Real-time qPCR for assessment of minimal residual disease in acute myeloid and lymphoid leukemia

Metodă *real-time qPCR* pentru evaluarea bolii minime reziduale în leucemiile acute mieloide şi limfoide

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Abstract

Evaluation of minimal residual disease is of major importance in the course of acute leukemia treatment. There are a number of technologies used in this regard, all of them exhibiting different limitations. Real time qPCR is one of the main technologies enabling gene expression analysis and is well suited for minimal residual disease monitoring of acute leukemia patients. cDNA of eight most common fusion genes was cloned - PML-RARα, TEL-AML1, AML-ETO, E2A-PBX1, SIL-TAL1, CBFβ-MYH11, MLL-AF4, BCR-ABL1. Serial dilution of cloned plasmids were prepared and used as standards for real-time qPCR. Sensitivity and overall performance of the method was evaluated as previously described. Overall testing demonstrated robustness of TaqMan technology for gene expression analysis. High reproducibility with low levels of both inter and intra run variation was obtained. Sensitivity exhibited by the assay was comparable to nested PCR which allowed early relapse detection for a number of patients.

Keywords: *real-time qPCR, acute leukemia, minimal residual disease.*

Rezumat

Cuantificarea bolii minime reziduale (BMR) este un domeniu de mare interes în studiul şi tratamentul leucemiilor acute. Urmărirea BMR permite evaluarea răspunsului la tratament a pacienţilor după curele de inducţie şi separarea lotului de pacienţi rezistenţi la terapie. De asemenea, urmărirea BMR permite despistarea timpurie a recăderii. Evaluarea BMR se face prin mai multe metode, fiecare oferind avantaje şi dezavantaje specifice. Metoda Real Time Quantitative PCR (RT-qPCR) este tehnologia cel mai des folosită pentru evaluarea

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expresiei genice şi este o metodă robustă şi simplă pentru determinarea BMR. Pentru evalurea BMR în laboratorul nostru a fost implementată o metodă de RT-qPCR ce permite determinarea BMR folosind ca ţintă moleculară expresia a 9 gene de fuziune, cel mai des întâlnite în leucemiile acute, atât limfoblastice cât şi mieloblastice: MLL-AF4, MLL-AF9, BCR-ABL1, TEL-AML1, AML1-ETO, CBFb-MYH11, PML-RARa, E2A-PBX1 si SIL-TAL1. RT-qPCR este o tehnologie robustă şi simplă pentru urmărirea BMR la pacienţii cu leucemie acută. Implementarea acestei metode a permis despistarea recăderii sau a rezistenţei la tratament la o parte din pacienţii investigaţi.

Introduction

Assessment of minimal residual disease (MRD) in the course of therapy is currently incorporated in all treatment protocols used for acute myeloid (AML) and lymphoid leukemia (ALL) (1-4). Clinical benefits of MRD monitoring include monitoring response to chemotherapy, early relapse detection and risk stratification based on different MRD levels at different treatment time points (5,6). Moreover, MRD studies have the potential to provide novel information on disease progression, response and clinical efficacy of novel and established therapies and basic leukemia's biology (4,6).

There is a number of different strategies described in the literature for MRD monitoring - flow cytometry (7), real-time quantitative RT-PCR (RT-qPCR) and FISH analysis for detection of leukemia specific fusion genes (ex. AML1-ETO) (5,8,9), detection of clonal Ig or TCR receptor rearrangements (2,10-12), RT-qPCR detection of gene mutations (ex. NPM1, MLL-PTD, FLT3) (13-15).

Flow cytometry is a method of widest applicability, potentially being able to identify not only MRD but also any clone change related or unrelated to the therapy, which is not easily detectable by other methods (7,16). On the other hand, there is a need for a very high technical expertise for a reliable detection of 1 leukemic cell in 10^4 normal cells, which is not applicable to all monitoring centers. Also instability of antigenic expression on leukemic cells (lineage switch or loss of antigens) during or after the treatment is limiting applicability of this technique (5,16).

FISH and cytogenetics, as methods for monitoring of MRD, are valuable techniques but present a very limited sensitivity which hinders their applicability for sensitive MRD detection (1).

