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Improvement of molecular monitoring of residual disease in leukemias by bedside RNA stabilization

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The sensitivity of assays designed to monitor minimal residual disease (MRD) by RT-PCR in leukemia depend on quality and quantity of RNA derived from peripheral blood (PB) and bone marrow (BM) leukocytes. Shipment of material may lead to RNA degradation resulting in a loss of sensitivity and, potentially, false negative results. Furthermore, degradation may lead to inaccurate estimates of MRD in positive specimens. We sought to determine feasibility and efficacy of a novel blood collection and processing system which is based on integrated RNA stabilization at the time of phlebotomy (PAXgene Blood RNA Kit) by comparison with standard methods of RNA extraction (cesium chloride gradient ultracentrifugation and RNeasy Mini Kit) using unstabilized EDTA anticoagulated PB. In 26 patients with chronic myelogenous leukemia (CML) on therapy, PB was processed after a storage time at room temperature of 2 and 72 h according to these protocols. BCR-ABL, total ABL and glucose-6-phosphate dehydrogenase (G6PD) mRNA transcripts of PB samples were quantified as a measure for response to therapy and RNA integrity. RNA yield expressed as the ratio of ABL transcripts after a storage time of 72 h/ABL transcripts after a storage time of 2 h at room temperature was significantly higher with the stabilizing method (median 0.40) compared to the RNeasy method using unstabilized PB (median 0.13, $P = 0.01$). Furthermore, ratios BCR-ABL/ABL after 72 *vs* 2 h still correlated well using the PAXgene method ($r = 0.99$, $P < 0.0001$) in contrast to the standard method which did not ($r = 0.65$, $P = 0.03$). Even investigation of complete cytogenetic responders with very low tumor burden showed a good correlation of ratios BCR-ABL/ABL compared to the reference method. Comparable results were achieved using G6PD transcripts as standard. We conclude that the new PAXgene stabilization method could improve RNA quality and the comparability of molecular monitoring within and between multicenter trials.
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Introduction

The discovery of specific molecular markers of leukemia permits molecular monitoring of residual disease after therapy on RNA level. Qualitative and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) methods have been developed and standardized.[1–3] Quality and concentration of RNA extracted from peripheral blood (PB) or bone marrow (BM) leukocytes play a crucial role for sensitivity of RT-PCR assays to monitor the efficacy of treatment in leukemia patients characterized by specific fusion transcripts. Transit times exceeding 48 h are associated with RNA degradation and a significant decrease of PCR sensitivity.[2] To preserve RNA from degradation, a novel blood collection system was introduced which is based on bedside RNA stabilization in a nucleic acid preservative (PAXgene Blood RNA Kit; PreAnalytiX, Hombrechtikon, Switzerland). This new system was compared to standard methods of RNA extraction (cesium chloride [CsCl] gradient ultracentrifugation[4] and RNeasy Mini Kit; Qiagen, Hilden, Germany) using unstabilized EDTA anticoagulated PB. We sought to determine (1) the influence of simulated transit conditions of 3 days on the sensitivity of quantitative RT-PCR assays and (2) the impact of bedside stabilization of PB on the integrity of RNA.

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Patients, materials and methods

Patients

Twenty-six patients (14 male, 12 female; median age 61 years, range 22–67) with BCR-ABL-positive chronic myeloid leukemia (CML) on therapy with imatinib (formerly STI571, Glivec; Novartis, Basel, Switzerland) were investigated after written informed consent. Patients were allocated into two groups: (1) patients lacking a major cytogenetic response to therapy at the time of analysis (Ph+ metaphases >35%, $n = 11$); (2) patients with ongoing complete cytogenetic response (Ph+ = 0%, $n = 15$). Cytogenetic analysis was performed according to standard protocols.[5] Prior to imatinib therapy stage of disease was in group 1: chronic phase (CP, $n = 6$), accelerated phase (AP, $n = 2$), myeloid blast crisis (BC, $n = 3$); and in group 2: CP ($n = 13$), AP ($n = 1$), myeloid BC ($n = 1$).

Procedure

After phlebotomy, 10 ml of PB were drawn directly into tubes containing stabilizing agent (PAXgene Blood RNA Tubes; PreAnalytiX) using a standard BD Vacutainer Blood Collection Set (Becton Dickinson, Franklin Lakes, NJ, USA). Consecutively, 10 ml EDTA anticoagulated unstabilized PB was taken. Each portion was divided into two equal parts, stored for 2 h and 72 h, respectively, until continuation of RNA isolation (Figure 1).

