

1-1-2004

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

HAKAN SAVLI

SEMA SIRMA

BALINT NAGY

MELİH AKTAN

GÜNÇAĞ DİNÇOL

See next page for additional authors

Follow this and additional works at: <https://journals.tubitak.gov.tr/medical>

 Part of the [Medical Sciences Commons](#)

Recommended Citation

SAVLI, HAKAN; SIRMA, SEMA; NAGY, BALINT; AKTAN, MELİH; DİNÇOL, GÜNÇAĞ; SALCIOĞLU, ZAFER; and ÖZBEK, UĞUR (2004) "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," *Turkish Journal of Medical Sciences*: Vol. 34: No. 2, Article 2. Available at: <https://journals.tubitak.gov.tr/medical/vol34/iss2/2>

This Article is brought to you for free and open access by TÜBİTAK Academic Journals. It has been accepted for inclusion in Turkish Journal of Medical Sciences by an authorized editor of TÜBİTAK Academic Journals. For more information, please contact academic.publications@tubitak.gov.tr.

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

Authors

HAKAN SAVLI, SEMA SIRMA, BALINT NAGY, MELIH AKTAN, GÜNÇAG DİNÇOL, ZAFER SALCIOĞLU, and UĞUR ÖZBEK

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

Hakan SAVLI¹, Sema SIRMA², Balint NAGY³, Melih AKTAN⁴, Günçağ DİNÇOL⁴,
Zafer SALCIOĞLU⁵, Uğur ÖZBEK²

¹Department of Medical Biology, Medical Faculty, University of Kocaeli, Kocaeli - Turkey,

²Department of Genetics, Institute for Experimental Medical Research (DETAE), İstanbul University, İstanbul - Turkey

³1st Department of Obstetrics and Gynaecology, Semmelweis University, Budapest - Hungary,

⁴İstanbul Medical Faculty, İstanbul University, İstanbul - Turkey,

⁵SSK Bakırköy Hospital, İstanbul - Turkey

Received: September 22, 2003

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-Retinoic 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/m², daily). Among these 6 patients, three 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 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 leukaemia *t*(15;17) patients*.

Patient	Age/sex	BP	WBC (10 ⁹ /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/m², 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	GTGGGTCAGTTCAGTGCC AGGACATTTGGTTCGCTTAG
AF-4	4q21	L13773	CCCATGGATGGTCAAGATCA TGGGTCATTTCTTCAGAATCTC

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 programme 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:

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

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).

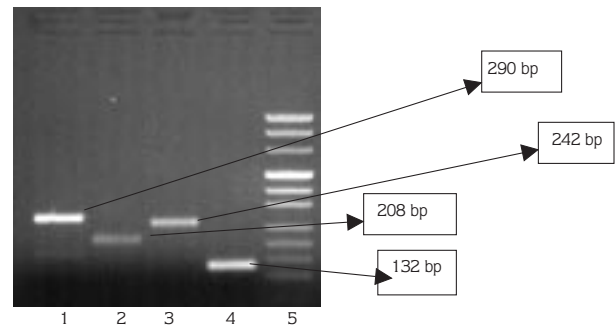


Figure. Reactions were analysed by agarose gel electrophoresis and revealed single amplification products of the predicted sizes (Lane 1, DEK gene 290 bp; Lane 2, AF4 gene 208 bp; Lane 3, FLI1 gene 242 bp; Lane 4, 40 RPS9 housekeeping gene 132 bp; Lane 5, Molecular weight marker MBI mix 8).

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 suggested 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.

References

1. Grignani F, Ferrucci PF, Testa U et al. The acute promyelocytic leukemia-specific PML-RAR alpha fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. *Cell* 74: 423-431,1993.
2. Benedetti L, Grignani F, Scicchitano BM et al. Retinoid-induced differentiation of acute promyelocytic leukemia involves PML-RAR alpha-mediated increase of type II transglutaminase. *Blood* 87: 1939-1850, 1996.

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.

Acknowledgement

This study was supported by a grant from the University of Kocaeli Research Fund.

