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ORIGINAL ARTICLE

Quantitative real time-polymerase chain reaction method in Bcr-Abl translocation diagnostics

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Summary

Purpose: *The quantitative real-time polymerase chain reaction (qRT-PCR) is used in the detection of molecular events involved in leukemogenesis, such as the Bcr-Abl gene translocation, the most important factor in the pathogenesis of chronic myeloid leukaemia (CML). The main aim of our study was to test the reproducibility, specificity and sensitivity of the qRT-PCR in the detection of Bcr-Abl gene translocation.*

Methods: *In complementary (c)DNA, isolated from K562 Bcr-Abl positive cell line, we performed qRT-PCR analysis with Bcr-Abl specific primers. For qRT-PCR analysis, we used serial dilutions of the newly synthesized cDNA in order to establish the detection threshold of this method.*

Results: *Using the specific primers for the Bcr-Abl translocation, we obtained the specific translocation product in cDNA sample of K562 human erythroid leukemia cell line. qRT- PCR showed significant sensitivity with the detection threshold for the Bcr-Abl fluorescent signal, which enabled the precise detection that was accurate within a 10-fold dilution range, and a dynamic range of 5 orders of magnitude.*

Conclusion: *The results of our study showed that the application of the qRT-PCR is the optimal method for the detection of Bcr-Abl gene translocation, characterized by high reproducibility, specificity and sensitivity.*

Key words: diagnostics, gene translocations, leukemia, qRT-PCR

Introduction

The significant progress in the domain of translational medicine resulted in the successful application of molecular data into clinical studies, enabling the precise molecular diagnostics and individualized targeted therapy of leukemia. However, data of epidemiological studies are discouraging. The mortality rate of acute leukemia is 5.2 cases per 100,000 in the general population, with survival rate at 5 years of 50% for the women, 75% for children and 35% for men in Europe [1].

Molecular alterations at the level of myeloid lineage cells, leading to malignant transformation and progression, are the result of accumulated mutations of genes coding for proteins, involved in cell growth and differentiation control [2]. Malignant myeloid lineage cells possess numerous genetic alterations, resulting as a consequence of different endogenous, as well as exogenous factors, inducing DNA damage and influencing the disease outcome [3].

The characteristic cytogenetic alteration in CML is the Philadelphia chromosome (Ph+), found also in samples of patients with acute lymphoblastic leukemia (ALL). In CML patients it is found in 95% of bone marrow cells, whereas the frequency in ALL patients is 3-5% in children and 15-25% in adults [4-7]. Ph chromosome is the result of reciprocal translocation of genetic material between the long arms of chromosomes 9 and 22 [t(9: 22)(q34: q11)] [6,8].

In all CML patients and in one third of ALL patients, the main breakpoint of the chromosome 22 is within the DNA fragment of 5800 base pairs (bp), designated as the "major breakpoint cluster region" (M-bcr). The messenger (m) RNA transcript of M-Bcr-Abl, is

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the fusion product of Bcr exons b2 and/or b3 and exon 2 of Abl (Abelson murine leukemia) protooncogene (b2a2 and b3a2). Reciprocal translocation and fusion of genetic material from different chromosomes result in transcriptionally active Bcr-Abl chimeric gene, and the fusion protein of 210 kDa, designated as P210. This protein product functions as active, deregulated, intracellular tyrosine kinase. *In vivo* experiments showed that the P210 protein is responsible for the development of acute lymphoproliferative disease in mice, thus, strongly supporting the hypothesis of its role in CML and ALL pathogenesis in humans [6,9].

In addition to fusion protein P210, in the majority of Ph+ ALL patients the breakpoint is situated in 5' region, within the first large intron of Bcr gene, resulting in the expression of fusion mRNA, designated as e1: a2. This transcription product is coding for the synthesis of chimeric P190 protein. In ALL patients, both transcripts (P190 and P210) may be detected, whereas in CML patients, the e1: a2 is very rarely detectable [6].

Numerous studies published in last two decades showed that the Bcr-Abl translocation is the most important factor in CML pathogenesis, due to tyrosine-kinase activity of the fusion mRNA product, responsible for malignant transformation of hematopoietic cells *in vitro* and *in vivo*. The activation of multiple cascade pathways of signal transduction in malignant cells stimulates the proliferation, reducing the growth-factor dependence, and inhibiting apoptosis. The expression of fusion Bcr-Abl protein provides to pluripotent hematopoietic stem cells and to their lineage the capacity of faster proliferation and clonal advantage, leading to clinical manifestations of CML [10].