RNA isolation

Three different methods of RNA isolation were used and compared. Stabilized PB samples were processed and RNA extracted according to the protocol of the PAXgene Blood RNA Kit. Total RNA from unstabilized PB samples was extracted by using the RNeasy Mini Kit (Qiagen) for group 1 patients. CsCl gradient ultracentrifugation (16 h at 36 000 r.p.m., Beckman L60 Ultracentrifuge, Rotor SW55 Ti, Palo Alto, CA, USA) was used for group 2 patients as standard method to enable RNA extraction from lysates of total WBC from 10 ml PB.

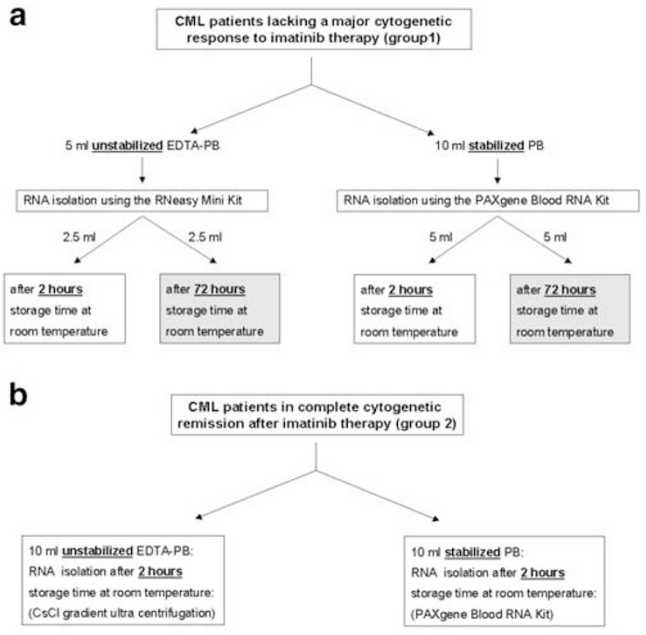

Figure 1 Processing of PB from CML patients (a) without a major cytogenetic response to imatinib therapy (group 1) and (b) with a complete cytogenetic response to imatinib therapy (group 2).

cDNA synthesis

Total RNA was transcribed to cDNA using random hexamer primers and MMLV-reverse transcriptase (GibcoBRL, Life Technologies, Gaithersburg, MD, USA) following a standard protocol.[6]

Quantitative reverse transcription polymerase chain reaction

BCR-ABL, total ABL, and G6PD transcripts were amplified using the LightCycler technology (Roche Diagnostics, Mannheim, Germany) and labelled with specific hybridization probes as described previously.[7]

Statistical methods

Quantitative variables were expressed as median and range (minimum to maximum). Correlation coefficients and *P* values were calculated according to Spearman. The impact of the PAXgene stabilization procedure on expression of housekeeping genes (ABL, G6PD) was analyzed by the Mann–Whitney test.

Results

Group 1: Quantification of BCR-ABL in patients lacking a major cytogenetic response

After 2 h of storage, the median ratio BCR-ABL/ABL was 22% (range 0.6–84) after PAXgene-mediated RNA stabilization and purification and 22% (range 1.4–79) using the standard method (RNeasy) without any stabilization component (NS, Figure 2, Table 1). The correlation of the ratios BCR-ABL/ABL of individual samples after 2 *vs* 72 h of storage was clearly better with the PAXgene system ($r = 0.99$, $P < 0.0001$, Figure 3a) compared to RNeasy extraction ($r = 0.65$, $P = 0.03$, Figure 3b). The samples of two patients even became BCR-ABL negative (false negative) after 72 h incubation without stabilization whereas BCR-ABL could still be reliably detected after 72 h with stabilization. Comparable results were obtained using G6PD as housekeeping gene. The correlation of the ratios BCR-ABL/G6PD after 2 *vs* 72 h of storage was clearly better with the stabilization method ($r = 0.95$, $P < 0.0001$) compared to the non-stabilization method ($r = 0.75$, $P = 0.01$, Table 1).

