Quantitative Intra-Individual Monitoring of BCR-ABL Transcript Levels in Archival Bone Marrow Trephines of Patients with Chronic Myeloid Leukemia

Oliver Bock, Ulrich Lehmann, and Hans Kreipe

From the Institute of Pathology, Medizinische Hochschule
Hannover, Hannover, Germany

We established a quantitative real-time RT-PCR assay for the detection of chimeric BCR-ABL transcripts in archival formalin-fixed bone marrow trephines, both acrylate-embedded and paraffin-embedded. This new methodology enables determination of transcript levels in direct comparison to histopathological findings and therapeutic interventions during the time course of the disease in a retrospective and a prospective manner. We found an excellent correlation between the quantitative molecular data and the morphological evaluation as well as the clinical outcome for a cohort of chronic myeloid leukemia patients ($n = 10$). To the best of our knowledge, this is the first study demonstrating the feasibility of large-scale quantitative expression analysis in archival bone marrow trephines for monitoring molecular markers over several years or even decades. (*J Mol Diagn* 2003, 5:54–60)

The discovery of the Philadelphia chromosome in 1960 was the first described consistent chromosomal abnormality associated with a specific type of leukemia.¹ A reciprocal translocation between chromosomes 9 and 22² leads to formation of the BCR-ABL hybrid gene.³

Beside histomorphological evaluation, karyotyping and fluorescence *in situ* hybridization (FISH) are well established cytogenetic methods to detect the Philadelphia chromosome and the BCR-ABL fusion gene. These techniques are used to confirm the initial diagnosis and to monitor the cytogenetic status of the chronic myeloid leukemia (CML) during the course of the disease.⁴ Recently, quantification of the BCR-ABL fusion transcripts in peripheral blood mononuclear cells (PBMC) and bone marrow aspirates of patients with CML using RT-PCR has been shown to be suitable for monitoring the response to therapeutic interventions such as bone marrow transplantation (BMT), peripheral blood stem cell transplantation (PBSCT), interferons, and selective tyrosine kinase inhibitors.⁵ Furthermore, the molecular level of BCR-ABL transcripts prior, during, and after therapeutic efforts seems to predict the clinical outcome of the patients and

thus, qualitative and quantitative detection of these transcripts from PBMC became a standard in clinical studies.^{6,7}

Most, if not all, of the bone marrow trephines of patients suspicious for hematological disorders are formalin-fixed and acrylate-embedded (FFAE) or paraffin-embedded (FFPE) samples. We established a real-time RT-PCR (TaqMan), which allows the quantification of the intra-individual b2a2^{BCR-ABL} and b3a2^{BCR-ABL} transcript levels in FFAE and FFPE bone marrow trephines of patients with CML. This new approach enables large-scale retrospective and prospective quantitative analysis of both transcripts in correlation to treatment efficiency and histopathological features. Furthermore, this application might be transferred to other disorders and genes.

Materials and Methods

Cell Lines

Leukemic cell lines BV-173 (b2a2^{BCR-ABL}), K562 (b3a2^{BCR-ABL}), and HL-60 (BCR-ABL negative) were obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany and were grown as described.⁸

Peripheral Blood Mononuclear Cells

PBMC from CML patients were separated from 20 ml heparinized peripheral venous blood by Ficoll gradient using Leucosep tubes (Greiner, Germany).

Bone Marrow Trephines

In the Institute of Pathology, Medizinische Hochschule Hannover, bone marrow trephines were fixed in 0.1 mol/L K-acetate (Merck, Darmstadt, Germany)/0.5% glutaraldehyde (SERVA, Heidelberg, Germany)/1.1% formaldehyde (Merck) for at least 18 hours, decalcified using EDTA (Applichem, Darmstadt, Germany) (pH 7.5) for 48

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Address reprint requests to Oliver Bock, M.D., Institute of Pathology, Medizinische Hochschule Hannover, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany. E-mail: Bock.Oliver@MH-Hannover.de.