Real-time quantitative RT-PCR is one the main technologies enabling gene expression analysis today (17). Its use for MRD monitoring in leukemia is frequently cited in literature due to its high sensitivity, robustness and relative simplicity of implementation (5,17,18). RT-qPCR is a very reliable method given that large standardization studies were performed and its limits are well understood (5). For MRD detection, many RT-qPCR targets have been proposed, mainly due to lack of a universal molecular marker which can be applied for all leukemia cases. Such targets include: fusion genes (ex. PML-RARα) (5), mutations in certain genes (ex. NPM1) (13), aberrant gene overexpression (ex. WT-1) (20) and detection of clonal Ig or TCR receptor rearrangements (10).

In this article we describe the implementation of a method for MRD monitoring by detection of fusion gene expression using RT-qPCR. This method is applicable to 30-40% of both AML and ALL in children and adult cases (5,18).

Material and methods

Patients and samples

All samples were from patients referred to Fundeni Clinical Institute, Department of Pediatrics and Department of Hematology, and to Coltea Hospital, Department of Hematology, between January 2008 and September 2011. A total number of 70 patients were identified as having fusion genes that can be analyzed by our method – 14 cases of PML-RARα, 5 cases of

CBFβ-MYH11, 10 cases of AML1-ETO, 13 cases of TEL-AML1, 15 cases of BCR-ABL1, 10 cases of MLL-AF4, 2 cases of E2A-PBX1 and 1 case of SIL-TAL1. Additionally, 120 follow up samples from some of these patients were available for MRD monitoring. Study protocol was approved by our institution's Ethics committee and informed consent was obtained for all the clinical samples.

RNA extraction

We used blood for RNA extraction, as it was shown that blood exhibits comparable fusion gene expression to bone marrow and sampling is a lot less invasive (5, 18). All samples were taken and brought to the laboratory on the same day. All other sample manipulations were made immediately after arrival or stored for a maximum of 24h at 4^0C. White blood cells were separated by lysis of red blood cells using RBC (Red Blood Cell) lysis buffer prepared in house (for 1L of 10x solution dissolve 8.26 g ammonium chloride (NH_4Cl), 1 g potassium bicarbonate ($KHCO_3$) and 0.037 g EDTA in ddH_2O). Cells were pelleted at 3000 rpm for 10 min, and washed once with PBS 1x. After washing, the pellet was resuspended in 1 mL of PBS and cells counted on COULTER® LH 750 Hematology Analyzer (Beckman Coulter) and aliquoted 20 000 000 cell /per tube. Cells were pelleted at 3000 rpm for 10 min. and supernatant removed. One mL of TRIZOL reagent per tube was added and cells were lysed by passage through a fine syringe needle. RNA was extracted following manufacturer's protocol. RNA quantity and purity was assessed using NanoDrop 1000 spectrophotometer. Final RNA concentration varied between 150 ng/μL and 2600 ng/μL. RNA purity was assessed using A260/A280 ration which was between 1.9-2.0 for most of the samples (23). Results for samples which exhibited lower A260/A280 ratios were interpreted with care.

Reverse transcription

RNA was reverse transcribed as modified from (5), using MMLV reverse transcriptase (Sigma): 4 μg of RNA in 30 μl of H2O were incubated at 65°C for 10 min and other reagents were added to final volume of 40 μl: RT buffer, DTT for a final concentration of 10 mM, random hexamers for a final concentration of 25 μM, RNAasin 20 units (Promega), RT enzyme MMLV 200 units (Sigma) and dNTP for a final concentration of 1 mM (Qiagen). Reaction mixture was incubated for 2h at 37^0C and MMLV was denatured at 95^0C for 5 min.

Fusion gene identification

Fusion genes were identified using primers previously described (21).