The ratio of ABL transcript levels of samples stored for 72 *vs* 2 h was significantly higher after RNA stabilization provided by the PAXgene system (median 0.40, range 0.18–1.00) as compared to the RNeasy system without stabilization (median 0.13, range 0.03–0.71, $P = 0.01$). This difference was confirmed using G6PD as an alternative housekeeping gene. The ratio of G6PD transcripts after 72 *vs* 2 h with the stabilization method (median 0.44, range 0.18–1.00) was significantly higher compared to the standard method using unstabilized PB (median 0.03, range 0.01–0.12, $P = 0.001$) (Table 1).

Group 2: Quantification of minimal residual disease in complete cytogenetic responders

In order to determine the sensitivity of the assay complete cytogenetic imatinib responders were analyzed. In this patient group the PAXgene system was compared with conventional CsCl gradient ultra centrifugation. After processing equal volumes of PB (10 ml) a good correlation of the ratios BCR-ABL/ABL respectively BCR-ABL/G6PD was found between both methods (BCR-ABL/ABL $r = 0.81$, $P = 0.02$, Figure 4; BCR-ABL/G6PD $r = 0.85$, $P < 0.0001$), even though the yield of RNA was about two-fold lower with the PAXgene system, as expressed as the ratio of ABL respectively G6PD transcripts

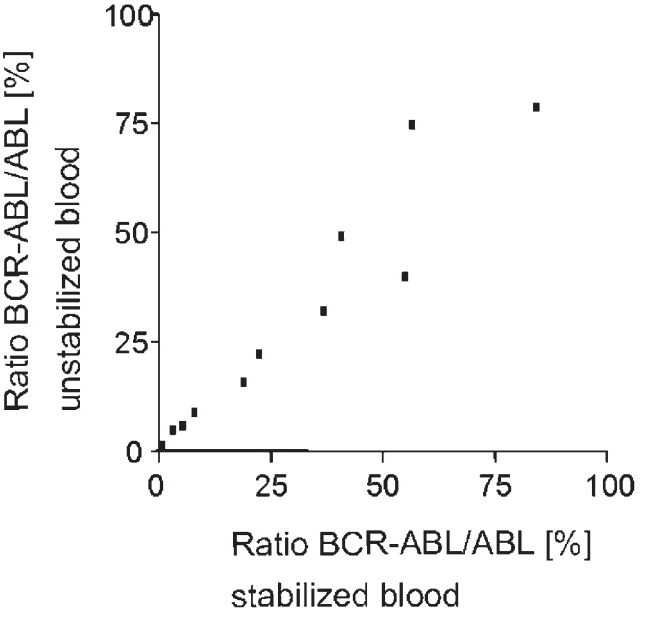

Figure 2 Correlation of the ratios BCR-ABL/ABL in group 1 patients after 2 h of storage: PAXgene RNA stabilization *vs* unstabilized EDTA anticoagulated blood (RNeasy method) ($n = 11$; $r = 0.95$, $P < 0.0001$).

Table 1 Comparison of quantitative RT-PCR results after using the PAXgene system or conventional methods

	Stabilized blood	*Unstabilized blood*
Group 1 (CML patients without a major cytogenetic response), n = 11		
Median ratio after 2 h (range) [%]		
BCR-ABL/ABL	22 (0.6–84)	22 (1.4–79)
BCR-ABL/G6PD	0.61 (0.01–3.38)	0.51 (0.02–2.95)
Correlation of the ratios 72 *vs* 2 h of storage		
BCR-ABL/ABL	$r = 0.99$, $P < 0.0001$	$r = 0.65$, $P = 0.03$
BCR-ABL/G6PD	$r = 0.95$, $P < 0.0001$	$r = 0.75$, $P = 0.01$
Median transcript ratio 72 h/2 h of storage		
ABL transcripts (range)	0.40 (0.18–1.00) $P = 0.01$	0.13 (0.03–0.71)
G6PD transcripts (range)	0.44 (0.18–1.00) $P = 0.001$	0.03 (0.01–0.12)
Group 2 (CML patients in complete cytogenetic remission), n = 15		
Correlation of the ratios		
BCR-ABL/ABL		$r = 0.81$, $P = 0.02$
BCR-ABL/G6PD		$r = 0.85$, $P < 0.0001$
PAXgene method *vs* CsCl gradient ultracentrifugation		
Median ABL transcript ratio (range)		0.36 (0.16–0.72)
Median G6PD transcript ratio (range)		0.66 (0.23–1.69)
(PAXgene method/CsCl gradient ultracentrifugation)		

derived with PAXgene/CsCl gradient ultracentrifugation (ABL median 0.36, range 0.16–0.72; G6PD median 0.66, range 0.23–1.69) (Table 1).