Table 1. Primers and Probes for Intra-Individual Monitoring of BCR-ABL Transcript Levels

Primers + Probes	5'-Sequence	
β -GUS forward	CTCATTGGAATTTTGCCGATT	
β -GUS reverse	CGAGTGAAGATCCCCTTTTTA	
β -GUS Probe	TGAACAGTCACCGACGAGAGTGCTGG	87bp
b2a2 ^{BCR-ABL} forward	ATCCGTGGAGCTGCAGATG	
b2a2 ^{BCR-ABL} reverse	CGCTGAAGGGCTTCTTCCTT	
b2a2 ^{BCR-ABL} Probe	CCAACTCGTGTGTGAAACTCCAGACTGTCC	96bp
b3a2 ^{BCR-ABL} forward	GAGTCTCCGGGGCTCTATGG	
b3a2 ^{BCR-ABL} reverse	GCCGCTGAAGGGCTTTTGAA	
b3a2 ^{BCR-ABL} Probe	TCTGAATGTCATCGTCCACTCAGCCACT	85bp

to 72 hours, and then methylacrylate (Merck)-embedded. Since 1998, the embedding procedure changed to paraffin (Medite, Burgdorf, Germany) in our institution. Calculation of the BCR-ABL transcript level was performed applying the $\Delta\Delta C_T$ -method (see below).

Main criteria for the selection of the cases was an initial diagnosis of CML with cytogenetical detection of the Ph chromosome and BCR-ABL positivity (>10%) in the FISH analysis. Another 85 FFAE and FFPE bone marrow trephines were retrieved from the Bone Marrow Registry of our institution to evaluate the constitutive expression level of the housekeeping gene β -glucuronidase (β -GUS).

RNA Extraction

Total RNA from cell lines (1.0×10^7 cells) and PBMC (0.8 to 1.0×10^7 cells) was isolated using the TRIzol reagent (Gibco BRL, Germany) according to the manufacturer's instructions. An optimized protocol was previously described⁹ for the isolation of total RNA from FFAE and FFPE bone marrow trephines. Both types of biopsies are treated identically. Briefly, three to five 10- μ m sections were cut from each acrylate or paraffin block and transferred into a 1.5-ml reaction tube. The sections were incubated at 55°C in a vigorously agitating thermoshaker in a solution containing 4 mol/L guanidinium isothiocyanate, 30 mmol/L TrisHCl (pH 7.6), 2% sarcosyl, 0.1 mol/L β -mercaptoethanol, and 5 mg Proteinase K overnight. After separation of undigested paraffin or acrylate by centrifugation and organic extraction, total RNA was precipitated from the aqueous phase with isopropanol and glycogen as an RNA-carrier for at least 24 hours. Afterward, the RNA concentration was measured spectrophotometrically. The yield of extracted total RNA depends on the biopsy size and approximately 3 to 30 μ g RNA were routinely obtained. Negative controls were obtained by extracting areas from paraffin blocks without tissue sample followed by cDNA synthesis and PCR reactions.

cDNA Synthesis

Total RNA (1 μ g) was transcribed into the complementary DNA using 250 ng random hexamers (Amersham Phar-

macia) and 200 units of SuperScript II Rnase⁻ reverse transcriptase (Invitrogen, Karlsruhe, Germany) in a volume of 20 μ l following the manufacturer's protocol. Negative controls were obtained by adding water instead of reverse transcriptase.

RT-PCR, Real-Time RT-PCR

Real-time RT-PCR was performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). PCR primers and TaqMan probes to amplify and detect the b2a2 and the b3a2 variant of BCR-ABL and the housekeeping gene β -GUS were designed using the Primer Express software version 1.0 (Applied Biosystems), as follows: b2a2^{BCR-ABL} and b3a2^{BCR-ABL} forward primer were placed in BCR-exon 12 and BCR-exon 13, respectively. Both reverse primers in ABL-exon 2 were placed to cover the fusion region of b2a2 and b3a2, respectively. The TaqMan probes b2a2, b3a2, and β -GUS were placed downstream of the forward primer (Table 1). The probes were purchased labeled with 6-carboxy-fluorescein (FAM) as the reporter dye and 6-carboxy-tetramethyl-rhodamine (TAMRA) as the fluorescent quencher. TaqMan probes were 3[prime]-phosphorylated to prevent elongation during PCR.