Cloning of plasmid standards and standard dilution preparation

For standard curve preparation, fusion genes were cloned using TOPO TA cloning kit (Invitrogen) with pCR2.1 TOPO vector and TOP10 competent cells. Fusion genes were amplified using primers previously described primers (21) and amplified using HighFidelity HotStart Taq (Qiagen) using standard protocol. There were a total of 11 amplicons cloned corresponding to PML-RARα bcr1 and bcr3 transcript, TEL-AML1, AML-ETO, E2A-PBX1, SIL-TAL1, CBFβ-MYH11 type A, MLL-AF4 e11-e4 and e9-e4, BCR-ABL1 e1-a2 and b3-a2. For BCR-ABL1 b3-a2 transcript, primers for amplification were BCR-b1-A (21) and ENR1063 (18) thus this plasmid can be used as standard for both M-bcr and ABL1 amplification. Amplicons were run on 1.5 % agarose gel in 1x TAE buffer for 1h at 100V. Bands were excised from gel using a razor blade and purified using Wizard® SV Gel and PCR Clean-Up System (Promega) following manufacturer's protocol. Extracted DNA quantity was measured on a NanoDrop 1000 instrument.

For cloning, 4μL of purified amplicon was added to 1 μL of Salt Solution and 1 μL of pCR2.1 TOPO vector and incubated 30 min. at room temperature. For transformation of TOP10 competent cells, 2μL of cloning reaction product was added to one tube of One Shot Chemically competent cells and incubated on ice for 5 min. Further, cells were heat-shocked for 30 seconds at 42^0C on a water bath and immediately transferred

on ice. 250 µL of room temperature S.O.C. medium was added to each tube and incubated for 1h at 37^{0}C at 200 rpm on a shacking incubator. After incubation, 50 µL of medium was spread on LB agar plates. LB agar plates were prepared as previously described and supplemented with 50 µg/mL of ampicillin (22). 40 µL of 40 mg/mL of X-gal (Sigma) in dimetyhylformamide (DMF) was spread on each plate and incubated for 1 h at 37^{0}C before use. Plates were incubated over night at 37^{0}C and 5 white colonies for each fusion gene were selected and grown in 5 mL of LB medium overnight at 37^{0}C with shacking (22). Plasmids were purified using included extraction kit and insert was confirmed using primers used for cloning and M13 forward and reverse primers provided with the kit. Two positive plasmids per fusion gene were sequenced using M13 forward and reverse primers and the sequence analyzed to confirm correct insert. Colonies that contained the correct insert were grown in LB medium for 1h at 37^{0}C at 200 rpm and 1 mL of medium was transferred to 250 mL of LB and grown overnight at 37^{0}C at 200 rpm. Plasmids were prepared using Plasmid Maxi Kit (Qiagen) following manufacturer's protocol. Final quantity of extracted plasmids varied between 100 and 200 µg. Presence of insert was checked once again by PCR using cloning primers (21).

Plasmids were digested using Spe1 enzyme (New England BioLabs) – 1 µg of plasmid in 1x NEBuffer 4, supplemented with 100 µg/ml BSA and 5 U of enzyme. Reaction was incubated overnight at 37^{0}C. Plasmids were purified using Wizard® SV Gel and PCR Clean-Up System following the manufacturer's protocol and quantified on a NanoDrop 1000 instrument. Linearization was checked by running 3 µL on 0.8% agarose gel in 1x TAE for 1h at 100V.

Copy number calculation was done as described (24):

$m = [n] \times [1.096 \times 10^{-21}$ g/bp]

where: n = plasmid size in bp, including insert; m = calculated mass of the plasmid; 1.096×10^{-21} base pair molar mass (average)

[Copy number of interest] x [plasmid molar mass] = [mass of plasmid needed]

By calculating how much plasmid we need, we can apply the following formula for dilution:

C1V1 = C2V2

where C1 is the concentration in copy number of the concentrated plasmid and V1 is the volume of the concentrated plasmid, C2 is the desired concentration and V2 volume of the diluted plasmid. We prepared first dilution of a stock solution of the plasmid of 10^8copies/µL, from which all the other dilutions were made. For preparation of dilution series we used *E. coli* tRNA 10 ng/µL (Roche). A dilution series for each plasmid was prepared: 2×10^0 copies/µL, 2×10^1 copies/µL, 2×10^2 copies/µL, 2×10^3 copies/µL, 2×10^4 copies/µL, 2×10^5 copies/µL and 2×10^6 copies/µL thus spanning 6 orders of magnitude which is sufficient to cover all the spectrum of biological variation of fusion gene expression (5,18).