Corresponding author:

Uğur ÖZBEK,

Istanbul University,

Institute for Experimental Medicine (DETAE),

Vakıf Guraba Cad. Capa 34280, İstanbul - Turkey

E-mail: uozbek@istanbul.edu.tr

3. Savli H, Aalto Y, Nagy B et al. Gene expression analysis of 1,25(OH)₂D₃ dependent differentiation of HL-60 cells a cDNA array study. *Brit J Haematol* 118: 1065-1070, 2002.
4. von Lindern M, Fornerod M, van Baal S et al. The translocation (6;9), associated with a specific subtype of acute myeloid leukemia, results in the fusion of two genes, dek and can, and the expression of a chimeric, leukemia-specific dek-can mRNA. *Mol Cell Biol* 12: 1687-1697, 1992.
5. Domer PH, Fakharzadeh SS, Chen CS et al.. Acute mixed-lineage leukemia t(4;11)(q21;q23) generates an MLL-AF4 fusion product. *PNAS* 90: 7884-7888, 1993.
6. Ma C, Staudt LM. LAF-4 encodes a lymphoid nuclear protein with transactivation potential that is homologous to AF-4, the gene fused to MLL in t(4;11) leukemias. *Blood* 87: 734-745, 1996.
7. Li Q, Frestedt JL, Kersey, JH. AF4 encodes a ubiquitous protein that in both native and MLL-AF4 fusion types localizes to subnuclear compartments. *Blood* 92: 3841-3847, 1998.
8. Yang BS, Hauser CA, Henkel G et al. Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets1 and c-Ets2. *Mol Cell Biol* 16: 538-547, 1996.
9. Ben-David Y, Giddens EB, Letwin Keryt et al. Erythroleukemia induction by Friend murine leukemia virus: insertional activation of a new member of the ets gene family, Fli-1, closely linked to c-ets-1. *Genes and Development* 5: 908-918, 1991.
10. Ben-David Y, Giddens EB, Bernstein A. Identification and mapping of a common proviral integration site Fli-1 in erythroleukemia cells induced by Friend murine leukemia virus. *PNAS* 87: 1332-1336, 1990.
11. Chomczynsky P, Sacchi N. Single step method of RNA isolation by acid guanidium thiocyanate-phenol chloroform extraction. *Analytical Biochemistry* 162: 156-159, 1987.
12. Kusec R., Laczika K, Knöbl P et al. AML1/ETO fusion mRNA can be detected in remission blood samples of all patients with t(8;21) acute myeloid leukemia after chemotherapy or autologous bone marrow transplantation. *Leukemia* 8: 735-739, 1994.
13. Zhong H, Simons JW. Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. *Biochemical and Bio Biophys Res Comm* 259: 523-526, 1999.
14. Bhatia P, Taylor WR, Greenberg AH et al. Comparison of glyceraldehyde-3-phosphate dehydrogenase and 28S-ribosomal RNA gene expression as RNA loading controls for northern blot analysis of cell lines of varying malignant potential. *Analytical Biochem* 216: 223-226, 1994.
15. Rajeevan MS, Ranamukhaarachchi DG, Vernon SD et al . Use of real-time quantitative PCR to validate the results of cDNA array and differential display PCR technologies. *Methods* 4: 443-451, 2001.
16. Baskaran K, Erfurth F, Taborn G et al. Cloning and developmental expression of the murine homolog of the acute leukemia proto-oncogene AF4. *Oncogene* 15: 1967- 1978, 1997.
17. Mills KI, Walsh V, Gilkes AF et al. High FUS/TLS expression in acute myeloid leukaemia samples. *Brit J Haematol* 108: 316-321, 2000.
18. Bartova E, Kozubek S, Kozubek M et al. The influence of the cell cycle, differentiation and irradiation on the nuclear location of the abl, bcr and c-myc genes in human leukemic cells. *Leuk Res* 24: 233-241, 2000.
19. Klemsz MJ, Maki RA, Papayannopoulou T et al. Characterization of the ets oncogene family member, fli-1. *J Biol Chem* 268: 5769-5773, 1993.
20. Zhang L, Eddy A, Teng YT et al. An immunological renal disease in transgenic mice that over-express Fli-1, a member of the ets family of transcription factor genes. *Mol Cell Biol* 15: 6961-6970, 1995.
21. Darby TG, Meissner JD, Ruhlmann A et al. Functional interference between retinoic acid or steroid hormone receptors and the oncoprotein Fli-1. *Oncogene* 15: 3067-3082, 1997.
22. Kappes F, Burger K, Baack M et al. Subcellular localization of the human proto-oncogene protein DEK. *J Biol Chem* 28: 26317-26323, 2001.