The real-time RT-PCR amplification was performed using a 96-well tray and optical caps (Applied Biosystems) with a final reaction mixture of 30 μ l containing both primers (250 nmol/L), probe (150 nmol/L), 0.75 units of Platinum Taq Polymerase (Invitrogen), 200 μ mol/L each of dATP, dCTP, dTTP, and dGTP in 1X Platinum Taq reaction buffer and 4 μ l cDNA. The reaction mixture was preheated at 95°C for 5 minutes, followed by 45 cycles at 95°C for 15 seconds, and 60.5°C for 1 minute.

The time point, expressed as a cycle number, where the fluorescence signal crosses a statistically defined threshold, is called C_T -value (for "threshold cycle"). This value is directly proportional to the starting copy number and is used for all further calculations (see below). For the monitoring and relative quantification of the BCR-ABL transcript levels in FFAE and FFPE bone marrow trephines and PBMC, real-time RT-PCR experiments were performed in three separate runs.

Relative Quantification

For the relative quantification of BCR-ABL expression, the $\Delta\Delta C_T$ -method was used as described previously.^{10,11,12} The transcript level of the target gene is always compared to that of the reference gene for which a constant expression has been demonstrated. Throughout this study the reference transcript was β -GUS. The relative transcript level is expressed as the difference of the C_T -values: $C_T[\text{housekeeping, sample 1}] - C_T[\text{target, sample 1}] = \Delta C_T[\text{sample 1}]$. The relative transcript level of the BCR-ABL gene (b2a2 or b3a2) at the time point of diagnosis was set to 100% for this patient ($\Delta C_T[\text{reference}]$). All transcript levels of biopsies taken later from this patient were calculated relative to this reference point. Assuming equal reaction efficiencies close to 100% for the target and reference gene (as shown in Figure 1, E and F) one can calculate the fold change of transcript level in a given biopsy relative to the reference point as follows (from which the name of the method is derived):

$$\text{Fold change} = 2^{-\Delta\Delta C_T},$$

where $\Delta\Delta C_T$ stands for: $\Delta C_T[\text{reference}] - \Delta C_T[\text{biopsy}]$.

In words: if the ΔC_T -value of a biopsy is, eg, three cycles smaller than the ΔC_T -value of the reference sample ($\Delta\Delta C_T = -3$), this biopsy contains eight times more transcript ($2^{-(-3)}$) of the target gene compared to the reference sample.

For a comprehensive and recent review of the equations and assumptions underlying this quantification algorithm, which was initially described in 1997 in the User Bulletin No. 2 from Applied Biosystems, see reference 13.

Efficiencies of Real-Time RT-PCR

The efficiency of the PCR can be directly calculated from the slope of the amplification plot (log relative fluorescence/PCR-cycle) in the exponential phase using the following equation:

$$s = \log(1 + E)^{14}$$

Determination of reaction efficiency is routinely used as an internal quality control for adequate assay performance.

Results

Constitutive Expression of the Housekeeping Gene β -GUS in FFAE and FFPE Bone Marrow Trepines

We first analyzed the constitutive expression of the housekeeping gene β -GUS in 85 FFAE and FFPE bone marrow trephines of patients with chronic myeloproliferative diseases (CMPD) and control cases (17 cases of polycythemia vera, 11 cases of essential thrombocythemia, 20 cases of idiopathic myelofibrosis, 17 cases of CML, and 20 reactive cases) to assess the reliability of relative quantitative expression analysis in FFAE and FFPE bone marrow trephines of patients with CML. Quantitative real-time RT-PCR for the expression of β -GUS were performed in duplicate and statistical analysis revealed a mean C_T value of 29.9 with a 95% confidence interval of [29.7; 30.2] demonstrating a constant expression of β -GUS in all samples included in this study.

