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Quantification of The FLI1 Gene Expression By Real-Time Quantitative RT-PCR

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Abstract: In this study, quantification levels were investigated to define alterations in the expression of the FLI1 gene on acute promyelocytic leukemia (APL), which is characterized by a reciprocal t(15,17) translocation of fusing the PML gene to the retinoic acid receptor alpha (RAR alpha) gene. The FLI1 gene plays an important role in several signal transduction pathways, and is involved in the normal regulation of myeloid hematopoiesis and leukomogenesis. We used the real-time quantitative RT-PCR (LightCycler) with SYBR Green I dye method for the labeling and analysis of the quantification of FLI1 gene RT-PCR products. Ribosomal protein S9 (RPS9) was used as an internal control for the normalization of the results. FLI1 gene levels were found up-regulated in two PMLRARA fusion gene positive APL patients compared to bone marrow samples from four healthy donors. To our knowledge, this study is the first attempt to quantify the FLI1 gene in APL patients by real-time RT-PCR. SYBR Green I dye detection and product verification by melting curve analysis is a rapid, sensitive and specific method to validate the expression of the FLI1 gene. Based on our findings, this method should be considered to be a successful approach to gene statement analysis. The possible correlation of high expression levels of FLI1 in APL pathogenesis remains to be established.

Key Words: Gene expression, FLI1, PML-RARA, Real Time RT-PCR.

Introduction

There are several methods to quantify gene expression including northern blotting, (1), cDNA array (2), RNAse protection assay (3,4), and RT-PCR (5). cDNA array technology is able to calculate tens of thousands of genes in a single experiment, but is limited in use due to its high cost. Northern analysis is a method providing information about mRNA size and the RNA integrity. RNAse protection assay is very useful for discriminating between related mRNAs of the same size. Both of these technologies are valuable methods, but low detection sensitivity is a problem they share. The focus on increasing sensitivity has led to the development of highly complex procedures such as three-step nested RT-PCR (6), but these procedures increase the possibility of contamination and false positive results (7). Real-time quantitative RT-PCR is the most sensitive, rapid and reliable approach compared to conventional methods and cDNA array technology (2,8,9).

It was found that the FLI1 gene was down-regulated during vitamin D dependent differentiation of acute myeloid leukemia cell line HL60 cells in a previous study using cDNA array technology and real-time quantitative RT-PCR (10). This finding indicated that an overexpressed FLI1 gene may play a role in acute promyelocytic leukaemia (APL) pathogenesis. The role of the FLI1 gene in myeloid leukemogenesis and normal regulation of myeloid hematopoiesis has been shown also in previous studies by other authors (11,12).

Our aim was to perform statement analysis of the FLI1 gene in APL t(15;17) cases using real-time quantitative RT-PCR (LightCycler, Roche Diagnostics GmbH, Germany). To our knowledge, this study is the first attempt at the quantification of the FLI1 gene in APL patients by real-time quantitative RT-PCR.

Materials and Methods

Patient Samples

This retrospective study is based on total RNA samples extracted from the bone marrow aspirates of two APL patients. The PMLRARA fusion gene was detected for diagnostic purposes in the Genetics Department of the Institute for Experimental Medicine, İstanbul University by RT-PCR analysis, and samples were collected from the Adult Hematology/Oncology Division of

İstanbul Medical Faculty, İstanbul University. The control group consisted of pooled bone marrow aspirates from four healthy volunteer.

RNA Isolation

Mononuclear cells from bone marrow aspirates were isolated by ficoll-hypaque centrifugation. Total RNA was extracted by using the guanidinium thiocyanate-phenol-chloroform extraction method as previously described (13). The quality of the RNA was checked by agarose gel electrophoresis. cDNA was synthesized from 1 µg of total RNA by using random hexamers (14).

Validation of Relative Gene Expression by Quantitative Fluorescent PCR

DNA Master SYBR Green 1 mix (Roche, Mannheim, Germany) was used with 2 µl of cDNA and with 10 pmol of the primers. PCR was performed on a "LightCycler", a rapid thermal cycling instrument of Roche (Roche Diagnostics GmbH, Germany), in capillary glass tubes with the LightCycler-Fast Start DNA Master SYBR Green I kit (Roche). During the amplification, SYBR Green was binding to double stranded PCR products and so the fluorescence signal synchronically increased with the increasing amount of the product. Work was always carried out on desktop coolers (pre-cooled to 4 °C). The master mixtures were prepared exactly according to the recommendations of the manufacturer, except for the concentrations of Mg^{+2}, primers and volume of cDNA. Final concentrations in the reaction mixtures of these were Mg^{+2}, 2.5 mM and primers, 50 pmol each. The accession numbers, chromosomal locations and sequences of gene-specific primers are shown in Table 1. The amplification program consisted of 1 cycle of 95 °C with a 60 s hold, followed by 45 cycles of 95 °C with a 10 s hold, annealing temperature at 55 °C with a 5 s hold, and 72 °C with a 20 s hold. Amplification was followed by melting curve analysis using the program run for one cycle at 95 °C with a 0 s hold, 65 °C with a 10 s hold, and 95 °C with a 0 s hold at step acquisition mode. A negative control without cDNA template was run with every assay to assess overall specificity. Each assay included duplicate reactions for each dilution and was repeated. Standard curves were obtained by using serial dilutions of the beta-globulin gene (DNA Control kit, Roche) according to the supplier's instructions. The concentration of each gene was determined on the basis of a kinetic approach using the LightCycler software. The obtained gene expression values were normalized using housekeeping gene RPS9 levels of pooled bone marrow aspirates from four healthy subjects. Melting curve analysis and the gel electrophoresis of the products validated the reactions. Ratios were calculated using the following formula:

