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ORIGINAL ARTICLE

Expression Analysis of DEK, AF4 and FLI1 Genes in All-Trans-Retinoic Acid (ATRA) Treated Acute Promyelocytic Leukaemia *t*(15;17) Patients by Quantitative Real-Time PCR

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Abstract: All-trans retinoic acid (ATRA) sensitivity of acute promyelocytic leukaemia (APL) cells is strictly dependent on the presence of *t*(15;17), but the molecular background of this sensitivity remains obscure. We showed the down- regulation of DEK, AF4 and FLI1 genes in the vitamin D treatment of APL cell line HL-60 , using cDNA array technology in our previous study. This finding prompted us to investigate the expression of these genes in APL patient samples. The effect of ATRA was studied in 6 APL patients carrying *t*(15;17). Two samples from each patient were compared with a primary diagnostic sample and a sample taken at remission. Frozen RNA samples were obtained from bone marrow aspirates and converted to cDNA, and then quantitative real- time PCR was performed. Among the traits of these 3 genes, the over-expression of FLI1 was particularly remarkable. The findings suggest that FLI1 over expression may be involved in APL and that it can be corrected after remission induction. Whether or not ATRA treatment has any effect on these genes may be studied in an experimental model in order to find new potential targets for rational drug discoveries.

Key Words: DEK, AF4, FLI1, Acute Promyelocytic Leukemia, All-Trans-Retimic Acid

Introduction

Acute promyelocytic leukaemia (APL) is the first human malignancy to be effectively treated with a cell differentiation inducer, all-trans-retinoic acid (ATRA). This disease is characterised by an arrest of granulocytic differentiation and a reciprocal translocation (15;17) fusing the PML gene to the retinoic acid receptor alpha (RAR alpha) gene. In vivo ATRA is capable of causing clinical remission in about 90% of APL cases but the molecular background of this treatment is not very clear. Although little is known about the initial events occurring after ATRA treatment in APL, recent evidence has indicated the direct involvement of PML-RAR alpha in mediating this response. ATRA induced differentiation of APL cells is strictly dependent on the presence of PML-RAR alpha (1,2)

The biological responses of ATRA and vitamin D are mediated by binding nuclear receptors belonging to the same receptor superfamily. We showed the down-regulation of DEK, AF4 and FLI1 genes in the vitamin D dependent differentiation of APL cell line HL -60 cells, using cDNA array technology in our previous study (3). This finding prompted us to investigate the expression levels of these 3 genes in APL patient samples. The DEK on 6p23 forms a fusion gene with the CAN (9q34) in a subset of patients with acute myeloid leukaemia (4). Despite significant disease associations, the physiological function of DEK has yet to be determined. AF4 is a fusion partner with MLL in t(4,11) acute lymphocytic leukaemia (5). The function of the AF4 gene remains poorly understood but it may play a role in regulating transcription (6,7). The FLI1 gene belongs to the Ets

transcription factors family. This family plays an important role in several signal transduction pathways (8-10).

In this study, our aim was to describe the expression of DEK, AF4 and FLI1 genes in promyelocytic cells isolated from bone marrow aspirates of patients with APL at the time of diagnosis and after achieving complete haematological remission following ATRA. The study includes 6 APL *t*(15;17) patients treated with ATRA. Two samples from each patient were compared: a primary diagnostic sample and a sample taken at remission. Promyelocytes were less than 5% in remission samples. RNA samples were converted to cDNA. Then quantitative real-time PCR (LightCycler), which is a recently developed method for the rapid and sensitive detection of gene expression, was used for quantification.

Materials and Methods

Patient Samples

This retrospective study was based on bone marrow aspirates from 6 APL patients having *t*(15;17) and treated with ATRA (45 mg/m^2, daily). Among these 6 patients, thee were adults (average age: 48) and 1 child (2). The APL diagnosis was based on FAB criteria. All patients were positive for PML-RAR alpha fusion transcripts detected by RT-PCR (Table 1). The sample of the child was obtained in from Paediatric Haematology-Oncology Section of SSK Bakırköy Hospital, İstanbul, Turkey. The samples of the adult patients were obtained from the Adult Haematology-Oncology Section, Department of Internal Medicine, Medical Faculty, İstanbul University. All treatments were given between 1998 and 2001.

RNA Isolation and DNase I treatment

Mononuclear cells from bone marrow aspirates were isolated by Ficoll-Hypaque centrifugation. Total RNA was extracted by guanidinium thiocyanate-phenol-chloroform extraction as previously described (11). RNA samples were treated with DNase I (Boehringer Mannheim, Mannheim, Germany) to remove the genomic DNA contamination in the preparations. The quality and integrity of the RNA were checked by electrophoresis using 1% agarose gel with ethidium bromide staining and UV transillumination. RNA concentrations were measured by spectrophotometer at 260 nm. cDNA was synthesised from 1 µg of total RNA by using random hexamers (12).