Linearity

To determine the linearity of the quantitative assay, we performed dilution experiments in which cDNA (corresponding to 50 ng total RNA) from BCR-ABL-positive cell lines (BV173, K562) was diluted step-by-step 1:5 (10 ng), 1:25 (2 ng), 1:125 (400 pg), 1:725 (80 pg) and 1:3625 (16 pg). Figure 1A demonstrates a constant ratio of the reference gene and the target gene ($b2a2^{\text{BCR-ABL}}$ or $b3a2^{\text{BCR-ABL}}$) down to the threshold of detection.

Variance

We performed quantitative PCR in duplicate on 3 successive days to assess the interassay variance. Figure 1B shows the $\Delta C_T^{(\beta\text{-GUS} - \text{BCR-ABL variant})}$ values for two different FFPE bone marrow trephines (1 + 2) and two different PBMC samples (3 + 4). The coefficient of variation ranged from 9.7% to 21%.

Figure 1. A: Dilution experiments of b2a2 (red regression line) as well as b3a2 (blue regression line) transcript variants from BCR-ABL-positive cell lines and determination of each $\Delta C_T^{(C_T - \beta - \text{GUS} - C_T^{\text{BCR-ABL}})}$ by relative quantification revealed the linearity of the quantitative PCR. The slope for the regression lines were $s = -0.05$ and $s = -0.11$ for b2a2 and b3a2, respectively, demonstrating no correlation between expression level and factor of dilution. **B:** Interassay variance was determined by relative quantification of the BCR-ABL transcript level in two different FFPE bone marrow trephines (1 + 2) and two different PBMC samples (3 + 4). Quantification was performed in duplicate on 3 successive days illustrated by different symbols. The coefficient of variation (CV) were 17.6% and 13.1% for the FFPE bone marrow trephines 1 + 2, respectively. We calculated for FFPE bone marrow trephines a variance of 1.3 cycles corresponding to a factor of 2.4 after conversion to linear values (CV, 31.0%). The PBMC samples 3 + 4 revealed a CV of 21% and 9.7%, respectively. **C and D:** By calculating the efficiency from the slope of the amplification plot in the exponential reaction phase ($s = \log(1 + E)$), the representative regression plots of the real-time RT-PCR for $b2a2^{\text{BCR-ABL}}$, $b3a2^{\text{BCR-ABL}}$, and the housekeeping gene β -GUS revealed PCR-efficiencies of $>95\%$. All regression lines in these diagrams are nearly in parallel indicating very similar reaction efficiencies. **C** demonstrates the regression plots of the b3a2 transcript in PBMC ($s = 0.30$; $E = 99\%$), FFPE ($s = 0.31$; $E = 99\%$), and FFAE ($s = 0.29$; $E = 95\%$) bone marrow trephines. **D** demonstrates the PCR efficiencies of β -GUS ($s = 0.29$; $E = 95\%$), b2a2 ($s = 0.34$; $E = 100\%$), and b3a2 ($s = 0.29$; $E = 95\%$) in bone marrow trephines which were processed in a FFPE manner. **E:** Analysis of a dilution of K562 cells (BCR-ABL positive) in a constant background of 10^6 HL-60 cells (BCR-ABL negative). One BCR-ABL-positive cell is detectable in a background of 10^6 BCR-ABL-negative cells as demonstrated for the $b3a2^{\text{BCR-ABL}}$ transcript. **F:** Analysis of a dilution of BV173 RNA (BCR-ABL positive) in a constant background of $1 \mu\text{g}$ of HL-60 RNA (BCR-ABL negative). A very good linearity for the detection of $b2a2^{\text{BCR-ABL}}$ mRNA can be observed down to a dilution of 10 pg BV173 RNA in $1 \mu\text{g}$ HL-60 RNA.