Real-time quantitative PCR (RT-qPCR)

RT-qPCR reaction was modified from (5,18). For a final volume of 20 µL, 10 µL of LightCycler 480 Probes Master, 2 µL of either cDNA or plasmid standard, 300nM of primers and 200nM of probe were added as previously described (5,18).

RT-qPCR program was: 10 min. at 95^{0}C followed by 45 cycles of 15 sec. at 95^{0}C and 30 sec. at 60^{0}C. Initially we used the published PCR program (5) which was modified by us to reduce PCR cycling time, with comparable results with initial protocol (the same value of Cq was obtained with both protocols for the same standards and samples); also, 45 cycles where enough even for 1 copy identification. All standards were run in triplicate and samples in duplicate (both for ABL1 and specific fusion gene) and 2 NTC (no template controls) were added per plate. RT-qPCR was repeated for samples in which a difference of more than 0.5 Cq between replicates was found. For low copy number samples, if one of the replicates was negative and the other positive, RT-qPCR was repeated. A sample was con-

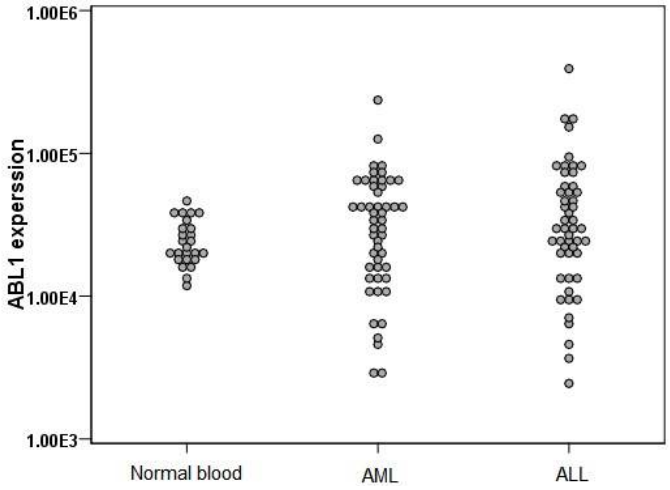

Figure 1. ABL1 level of expression in 25 samples of normal blood, 50 AML and 50 ALL samples.

sidered positive if both or only one replicate was positive in the second run, otherwise the sample was considered negative (5).

All RT-qPCR experiments were run on a LightCycler 480 II (Roche). The analysis was performed using the second derivative maximum method (LightCycler 480 analysis software version 1.5). In our experience this method provided the most consistency between runs. Using standard curves we obtained a mean slope value -3.43 (S.D. = 0.1), mean efficiency of 1.953 (S.D. = 0.033) and Y intercept 38.5 (S.D. = 1.3) (calculated for ABL1 and all fusion genes). The assay was linear for the 6 orders of magnitude tested (between 4 and $4x10^6$ copies/reaction) for all fusion genes and ABL-1.

The sensitivity of the assay was high, allowing the amplification of 4 target copies per reaction in all replicates for all fusion genes (using plasmid standards) thus being near theoretical limit of detection of 3 (LOD = 3) (27,25). As shown in (5), this assay allows the detection of fusion gene transcript down to a dilution of 1 in 10^5.

The reproducibility was assessed by Cq variation for 40000 copies/reaction for ABL1 and a mean Cq of 23.55 was obtained with S.D.=0.22 for inter run variation and S.D.=0.04 for intra run variation.

Result normalization – all results for fusion gene expression were normalized to 10^4 copies of ABL1. ABL1 expression was evaluated in 25 normal samples, 50 AML and 50 ALL samples (*Figure 1*) with corresponding median values of 21400, 34625 and 29475 copies/ 200 ng of total RNA respectively. As shown in *Figure 1,* ABL-1 exhibited comparable expression level for normal and pathological tissue with more variation in leukemic cells (18).

Reference ABL1 expression was calculated as previously described (18). Reference range was defined as the median of ABL1 expression and 2 limits as the 3rd and 97th percentile. This allowed the identification of poor quality samples and of samples in which ABL1 expression level was likely overestimated (18). Median expression of ABL1 in normal, AML and ALL cells (for a total of 125 samples) was 28400 copies with expression in 3rd percentile of 4410 copies and 97th percentile 153000 copies.

