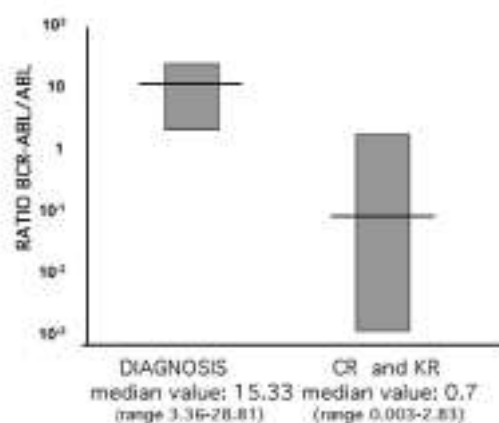


Figure 2. *Bcr-abl/ABL-1a* ratios, as evaluated by real-time PCR, of samples from Ph⁺ CML patients at diagnosis [group (a), Table 3] or during clinical remission and karyotypic remission after allogeneic bone marrow transplantation [group (b), and some patients of group (c) Table 3].

(inter-assay comparison, day-to-day variation with new mixtures of reagents) resulted in a CV of $r=0.01$, the cycle threshold crossing points having a CV of $r=0.01$.

Correlation between real-time quantification of *bcr-abl/ABL* ratio and karyotypic status

Figure 2 summarizes the results of 59 BM samples retrospectively studied by real-time RT-PCR. All had positive ABL quantification, with amounts of transcript ranging from 38 to 93,544 (median 4,217). The median *bcr-abl/ABL-1a* ratio at diagnosis (34 patients, 36 samples) was 15.334 (range 3.361-28.810) (Table 3a). At clinical remission, 7 patients were also in cytogenetic remission (UPN164, UPN194, UPN249, UPN262, UPN274, UPN292, UPN301; 8 BM samples) and 7 patients were mostly with minimal residual disease by cytogenetic analysis (UPN141, UPN190, UPN199, UPN214, UPN267, UPN285, UPN288; 21 BM samples) (Table 3b and c). The median

Table 4 (a), (b), (c). Molecular and cytogenetic evaluation of clinical samples of CML patients.

CML						
Cases studied	Phase of disease	Ph+%	Quantitative competitive RT-PCR analysis for <i>bcr-abl</i>	Quantitative real-time RT-PCR analysis for <i>bcr-abl</i>	Quantitative real-time RT-PCR analysis for <i>bcr-abl/ABL-1a</i> ratios	Qualitative RT-PCR analysis for <i>bcr-abl</i>
(a) Cases from 1 to 34	Chronic phase 1 and diagnosis	100	135,418 (range 178,278-48,938)	157,690 (range 4,898- 1,778,278)	median 15,334 (range 3.36-28.81)	+ all (positive)

This table shows comparison between quantitative competitive and real-time quantification RT-PCR methods for *bcr-abl*: All samples from CML patients were studied at diagnosis (group a). Either quantitative competitive (as described in ref. #15) or real-time RT-PCR analyses for *bcr-abl* were expressed as *bcr-abl* transcript for 1mg of total RNA. *Bcr-abl/1a* ratios are expressed as described in Design and Methods: qualitative PCR analysis was considered positive (+) or negative (-).

bcr-abl/ABL-1a ratio at clinical remission on 25 samples was 0.9 (range 0.003-26.1) showing a decrease of the transcript from diagnosis to CR. Fourteen samples from ten patients (UPN164, UPN194, UPN249, UPN262, UPN274, UPN292, UPN301, UPN141, UPN267 and UPN285) (Table 3b and c) showed complete karyotypic remission: the median value of *bcr-abl*/ABL-1a ratio was 0.7 (range 0.003-2.83) (Figure 2). A highly significant correlation was seen between the proportion of Ph⁺ metaphases determined by cytogenetics and the *bcr-abl*/ABL ratios determined by the Taqman ($p < 0.01$).

Real-time RT-PCR is more sensitive than qualitative RT-PCR

Real-time RT-PCR revealed the presence of *bcr-abl* transcripts in all 14 patients in CR who turned out to have MRD, whereas only 5 (UPN141, UPN190, UPN199, UPN214 and UPN288) were found to be positive at qualitative RT-PCR (Table 4): this suggests that real-time RT-PCR is highly specific, and indicates that it is significantly more sensitive than qualitative RT-PCR ($p = 0.001$).