A) Linearity

B) Variance

Efficiency

C) One target - three sample types

D) Three targets - one sample type

Sensitivity

E) Dilution of cells

F) Dilution of RNA

Real-Time RT-PCR Efficiencies in Archival Bone Marrow Trepines

The real-time RT-PCR efficiencies for the transcripts $b2a2^{BCR-ABL}$, $b3a2^{BCR-ABL}$, and β -GUS were nearly identical (>95%) regardless whether FFAE, FFPE bone marrow trephines or freshly isolated PBMCs were analyzed (Figure 1, C and D). This enables the direct comparison of reaction efficiencies and transcript levels in differently processed samples in case no alternative is available. During the change from arcylate-embedding to paraffin-embedding, several trephines were split and one half was embedded in arcylate, the other one in paraffin. This allowed the direct comparison of transcript levels in different embedded halves of the very same biopsy. Ten cases for which split biopsies existed were retrieved from the archives and analyzed as described above. The measured reaction efficiencies and ΔC_T -values turned out to be very similar (data not shown).

Sensitivity

BCR-ABL-positive cells (BV173 and K562) were diluted serially seven times from 1×10^6 cells to 1 single cell in a constant background of 1×10^6 BCR-ABL-negative cells (HL-60). After extraction of RNA, synthesis of cDNA and quantitative real-time PCR, a detection sensitivity of 1 BCR-ABL-positive cell in a background of 1×10^6 BCR-ABL-negative cells could be determined for both $b2a2^{BCR-ABL}$ and $b3a2^{BCR-ABL}$ (Figure 1E). When RNA from BV-173 and K562 cells were diluted serially, BCR-ABL positivity was detected to the corresponding amount of 10 pg and 25 pg total RNA for $b2a2^{BCR-ABL}$ and $b3a2^{BCR-ABL}$ transcripts, respectively (Figure 1F).

Molecular Monitoring of BCR-ABL Transcript Levels in FFAE and FFPE Bone Marrow Trepines of Patients with CML by Quantitative Real-Time RT-PCR

To monitor the intra-individual BCR-ABL transcript level in FFAE and FFPE bone marrow trephines, we retrospectively analyzed 60 bone marrow trephines of 10 patients with CML from the Bone Marrow Registry at the Institute of Pathology and generated the corresponding intra-individual expression of BCR-ABL transcripts in correlation with histopathological findings and therapeutical intervention. Figures 2 to 4 show three representative time courses of the BCR-ABL expression load in patients with CML after the initial diagnosis. In total, 10 time courses were analyzed. The BCR-ABL dynamics were analyzed retrospectively for the time courses of disease ranging from 2 to 10 years. The molecular analysis and the calculation of transcript levels were performed without prior knowledge of the histopathological findings and therapeutic interventions. Each plot of the BCR-ABL expression in a CML patient represents the mean data obtained in three independent real-time RT-PCR assays. Quantitative results based on the $\Delta\Delta C_T$ -method as described in

Figure 2. After the initial diagnosis in 1994, the patient received a PBSCT in 1997. At this time point the BCR-ABL transcript level has fallen to 50%. Two years later, the disease accelerated with an increasing BCR-ABL load up to 250%. The patient died in the blast crisis 3 months later. Prior, the analyzed FFPE bone marrow trephine revealed another increase in the BCR-ABL transcripts (Quantitative results based on the $\Delta\Delta C_T$ -method as described in Material and Methods, basic value for BCR-ABL level at time point of diagnosis was set to 100%).

Material and Methods, basic value for BCR-ABL level at time point of diagnosis was set to 100%.

Discussion

In the present study we performed, to the best of our knowledge for the first time, quantitative intra-individual monitoring of BCR-ABL fusion transcripts in FFAE and FFPE bone marrow trephines of patients with CML based on a recently optimized one-step extraction protocol of RNA from archival biopsies.⁹ The method will enable the detection and quantification of all transcripts of interest in bone marrow biopsies embedded typically in paraffin or arcylate. This allows direct correlation of morphological and

Figure 3. Illustration of the BCR-ABL transcript level of a patient with a long-term course of a CML. After the diagnosis in 1991 and the beginning of therapy with interferon- α , the BCR-ABL load declined sharply. During the following 10 years, the BCR-ABL level remained nearly constant. Also, the histomorphological evaluation revealed nearly no morphological changes. (Quantitative results based on the $\Delta\Delta C_T$ -method as described in Material and Methods, basic value for BCR-ABL level at time point of diagnosis was set to 100%).