Sensitivity was calculated as described (5): SENS= -log10(NCN) - log10(CGCN), where NCN = normalized copy number of fusion gene transcript at presentation, CGCN = copy number of control gene at follow up. Sensitivity was expressed as 10^{SENS}.

Results and discussions

Cloned cDNA standards provide a reliable mean for standard curve calculation as opposed to RNA (5). In our setting, it allowed standard curve calculation with the mean slope of -3.43 (S.D. = 0.1), mean efficiency of 1.953 (S.D. = 0.033) and Y intercept 38.5 (S.D. = 1.3) calculated for ABL-1 and all FGs. These results are close to theoretical values – slope -3.32, efficiency 2 and Y intercept 36 (25,27), see *Figure 2*.

Calculation of ABL1 expression limits for this assay in our setting allowed lowering the number of standards used for ABL-1 quantification to

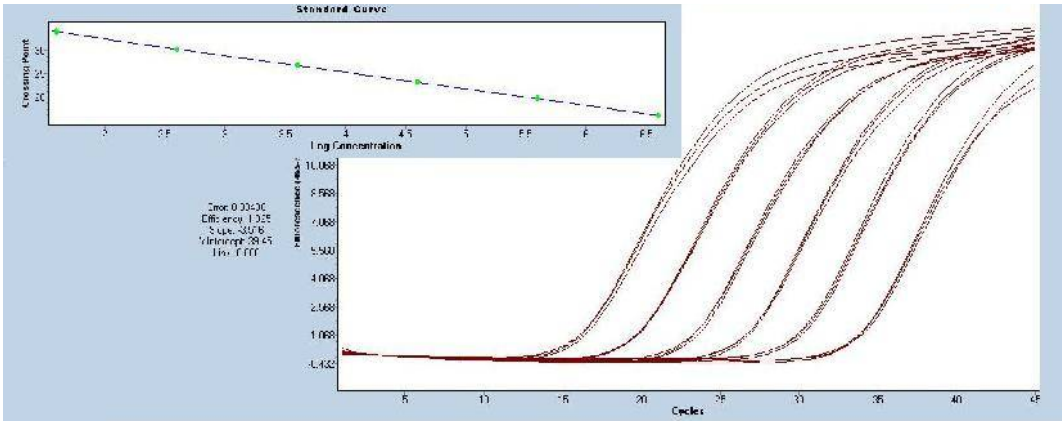

Figure 2. Amplification curve for 6 dilutions (40 to 4.000.000 copies/reaction) of ABL-1 plasmid standards are shown along with the standard curve (upper left corner)

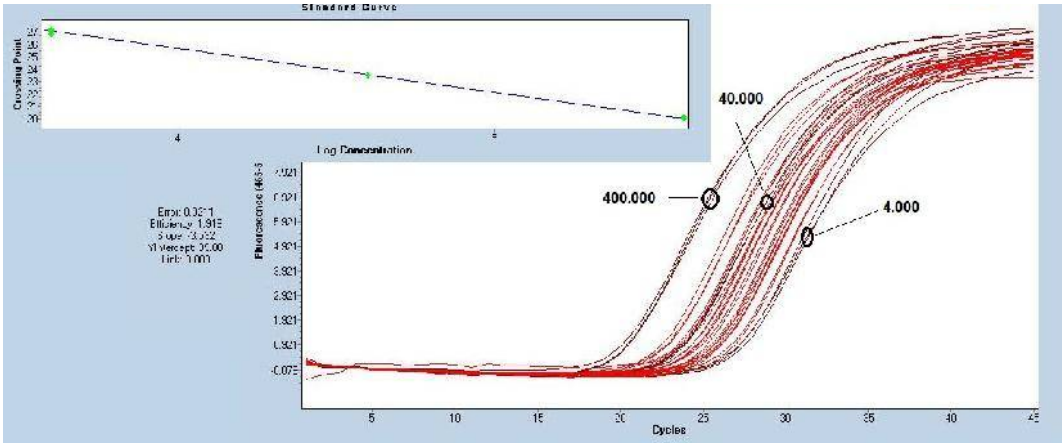

Figure 3. Standard curve calculation using 3 standard points. Red curves correspond to actual samples

the minimum of three – 4.000, 40.000 and 400.000 copies/reaction (*Figure 3*). Three standard points are sufficient for standard curve calculation and this interval is sufficient for ABL-1 quantification as it includes limits of ABL-1 expression between 4410 and 153.000 copies/reaction (18,27). This allowed for a slight cost reduction.