Discussion

In CML, cytogenetic analysis is still the standard technique for assessing the proportion of malignant BM cells after therapy and defining patients' response to treatment. A drawback of this technique is that BM samples are required in order to obtain metaphases. We^{15, 28-29} and others³⁰ have previously demonstrated the advantages of quantitative RT-PCR techniques for monitoring CML patients during or after treatment. Results from competitive RT-PCR correlate well with cytogenetic response in patients treated with α -IFN^{31,32} or with allo-transplantation.^{28,29} Variable numbers of *bcr-abl* transcripts persist in CML patients who achieve CR with α -IFN,²⁹ and levels of detectable MRD may vary by as much as two²⁹ or even four orders of magnitude.^{29,33} After allo-transplantation, rising or persistently high levels of *bcr-abl* mRNA can be detected prior to cytogenetic or hematologic relapse.^{29,34-38}

Quantitative data from competitive RT-PCR have been used to initiate donor lymphocyte transfusions for treatment after relapse, and to monitor response to therapy.^{39,40} Real-time RT-PCR allows direct measurement of the amount of RT-PCR product during the amplification process and provides a far more accurate indication of the initial number of targets. Following other early reports of real-time RT-PCR for the detection and quantification of *bcr-abl* transcripts using the TaqMan system with a double fluorescence labeled probe,⁴¹⁻⁴⁴ for the present study we set up a novel primer/probe combination to provide a similar system bearing some particular characteristics. We tested our technique on 65 BM samples taken from 48 CML patients. The use of a probe matching ABL exon 2 sequences in combination with appropriate primers allows the detection of the two most common *bcr-abl* transcripts,^{2,13} but could also be effectively applied in other rarer types of *bcr-abl* transcripts such as e1a2,²⁷ e6a2,⁴⁵ e8-int-a2,^{46,47} or c3a2

(e19a2).⁴⁸ A possible limitation of our system could be an inability to recognize and quantify some very rare *bcr-abl* transcripts that lack ABL exon 2 (b2a3 or b3a3).^{24,25,49}

The sensitivity of our single step real-time RT-PCR technique turned out to be almost as high as standard nested RT-PCR, and significantly higher than qualitative single step RT-PCR. To standardize *bcr-abl* mRNA levels with respect to variability in RNA and cDNA quality, we used ABL-1a transcripts as an internal control. Thus, amplification of ABL-1a cDNA sequences between exons 1a and a2 was done to produce appropriate control sequences expressed at similar levels as the target genes and to avoid non-specific cDNA amplification. The advantage of using ABL-1a as a control gene is that it can be quantified by the same probe and reverse primer as *bcr-abl*. However, we also tested the variability of the ABL quantification by comparing ABL-1a with β_2 -microglobulin and GAPDH transcript levels in samples of different qualities and with different levels of *bcr-abl* (data not shown). The levels of these three housekeeping genes correlated significantly, and similar results were obtained with *bcr-abl*/ABL, *bcr-abl*/ β_2 microglobulin and *bcr-abl*/GAPDH ratios with respect to percentages of Ph⁺ cells at cytogenetic analysis. These findings suggest that any of these three genes may be used as an internal standard. However, both β_2 -microglobulin and GAPDH level of expression turned out to be 5 to 10 times higher than that of ABL-1a, and we are currently investigating whether these differences could be relevant in patients who show clinical and cytogenetic responses.

We tested the reliability of our system by comparing it with an established method of quantification, namely competitive RT-PCR followed by CE.²⁸ The two methods gave virtually identical results. The correlation with cytogenetic analysis was also very good. In the future, the possibility of using peripheral blood samples for real-time RT-PCR²⁷ instead of the BM samples required for cytogenetic analysis could enable molecular monitoring to be done on a regular basis with a minimum of patient discomfort.

We conclude that the real-time RT-PCR technique described herein for quantification of *bcr-abl* is a rapid, sensitive and reliable method for monitoring CML patients before and after therapy. The method offers the opportunity to standardize the assay and to develop rigorous standards and controls. It could provide an effective and convenient substitute for cytogenetic monitoring. It is likely that real-time PCR will enable the quantitative analysis of MRD to become more widely available. This could guide therapeutic decision-making in CML patients treated either with allo-transplantation or α -IFN.

Contributions and Acknowledgments

MA was the principal investigator: she designed the study. BG set up PCR procedures and drafted the paper. NT was responsible for cytogenetic and molecular analyses.

VM, GR, CZ, CT, SB, EO, SB, AdV, ET, GS, FP, MB were responsible for clinical management of patients. ST gave the final approval for submission. GM was responsible for ethical approval of the program, for funding and direct supervision. The order of authorship reflects the contribution given to the study.

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Potential implications for clinical practice

Real-time RT-PCR - such as the TaqMan assay set up by us - for quantification of *bcr-abl* is a rapid, sensitive and reliable method for monitoring CML patients before and after therapy (allotransplantation, α -IFN etc.), and should provide a major advance on cytogenetics in routine practice. Use of peripheral blood samples for real-time RT-PCR would spare patients the discomfort of the bone marrow biopsies necessary for cytogenetic analysis, and would facilitate more regular and frequent monitoring.

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