The acronym qRT-PCR (quantitative real-time polymerase chain reaction) indicates one of the most sensitive methods for the detection and quantification of mRNA in cells, tissues and organs. In order to detect the molecular events involved in the process of leukemogenesis, the qRT-PCR method was applied in our study. This method is based on cDNA synthesis and PCR amplification of the product, using the Bcr-Abl specific oligonucleotides (two-step qRT-PCR). The qRT-PCR method is a rapid technique, characterized by sensitivity, reproducibility, sensitivity and specificity, thus enabling the accurate molecular diagnosis of Bcr-Abl translocation [11].

Standardization and validation are necessary before routine use of an assay can occur, in order to control for inter- and intra-laboratory variations in results, to reduce laboratory errors and to detect critical loss of assay sensitivity [12]. Therefore, we aimed to test the serial dilutions of the cDNA originating from the human leukemic cell line K562, which expresses the fusion gene Bcr-Abl.

Methods

The synthesis of cDNA was performed using 1 μg of commercial RNA as a template. The RNA originated from human erythroleukemia K562 cell line, and cDNA was synthesized using the cDNA synthesis kit (Superscript III, Invitrogen, USA). For the reverse transcription, we used the combination of the oligo dT and random hexamers in order to achieve the optimal cDNA synthesis. For the qRT-PCR analysis, we performed serial dilutions of the newly synthesized cDNA in order to establish the detection threshold of this method. The dilution was within a 10-fold range, with a dynamic range of 6 orders of magnitude.

Both couples of primers (the sequence of primers is shown in Table 1) were used in concentration of 1.2 μM each. The qRT-PCR analysis was performed using the instrument StepOne Plus (Applied Biosystems, USA); the standard software PCR product fluorescence detection and analysis (Applied Biosystems StepOne^{TM} Real-Time PCR software v. 2.0) was used.

DNA sequencing and subsequent *in silico* analysis of the products were performed using the PCR products amplified by qRT-PCR analysis. PCR products were purified using BioGel P-100 (Bio-Rad laboratories, USA). To 2 μl sense or antisense sequencing primer (1.5 μM) and 3 μl Bigdye terminator mix (Applied Biosystems, Foster City, CA, USA), 1 μl purified PCR products was added. The amplification consisted of an initial 5 min denaturation step at 96° C, 25 cycles of 10 sec of denaturation at 96° C, and a 4 min annealing/extension step at 60° C. The purified reac-

Table 1. The sequence of oligonucleotides used for qRT-PCR analysis

Oligonucleotide	*Sequence*	*Gene*
B2A_q_F	ACAGCATTCCGCTGACCATCAATAAG	Bcr
CA3_q_R	TGTTGACTGGCGTGATGTAGTTGCTTG	Abl
3'GAPDH_f	TCTCCTCTGACTTCAACAGCGAC	Reference gene (GAPDH)
3'GAPDH_r	CCCTGTTGCTGTAGCCAAATTC	Reference gene (GAPDH)

tion products (G50 Sephadex spin column, Boehringer Mannheim) were sequenced on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Alignment analysis was performed with Genalys software [13].

Results

Using specific primers for the Bcr-Abl translocation, we obtained the specific translocation product in cDNA sample of K562 human erythroid leukemia cell line. Figure 1 shows the specific sequence, indicating the translocation t(9: 22)(q34: q11). The confirmation of the specificity of the sequenced PCR product was the *in silico* BLAST analysis, showing the specific hybridization of the obtained sequence with Bcr and Abl gene (chromosomes 22 and 9, respectively).

Our bioinformatic analysis of the sequenced PCR product showed that the use of the Bcr primer within the exon 13 (Figure 2) resulted in amplification of the predicted PCR fusion product, localized in the "major breakpoint cluster" region, with the additional result of the single nucleotide insertion in K562 cell line cDNA. This single adenine insertion created the frameshift mutation, resulting in the synthesis of alanine, instead of proline.

The software analysis of the qRT-PCR, performed with the serial dilutions of K562 cDNA, showed that the qRT-PCR is highly sensitive method, confirming its capacity to detect the Bcr-Abl product of low abundance.

Figures 3 and 4 show the amplification curves of the Bcr-Abl gene and GAPDH control gene in K562 cDNA. The curves for the amplification of Bcr-Abl gene and GAPDH in all cDNA dilutions were detected almost during the same amplification cycle, indicating that both amplicons were present in similar amounts. The amplified and detected products for Bcr-Abl and GAPDH were highly specific and well detected in all serial dilutions of K562 cDNA.

The accuracy of the qRT-PCR analysis is shown in Figure 5, with the Bcr-Abl/GAPDH quantity ratio, which was 1 ± 0.2 in all analyzed cDNA dilutions. For both Bcr-Abl and control house-keeping GAPDH gene, the intensity of the fluorescent signal above the signal detected from the non-template control was in the range of dilutions of samples between 10^{0}-10^{-5}.