ABL-1 expression limits are very useful indicators of sample quality, and results with ABL-1 concentration beyond those limits should be interpreted with care as those concentrations are very likely to be inaccurate and could result in over or under appreciation of the real FG expression, and can greatly affect the sensitivity of MRD determination (18).

FG expression was assessed with this method in 70 presentation and 120 follow-up samples. Expression of FGs was normalized versus ABL1 expression – FG/10^4 ABL1 (*Figure 4)*.

Most of the variation in fusion gene expression is exhibited by AML1-ETO, with MLL-AF4, PML-RARα and TEL-AML1 being at the opposite end. This can be in part explained by the fact that gene expression was calculated for peripheral blood, and blast cell percentage was not used for correction (the data

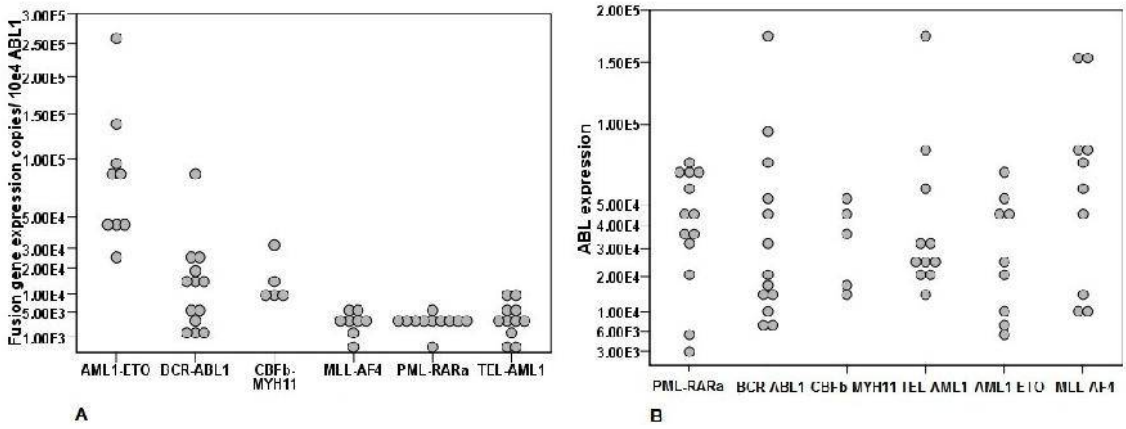

Figure 4. A. Fusion gene expression normalized to 104 ABL1 (E2A-PBX1 and SIL-TAL1 not shown due to their low prevalence). **B.** ABL1 gene expression in respective samples from A showing relative homogeneous expression in samples with different fusion gene types.