$$\text{Ratio} = \frac{\text{The measured expression of the FLI1 gene in patients / The measured expression of the housekeeping RPS9 gene in patients}}{\text{The measured expression of the FLI1 gene in pooled healthy bone marrow samples / The measured expression of the housekeeping RPS9 gene in pooled healthy bone marrow samples}}$$

Results

FLI1 gene expressions were investigated in two t(15;17) APL patients. The first patient had a bcr1/2 type breakpoint on the PML gene and FLI1 gene levels were found eight times up- regulated. The second patient had a bcr3 type breakpoint on the PML gene and FLI1 gene levels were found 24 times up-regulated compared to the pooled bone marrow samples of four healthy donors (Table 2).

Amplification reactions demonstrated the gradual reduction in fluorescence as temperature increases. The rapid falls indicate the specific products that melt at specific temperatures as 85 °C for FLI1 and 86 °C for RPS9. Gene specific amplifications were demonstrated with melting curve data (Figure 1) and the presence of PCR products of the expected size in agarose gel electrophoresis (Figure 2).

Table 1. Primer sequences of the studied genes.

Genes	Chromosomal Location	Accession Number (GenBank)	Primer Sequences
RPS9 (Housekeeping)	19q13.4	U14971	CGTCTCGACCAAGAGCTGA GGTCCTTCTCATCAAGCGTC
FLI1	11q24.1-q24.3	M93255	CCACACTGGTGACACAGGAG TCTTTGACACTCAATCGTGAGGA

Table 2. Validation of relative gene expression by quantitative real-time RT- PCR (compared to bone marrow samples of four healthy donors).

Patients	Age/sex	Break Point	FLI1 ratio
1	44 (M)	bcr1/2	8
2	26 (F)	bcr3	24

*Ratio = (The measured expression of the FLI1 gene in patients / The measured expression of the housekeeping RPS9 gene in patients) / (The measured expression of the FLI1 gene in pooled healthy bone marrow samples / The measured expression of the housekeeping RPS9 gene in pooled healthy bone marrow samples)

Discussion

In this study we analyzed the expression levels of FLI1 gene in two APL patients who carry t(15;17). We used the real-time (kinetic) RT-PCR with SYBR Green I dye method for the labeling and quantification analysis of PCR products. Our method is considered rapid, sensitive and specific to validate gene expression (15).

At present, the normalization of the amplification product is the main problem of quantitative RT-PCR. Quantification errors are easily obtained by variations in the amount of starting material between different samples. The main approach for minimizing these errors is through using cellular RNA as an internal reference

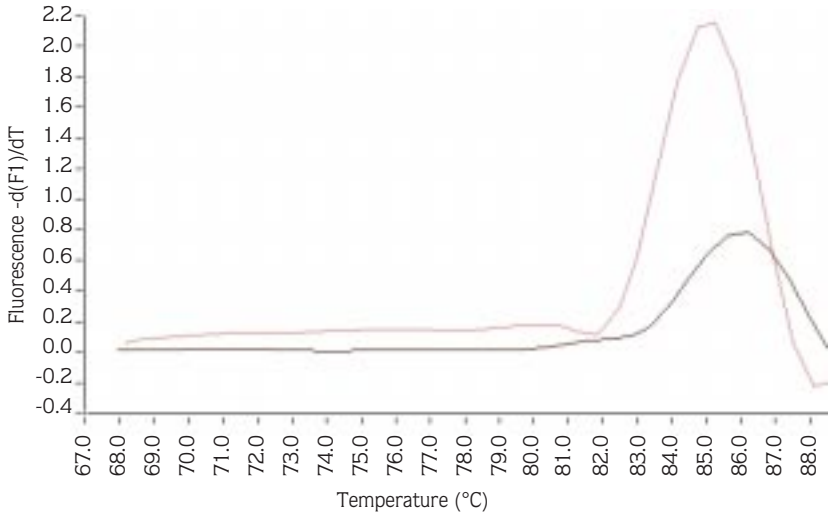

Figure 1. Specific amplifications of FLI1 and 40SR9. Melting curve analysis of a FLI1 and RPS9 amplification reactions demonstrating the gradual change in fluoresence as temperature increases. The rapid falls indicates the specific products melt at specific temperatures, 85 °C for FLI1 and 86 °C for RPS9.