Discussion

Optimum sample quality is a crucial prerequisite for molecular monitoring of leukemia patients on therapy.[1,8] Central molecular monitoring within multicenter trials is advantageous due to good intralaboratory stability of the individual PCR method used compared to the difficult interlaboratory comparability of quantification results in case of decentral analyses.[3,9,10] This applies not only to CML but to all leukemias with specific fusion transcripts to be quantified by RT-PCR. In CML, which is regarded as a model disease for molecular monitoring, a significant proportion of patients reach complete cytogenetic remissions after allogeneic stem cell transplantation, interferon alpha, or imatinib therapy. Quantitative RT-PCR is regarded as the optimal assay to monitor residual disease in this situation.[2,4,5,11–13] Sensitivity of the assay critically depends on a sufficient amount of non-degraded RNA in the sample after transit to the laboratory. Until now several RNA isolation methods with different capacities of processable cell numbers have been in use, eg the RNeasy Mini Kit or comparable methods with an upper test limit of 1×10^7 white blood cells (WBC). To reach higher sensitivities for MRD diagnostics the laborious CsCl gradient ultracentrifugation has been employed which allows RNA isolation from about 5×10^7 WBC.[4] A drawback of both methods is the delayed start of blood processing in the specialized laboratory after transit associated with RNA degradation. Previously, we found that the number of ABL transcripts per volume cDNA as a marker for undegraded RNA in PB samples of humans show a rapid decrease down to a median of 13% after 3 days without stabilization.[14] The novel PAXgene Blood RNA Kit offers the advantage of shipping material stabilized during blood collection. Apart from inhibiting RNA degradation the system has recently been shown to prevent *ex vivo* gene induction in PB samples after storage at room temperature.[15] Our data support the notion of superiority of the new assay *vs* conventional methods based on inhibition of RNA degradation. We found that the PAXgene method provides good RNA stability particularly for samples in transit for up to 3 days. RNA stability is significantly higher after 72 h storage at room temperature with the PAXgene system than with unstabilized EDTA anticoagulated PB. After 72 h storage ratios BCR-ABL/ABL and BCR-ABL/G6PD are more stable using the PAXgene method compared to unstabilized EDTA anticoagulated PB.

Stability of mRNA may vary over several orders of magnitude. In higher cells some mRNAs are degraded with a half-life of a few minutes, while in the same cell, other mRNAs are degraded with half-lives over 24 h.[16] mRNA decay can either be initiated by shortening of the poly(A) tail followed by decapping and exonucleolytic degradation of the transcript or by direct endonucleolytic cleavage[17] or by other mechanisms.[18] Examination of tissue stabilization with RNAlater (Ambion, Austin, TX, USA) for skin and tumor biopsies showed that a sufficient amount of RNA could be extracted after 24 h of storage at room temperature and subsequent storage at 4ºC. However, the differences were not quantified.[19] To our knowledge the present study is the first systematic look at degradation of human mRNA over time under controlled conditions in PB samples.

We conclude that bedside RNA stabilization using the PAXgene system is advantageous for RT-PCR-based methods to monitor residual disease in leukemias. The expression level of leukemic fusion transcripts differs between entities. However, the new method would be useful even in leukemias with a high expression level of fusion transcripts, such as AML1-ETO. All improvements in RNA stabilization and extraction would contribute to a more sensitive MRD detection in leukemias with low-level expression of fusion transcripts, such as acute promyelocytic leukemias.

The system would be of particular interest for multicenter therapeutic trials with centralized molecular diagnostics. The novel approach may help to improve the comparability of qualitative and quantitative RT-PCR results within and