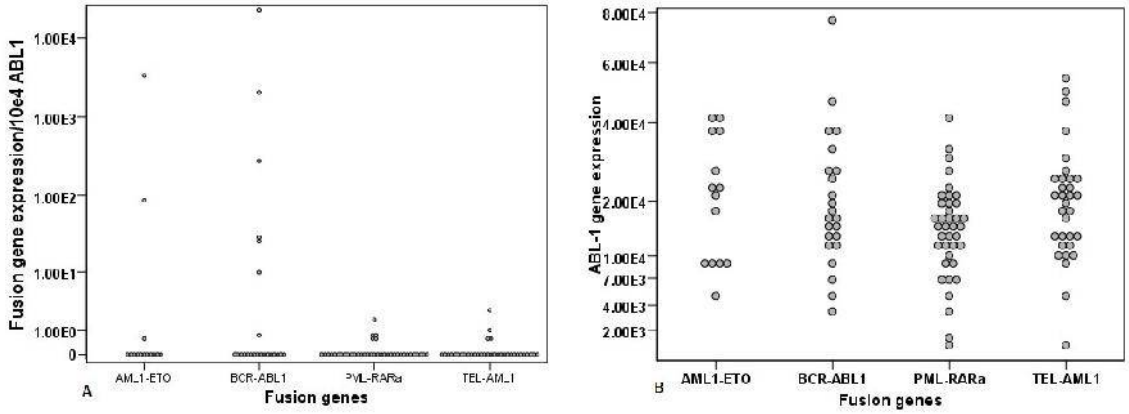

Figure 5. A. Fusion gene expression for follow up samples normalized to ABL-1. **B.** ABL-1 gene expression for respective samples showing uniform ABL-1 expression pattern. Results are shown only for a part of FGs for which many data point are available (for the rest of 4 fusion genes only 12 data points are available).

was not available for all patients) (5). On the other hand, ABL-1 gene expression was relatively uniform for this set of patients, which demonstrates once again the feasibility of the use of ABL1 as a control gene for normalization. Gene expression levels of all FGs were within the described limits (5).

For 121 follow-up samples available for analysis, results for 109 are shown in *Figure 5*.

Follow-up results were also correlated with nested PCR results. In general a good correlation was obtained, with no differences for BCR-ABL1 (23 samples), MLL-AF4 (2 samples), E2A-PBX1 (2 samples), AML1-ETO (14 samples), SIL-TAL1 (8 samples). In 4 cases, a negative result was obtained with this assay as compared to nested PCR for TEL-AML1, and 1 positive that was negative with nested PCR (from a total of 34 samples). For PML-RARa, in 6 cases a negative result was obtained and in 2 cases RT-qPCR was positive and nested PCR negative (from a total of 38 samples). No follow up sam-

ples were available for CBFβ-MYH11. These discrepancies can be partially explained by lower ABL-1 expression for samples which are negative in real-time PCR and higher nested PCR sensitivity. On the other hand, positives which are not reflected in nested PCR can be explained by the fact that real-time PCR is performed in duplicate whereas nested PCR was performed in single tube and, due to random variation at low copy number, such differences can occur.

Clinical correlation between positives and disease evolution was observed, especially in cases with higher copy number. In general, positivity was strongly correlated with relapse or chemotherapy resistance.

Sensitivity of the assay was determined and a mean SENS of 4.05 (S.D.=0.69, interval of variation between 2.63 and 5.37) was obtained. As expected, the highest sensibilities were obtained for AML1-ETO (as it has the highest expression ratio versus ABL-1) and the lowest were for SIL-TAL1 (as it has the lowest expression ratio versus ABL-1) (5).

As described (5) for BCR-ABL1 expression, we also obtained levels of FG expression higher than 1 in 6 samples which theoretically could not happen as ABL-1 assay also amplifies BCR-ABL-1 fusion gene. As explained in (5), this probably is one of the limitations of RT-qPCR, as small differences in efficiency of FGs versus ABL-1 amplification can add up to important differences after 25-30 cycles of PCR amplification (25).

Conclusions

As demonstrated by the use of cloned cDNA standards, the qPCR component of this RT-qPCR assay is very robust. Cq cycle for all ABL-1 runs exhibited very small overall variation in amplification efficiency both intra and inter run. The wide dynamic range of the assay, of at least six orders of magnitude, includes all the biological variation of fusion gene expression.

This assay exhibited a high level of sensitivity for the majority of fusion gene types. The control gene used is a very robust normalizer exhibiting comparable level of expression in normal, AML and ALL cells. This allowed the normalization for all variations which might be introduced in different steps of this assay.

Overall, RT-qPCR is a very useful method for MRD detection, having a high sensitivity, comparable to nested PCR. This allowed early relapse detection for a number of patients. MRD monitoring is an integrated part of the today's treatment protocols and a standardized RT-qPCR method should be used by all laboratories involved in leukemia patient investigation.

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Abbreviations list

ALL – Acute Lymphoid Leukemia
AML – Acute Myeloid Leukemia
CGCN - Copy number of control gene
FG – Fusion Gene
FISH –Fluorescent *In Situ* Hybridization
MRD – Minimal Residual Disease
NCN -Normalized copy number of fusion gene transcript
RT-qPCR – Real-Time Quantitative PCR
SENS – sensitivity

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