Validation of relative gene expression by quantitative fluorescent PCR

Standard curves were obtained by using serial dilutions of beta-globulin gene (DNA control kit, Roche, Mannheim, Germany). The work was always carried out on desktop coolers (pre-cooled to 4 °C). A DNA Master SYBR Green 1 mix (Roche, Mannheim, Germany) was used and the master mixtures were prepared exactly according to the recommendations of the manufacturer, except for the concentrations of Mg, primers and volume of cDNA. The final concentrations in the reaction mixtures of these were Mg 2.5 mM, primers 50 pmol each and cDNA 0.1 v/v. The accession number, chromosomal locations and sequences of the gene-specific primers are shown in Table 2.

PCR was performed on a LightCycler, a rapid thermal cycling instrument (Roche Diagnostics GmbH, Germany) in capillary glass tubes. The amplification programme

Table 1. Data of acute promyelocytic lekaemia *t*(15;17) patients*.

Patient	Age/sex	BP	WBC (109/L)	Hb (g/d)	Promyelocyte count at diagnosis	S (m)	FLI1 ratio	AF4 ratio	DEK ratio
1	19 (F)	bcr1/2	7000	10	18%	14+	6.7	1.4	0.86
2	69 (F)	bcr1/2	2600	10	50%	16+	3.48	1.56	1.98
3	44 (M)	bcr1/2	2500	5.1	95%	47+	7352	0.67	0.4
4	29 (M)	bcr3	39.500	6.7	80%	12+	34.482	110.903	8.7
5	2 (M)	bcr1/2	10.000	8	20%	n.a.	2500	1.6	5.7
6	54 (F)	bcr1/2	n.a.	n.a.	n.a.	n.a.	0.5	1.6	11.6

(Abbreviations: BP: Breakpoint, WBC: White Blood Cells, Hb: Haemoglobin, S (m): Survival (months), n.a.: Data is not available)

* All patients were treated with ATRA 45 mg/m^2, daily.

Table 2. Primer sequences of the studied genes.

Genes	Chromosomal Location	Accession Number (GenBank)	Primer Sequences
RPS9 (Housekeeping)	19q13.4	U14971	CGTCTCGACCAAGAGCTGA GGTCCTTCTCATCAAGCGTC
FLI-1	11q24.1-q24.3	M93255	CCACACTGGTGACACAGGAG TCTTTGACACTCAATCGTGAGGA
DEK	6p23	X64229	GTGGGTCAGTTCAGTGGC AGGACATTTGGTTCGCTTAG
AF-4	4q21	L13773	CCCATGGATGGTCAAGATCA TGGGTCATTTCCTTCAGAATCTC

consisted of 1 cycle of 95 °C with a 60-s hold, followed by 45 cycles of 95 °C with a 10-s hold, and annealing 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 programmne run for 1 cycle at 95 °C with a 0-second hold, 65 °C with a 10- s hold, and at 95 °C with a 0-s hold at the 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 levels of the housekeeping gene RPS9 were used as internal controls for the normalisation of RNA quantity and quality differences in all samples. RPS9 is a gene belonging to the ribosomal gene family and is considered more reliable than either of the classical housekeeping genes, in human and mouse malignant cell lines (13,14) . Ratios were obtained with the following formula:

$$\text{Ratio} = \frac{\text{Observed expression of the FLI1, AF4, DEK genes in non-treated samples / Observed expression of the housekeeping RPS9 gene in non-treated samples}}{\text{Observed expression of the FLI1, AF4, DEK genes in ATRA treated samples / Observed expression of the housekeeping RPS9 gene in ATRA treated samples}}$$

Results

Expression levels of the DEK, AF4, and FLI1 genes were determined by real-time quantitative PCR (LightCycler). Expression of the DEK, AF4 and FLI1 genes in diagnostic samples was compared to ATRA treated samples from 6 APL patients.

Ratios obtained, promyelocyte counts at diagnosis, breakpoints and some other clinical parameters are shown in Table 1. Gene specific amplifications of DEK, AF4, FLI1 and RPS9 genes were demonstrated with an agarose gel picture (Figure).