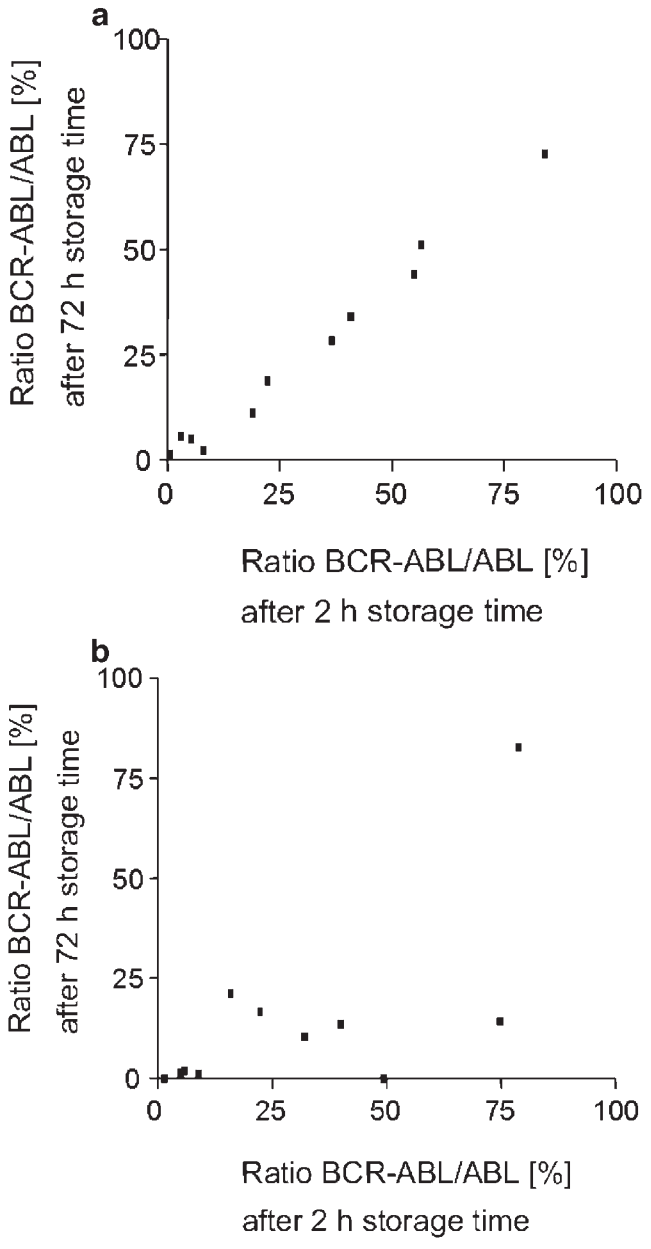

Figure 3 (a) Stabilized PB according to PAXgene protocol; correlation of the ratios BCR-ABL/ABL after 2 *vs* 72 h of storage ($n = 11$; $r = 0.99$, $P < 0.0001$). (b) Unstabilized EDTA anticoagulated PB according to RNeasy protocol; only borderline correlation of the ratios BCR-ABL/ABL after 2 *vs* 72 h of storage ($n = 11$; $r = 0.65$, $P = 0.03$).

between international study groups by providing RNA stability and a standardized pre-analytical solution.

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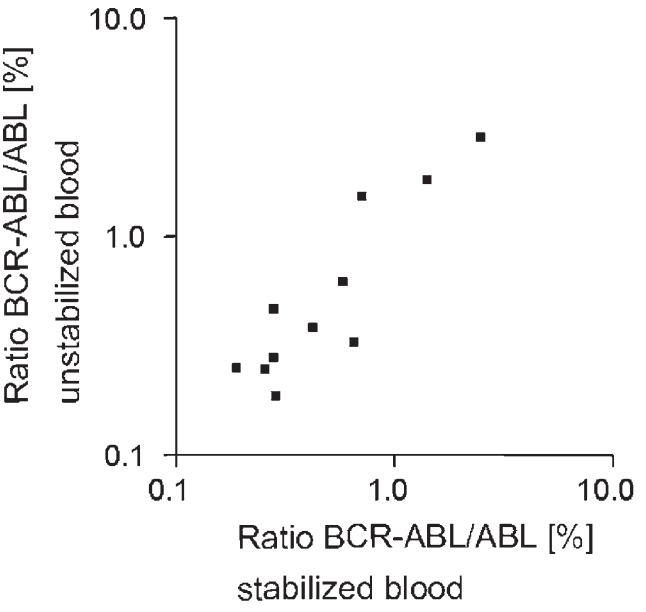

Figure 4 Correlation of the ratios BCR-ABL/ABL in group 2 patients after 2 h of storage: PAXgene RNA stabilization *vs* unstabilized EDTA anticoagulated blood (cesium chloride gradient ultra centrifugation) ($n = 15$; $r = 0.91$, $P < 0.0001$).

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