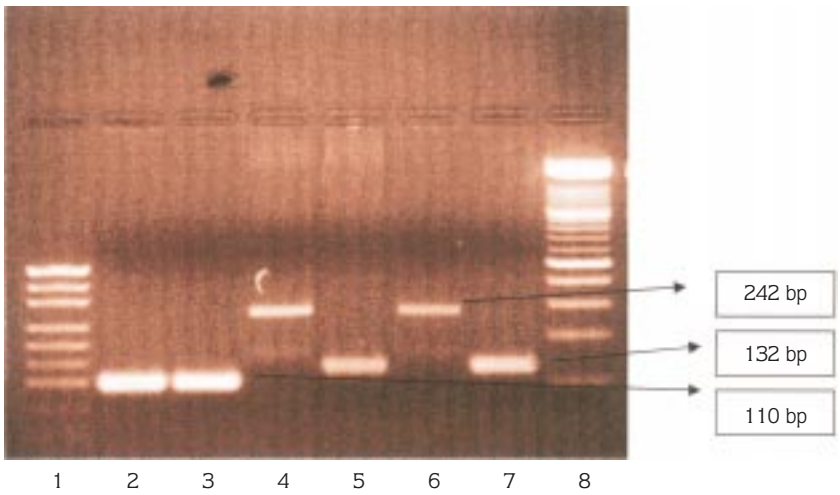

Figure 2. Reactions were analyzed by agarose gel electrophoresis and revealed single amplification products of the predicted sizes (Lane 1, Marker ; Lane 2,3. Beta globulin housekeeping gene*, 110 bp; Lane 4,6. FLI1 oncogene 242 bp; Lane 5,7. RPS9 housekeeping gene 132 bp; Lane 8, Marker.
* Beta globulin housekeeping gene from DNA control kit (Roche, Mannheim, Germany) used to obtain standard curves by serial dilutions.

(16). The ideal gene used for normalization should be expressed at a constant level in different tissues at all stages of development, and should not be affected by any type of disease. Housekeeping GAPDH (15), Beta actin (17) and ribosomal RNA are the genes most commonly used to normalize patterns of gene expression. rRNA sequences have been shown to be more reliable than either of the housekeeping genes in human and mouse malignant cell lines (18,19). For this reason, we used ribosomal protein S9 (RPS9) as an internal control for the normalization of our quantification results.

We optimized PCR reactions for comparative quantification of FLI1 and RPS9. It is important that these primers were working free of primer-dimer complexes in normal conditions. However, after each reaction, melting peak analysis is the step to be remembered, but not the agarose gel run of the products. The sequence of the work was as follows: a) cDNAs were diluted in eppendorf tubes to obtain a series, b) the primers were distributed in capillary tubes, c) the master mixture was prepared by mixing a sufficient amount of Mg into the ready-to-use mixtures, d) the master mixture was divided into 0.5 ml eppendorf tubes (always on the cooler). Mixing was achieved by pipetting and later the mixtures were distributed over the capillary tubes. This sequence was important to ensure the presence of equivalent amounts of sample cDNA for different primers and reactions.

ETS family members have been shown to play an important role in several signal transduction pathways (11). The human FLI1 gene is an ETS family member and is involved in malignancies in both humans and mice. The overexpression of FLI1 perturbs normal lymphoid cell function and apoptosis (20,21). FLI1 also prevented the rapid down-regulation of cyclin D2 and D3 expression normally observed during Epo-induced differentiation and delayed the down-regulation of several other genes involved in cell cycle or cell proliferation control. It has been suggested that the maintenance of high levels of cyclin D2 and D3 in FLI1 expressing erythroblasts could therefore also contribute to the deregulation of the normal control of the balance between proliferation and differentiation (22). Obtained results showed that the FLI1 gene is overexpressed in patients compared with that in healthy donors. These results were in parallel with the findings of a previous study which demonstrated the down-regulation FLI1 gene in vitamin D dependent differentiation of promyelocytic leukemia cell line HL 60 at 72 h of cultures, using cDNA array technology (10).

There are other quantification studies of the FLI1 gene in the literature, but most of these studies concern the quantification of the EWS-FLI1 fusion in Ewing's sarcoma family tumors. A significantly better treatment outcome was reported for a limited number of patients with localized disease when the tumors expressed a type 1 EWS-FLI1 fusion compared with tumors that expressed a non-type 1 EWS-FLI1 fusion. This raised the question of whether EWS-FLI1 gene fusion type might serve as a prognostic molecular indicator in this group of patients (23-25). On the other hand, Levanon et al. (26) obtained results related to the FLI1 gene while they were studying the architecture of the genomic locus encoding the human leukemia-associated transcription factor RUNX1/AML1. They found that the FLI1 homologous sequence contains a breakpoint of the t(11;22) translocation associated with Ewing's tumors, and may have a similar function in RUNX1. Taken together, these data suggest that the FLI1 gene also plays an important role in tumorigenesis in fusion forms with other genes.

Based on our findings, the real-time quantitative (kinetic) RT-PCR (LightCycler) method should be considered a successful approach for gene statement analysis and here we propose an optimized strategy for FLI1 gene detection. Larger studies are required to assess the impact of our approach to FLI1 gene expression analysis in myeloid leukomogenesis. The possible correlation of high expression levels of FLI1 to APL pathogenesis remains to be established.

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