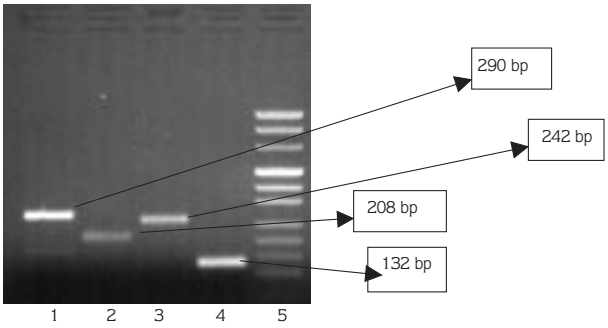

Figure. Reactions were analysed by agarose gel electrophoresis and revealed single amplification products of the predicted sizes (Lane 1, DEK gene 290 bp; Lane 2, AF4 gene 208 bp; Lane 3, Fli1 gene 242 bp; Lane 4, 40 RPS9 housekeeping gene 132 bp; Lane 5, Molecular weight marker MBI mix 8).

Discussion

In this study we analysed the levels of expression of DEK, AF4 and FLI1 genes in promyelocytes isolated from the bone marrow aspirates of 6 patients at the time of diagnosis and after achieving complete haematological remission following ATRA. We used real-time quantitative PCR (LightCycler), which is a sensitive and specific method of validating gene expression (15). In the literature, there are many studies about the differentiation effect of the ATRA treatment, including proto-oncogenes as c-MYC, $p21^{WAF1/CIP1}$ and $p19^{INK4D}$ as cell cycle inhibitors, BCL2 related genes as apoptosis

antagonists or novel genes as FUS (16-18). DEK, AF4 and FLI1 may also be affected by the ATRA treatment of APL in relation to the central process of differentiation and apoptosis. In this sense, our approach in evaluating the expression of these genes regulated by ATRA can be informative. Our study is the first attempt for the quantify the DEK, AF4 and FLI1 genes in ATRA treated *t*(15;17) APL patients.

The human FLI1 gene is an ETS family member and is involved in malignancies in both humans and mice. The over-expression of FLI1 perturbs normal lymphoid cell function and apoptosis (19,20). Darby et al have observed that FLI1 and the retinoic acid receptor (RARalpha) can reciprocally repress one another's transcriptional activation. It has been suggested that accumulation of FLI1 can oppose the transcriptional activity of hormone receptors in haematopoietic cells (21). In our experiments, FLI1 ratios were found very high in three cases (3-5) and promyelocyte counts at diagnosis 95%, 80%, 20%, respectively, but it was moderately over-expressed in case 2, the promyelocyte count of which was 50% at diagnosis. This observation makes this gene a good candidate for further studies.

The DEK gene on 6p23 forms a fusion gene with the CAN gene on 9q34 in acute myeloid leukaemia patients who carry a t(6; 9) (p23; q34) translocation (4). The breakpoints on chromosome 6 and those on chromosome 9 are clustered in a specific intron of the DEK and CAN genes, respectively; hence, the DEK/CAN fusion gene encodes an invariable transcript. The 5-prime part of the DEK gene is fused to the 3-prime part of the CAN gene with no interruption of the open reading frame. The kind of role DEK plays in leuokomogenesis after this translocation and/or alone in cell physiology has not been defined yet. Recently, Kappes et al. demonstrated that the major portion of the DEK is associated with chromatin in vivo and suggestted that it plays a role in chromatin architecture (22). We found that DEK gene levels were moderately over-expressed in 3 cases (cases 4,5 and 6). We suggest that this gene should be studied in larger groups of *t*(15;17) APL patients before making a decision about its role in ATRA dependent APL therapy.

We did not observe the down-regulation effect of the ATRA treatment on AF4 gene in 5 of the 6 patients. AF4 is a gene described as a fusion partner with MLL in *t*(4;11) acute leukaemia (5,6). Some authors suggest that AF4 plays an important role in the development of the haematopoietic, cardiovascular, skeletal and central nervous systems. A repeating pattern of AF4 expression in development is down-regulation with differentiation of a tissue. Among the cell types where this pattern of down-regulation is noted are B-lymphocytes. These findings raise the possibility that the disruption of normal AF4 function by translocation may contribute to leukemogenesis (16). The function of this gene remains poorly understood but it may play a role in regulating transcription (6,7). AF4 was moderately over-expressed in 5 cases (cases 1, 2, 4, 5 and 6) and highly over-expressed in case 4. This gene could be sensitive to ATRA treatment but our observations may extend to larger studies.

In conclusion, although DEK and AF4 genes were not very clearly expressed in relation to the promyelocyte counts of the patients, a clear correlation was found between FLI1 and leukaemia activity. Our findings suggest that FLI1 is involved with APL and their over-expression is corrected after remission induction. Whether ATRA has any effect on these genes may be studied in an experimental model in order to find new potential targets for rational drug discoveries.

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