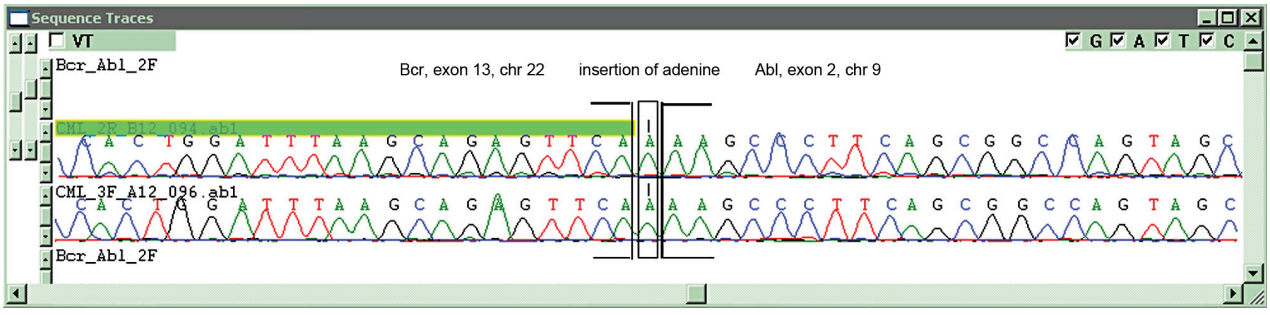

Figure 1. Specific Bcr-Abl translocation t(9: 22)(q34: q11), with the adenine insertion.

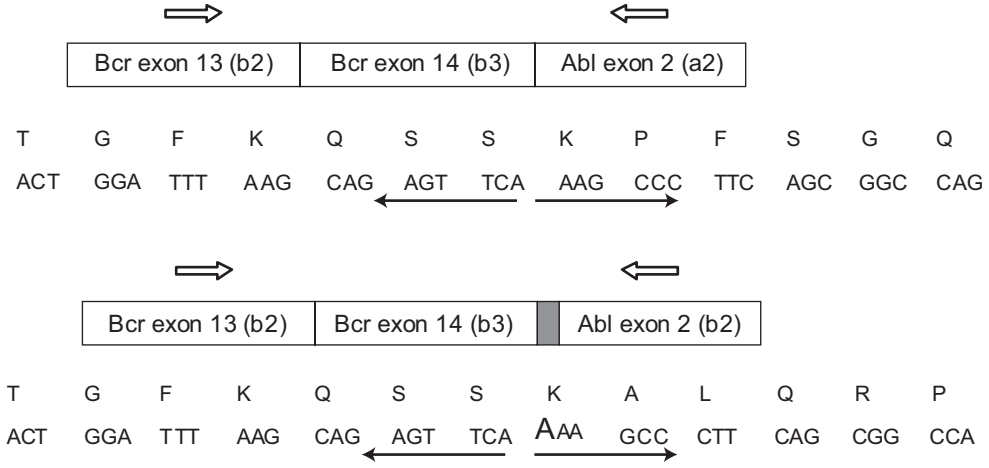

Figure 2. Position of primers in the Bcr and Abl gene exons.

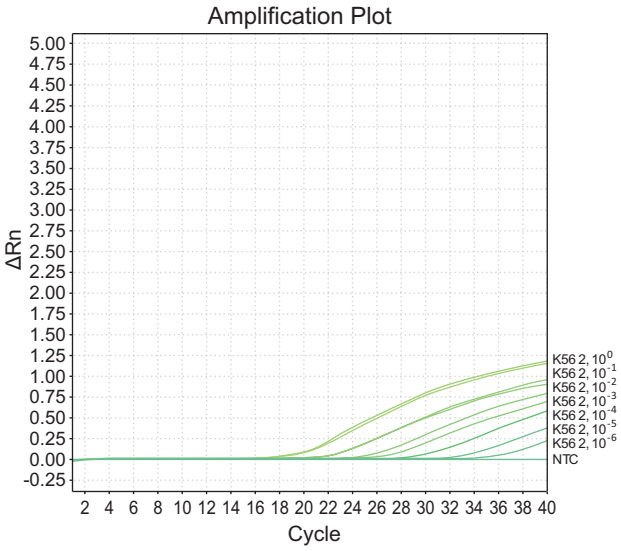

Figure 3. qRT-PCR analysis of Bcr-Abl gene in K562 cell line cDNA.