Figure 4. Illustration of the BCR-ABL transcript levels of a patient who received an intermittent therapy with busulfan. After a drastic decrease of the BCR-ABL load to approximately 1% of the initial level, slight fluctuations of the transcripts levels accompanied the time course of the disease. The last histomorphological evaluation revealed a severe hypoplastic hemopoietic bone marrow (BM) which led to severe pancytopenia. The patient died of complications due to an infection. (Quantitative results based on the $\Delta\Delta C_T$ -method as described in Material and Methods, basic value for BCR-ABL level at time point of diagnosis was set to 100%).

evaluation with quantitative molecular data. Recently, several studies emphasized that the detection of the BCR-ABL transcript is one of the hallmarks for diagnosis, monitoring, and, more recently, for the treatment of CML.^{7,15,16} Besides the conventional cytogenetic approach, the qualitative and quantitative detection of BCR-ABL transcripts in bone marrow aspirates and PBMC became standard in monitoring the molecular status of the disease and in predicting the individual prognosis. Therefore, several studies made the effort to quantify the amount of BCR-ABL transcripts in an absolute manner.^{16,17,18} We decided to quantify the BCR-ABL transcript level in a relative manner, because the absolute numerical quantification of transcripts has some important disadvantages. First, preparation of exact quantitative standards is laborious and a serious source of errors. Second, stable storage of quantitative standards is very difficult to achieve. And third, absolute quantification requires several standards for generating a calibration curve within each real-time PCR run and thereby reducing the throughput of the system and the capacity for analysis. In addition, the risk of contamination by plasmids encoding the BCR-ABL variants that are necessary for generating external standards is a serious risk for a diagnostic molecular laboratory. One of the main advantages of quantifying transcripts in a relative manner is the comparison of signals generated from identical cDNA preparations, excluding the variability of RNA preparation and the efficiency of the reverse transcriptase which is not known.^{12,19} As demonstrated in Figure 1, C and D, the real-time RT-PCR efficiencies are nearly identical. This allows the direct comparison of transcript levels from different sample types. In addition, we performed real-time RT-PCR for the BCR-ABL and β -GUS expression in bone marrow trephines that were divided and processed in a FFAE as well as in a FFPE manner. In these samples, the relative quantification of the BCR-ABL transcripts re-

vealed no differences. Moreover, for the first time we could demonstrate the almost constant expression of a housekeeping gene in a large series of archival bone marrow trephines representing very different disease states.

The data obtained demonstrated that archival bone marrow biopsies of patients with CML are an excellent source for the quantification of BCR-ABL variants. As expected, the transition from chronic phase to acceleration and fatal blast crisis is accompanied by a sharp increase in the amount of BCR-ABL transcript (Figure 2). These data could be correlated with histopathological findings in the identical sample. Patients with long-term courses of disease exhibit a more or less constant level of BCR-ABL transcripts after the initial response to treatment (Figures 3 and 4). These results complement other studies quantifying BCR-ABL transcripts in bone marrow aspirates and PBMC.^{20,21} Furthermore, the quantification of BCR-ABL transcripts from archival bone marrow biopsies allows correlation of the amount of BCR-ABL transcripts with a detailed morphological characterization including cell-type specific predominance, maturation status of the neoplastic cells, and level of fibrosis. In future prospective studies, it would be very interesting to compare transcript levels determined in fixed and embedded trephines with FISH and cytogenetic data from the very same patient (These data were only partially available for the patient cohort analyzed in this study). Because of the vast number of archival bone marrow samples collected in hematopathological institutions, the described method of quantitative molecular monitoring of transcripts now enables comprehensive retrospective studies for expression analysis of bone marrow-associated hematological disorders, which can be correlated to pathobiology, response to treatment, and clinical outcome.

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