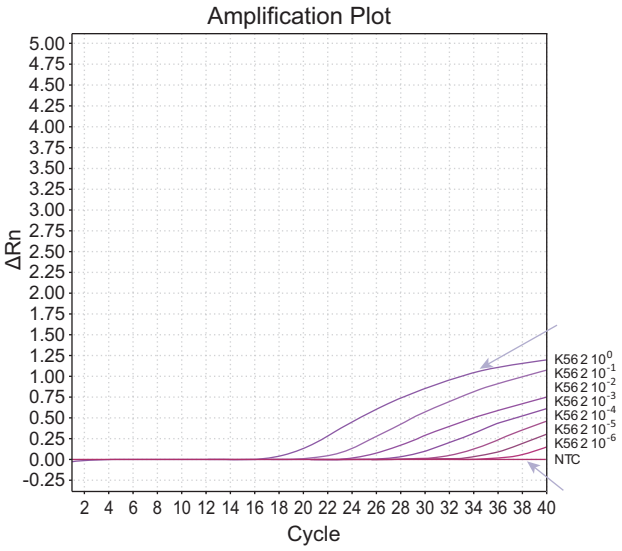

Figure 4. qRT-PCR analysis of GAPDH gene in K562 cell line cDNA.

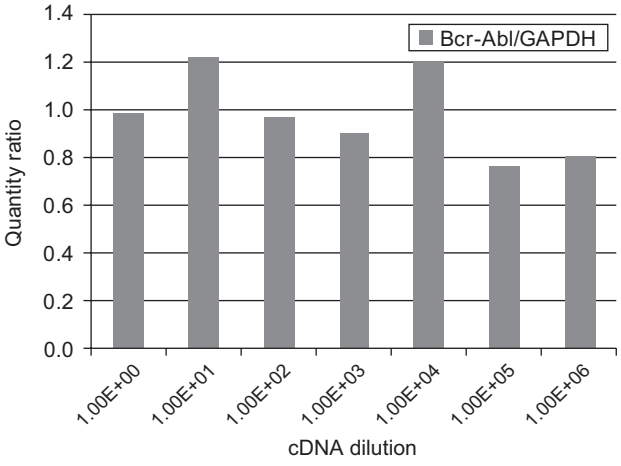

Figure 5. qRT-PCR analysis of Bcr-Abl/GAPDH quantity ratio in K562 cell line cDNA.

Discussion

The qRT-PCR method is the reference method for the quantification of Bcr-Abl fusion transcript in samples of patients with clinical and cytogenetic diagnosis of CML [14-16]. The synthesis and clinical use of imatinib mesylate was the first success of the translational medicine in the application of the results of precise molecular events involved in the reciprocal translocation in Ph+ CML, resulting in the Bcr-Abl fusion gene [17]. This gene is coding for the constitutively active tyrosine kinase, involved in the promotion of cell survival, proliferation and malignant transformation. The clinical use of imatinib in the chronic phase of CML resulted in the follow-up of 3 key parameters of patient response to targeted therapy: complete hematological response, complete cytogenetic remission and complete molecular response to therapy, defined as the elimination of Bcr-Abl fusion transcript from the patient blood and bone marrow samples, analyzed by the qRT-PCR method [10,18,19].

The reciprocal translocation of the long arms of chromosomes 9 and 22, resulting in Ph chromosome, is detected in more than 90% of CML patients and in 15-25% of patients with ALL. This translocation results in the transposition of c-Abl oncogene (9q34) to Bcr gene (22q11). The fusion Bcr-Abl transcript and its product are the specific markers for the diagnosis of leukemia, as well as for the monitoring of the therapeutic outcome and progression of disease. Numerous analyses, including our study, showed that the qRT-PCR enables the specific and sensitive detection of Bcr-Abl fusion product in Ph+ cells. Due to its significant sensitivity and specificity, qRT-PCR is a powerful tool for the detection of subclinical minimal residual disease or disease relapse, after bone marrow transplantation or tyrosine kinase targeted therapy.

Our result showing the single nucleotide insertion in K562 cell line cDNA may indicate that the response of K562, as an experimental *in vitro* model, to the treatment by tyrosine kinase inhibitors, may be different from the cells without this insertional mutation. On the other hand, this result further supports the need for careful and specific design of the primers, positioned within the sequence of both genes (Bcr and Abl), in order to enable the detection of fusion transcript in each specific translocational event.

The results of our study showed numerous ad-

vantages of molecular biology methods, namely the sequencing of PCR product and qRT-PCR in the characterization of molecular events involved in the Bcr-Abl translocation, as well as the precise localization of exons participating in the formation of the resulting fusion gene, which is in concordance with the results of Gullo et al. and Strnad et al. [20, 21]. qRT-PCR showed significant sensitivity, with the detection threshold for the Bcr-Abl fluorescent signal, which achieved the precise detection that was accurate within a 10-fold dilution range, and a dynamic range of 5 orders of magnitude.

According to our results, we may conclude that qRT-PCR is the original method for the "screening" of the molecular events involved in leukemogenesis of the cells of myeloid lineage. The use of qRT-PCR is characterized by high specificity and sensitivity. This method enables the detection of the fusion Bcr-Abl transcript, responsible for the Bcr-Abl translocation and leukemogenesis.

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