Quantification of All-Trans-Retinoic Acid (ATRA) Dependent Expression of CXCR4 Gene in Acute Promyelocytic Leukaemia

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ABSTRACT

CXCR4 is the receptor of CXC chemokine SDF-1 and may play a role in the homing of hematopoietic stem cells. We have investigated the CXCR4 gene expression during ATRA treatment in acute promyelocytic leukaemia (APL) patients. APL is a characteristic disorder with a specific translocation between PML and RAR alpha genes on chromosome 15 and 17. ATRA-induced differentiation of APL cells is strictly dependent on the presence of PML-RAR alpha. In our study, five APL patiens were involved. Two samples from each patient were compared to each other: Primary diagnostic sample and a sample taken at remission. Quantitative real-time PCR (LightCycler) has been used for quantification, which is a recently developed method for rapid and sensitive detection of gene expression. CXCR4 gene ratios were found under-expressed in cases 1 and 6 with blast counts at diagnosis 18%, and 20% but only moderately under-expressed in cases two and four where the blast count was at diagnosis 50%, and 80%. It was over-expressed only in case three where the blast count was 95%. These findings indicate that ATRA treatment might be effective in CXCR4 gene expression related to amount of the blast population in APL. Further gene expression studies would be helpful to understand how ATRA works on CXCR4 related molecular pathways in APL pathogenesis.

Key Words: Gene expression, CXCR4, APL, ATRA.

ÖZET

Akut Promiyelositik Lösemide ATRA Bağımlı CXCR4 Ekspresyonunun Kantitasyonu

CXCR4, CXC kemokin SDF-1'in reseptörüdür ve hematopoietik hücrelerin hominginde rol oynayabilir. Bu çalışmada akut promiyelositik lösemili (APL) hastalarda ATRA tedavisi sırasında CXCR4 gen ekspresyonunu araştırdık. APL, 15 ve 17. kromozomlardaki PML ve RAR alfa genleri arasında özgül bir translokasyonla karakterize-

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

dir. ATRA'ya bağlı APL hücre farklılaşması sıkı bir şekilde PML-RAR alfa varlığına bağlıdır. Bu çalışmada beş APL hastası alınmış ve her hastadan tanıda ve remisyonda alınan iki örnek birbirleri ile karşılaştırılmıştır. Hızlı ve duyarlı bir yöntem olan kantitatif gerçek zamanlı PCR C LightCycler kantifikasyon için kullanılmıştır. Tanıda promiyelosit oranları %18 ve %20 olan iki olguda CXCR4 gen oranları az eksprese olmuş, %50 ve %80 olan iki olguda ise orta derecede eksprese olmuş, blast oranı %95 olan bir olguda ise aşırı eksprese olmuştur. Bu bulgular ATRA tedavisinin, blast oranına bağlı olarak CXCR4 eksprese eden olgularda etkili olabileceğini ancak ATRA'nın CXCR4'le ilgili moleküler yollarda nasıl etki ettiğinin araştırılması gerektiğini göstermektedir.

Key Words: Gen ekspresyonu, CXCR4, APL, ATRA.

Turk J Haematol 2003;20(3): 153-159

Received: *23.09.2002* ***Accepted:*** *13.12.2002*

INTRODUCTION

Chemokines comprise a family of secreted proteins that activate a variety of cell types. There are two groups of chemokines. The CC chemokines usually act on monocytes. In contrast, the CXC chemokines generally act upon neutrophils. The CXC chemokine SDF-1 and its receptor CXCR4 mediate myelopoiesis. It has been concluded that CXCR-4 is expressed on CD34+ cells including more primitive, pluripotent progenitors, and may therefore play a role in the homing of hematopoietic stem cells[1,2]. Differentiation therapy of leukaemia whereby immature cells may be stimulated to their mature phenotype has in recent years aroused priority. The vitamin A derivative all-trans-retinoic acid (ATRA) can induce differentiation only in acute promyelocytic leukaemia (APL) while vitamin D derivatives can differentiate several types of AML cell lines such as HL-60, U937[3,4]. Interestingly, biological responses of vitamin D and ATRA is mediated by binding to nuclear receptors, which belongs to the same receptor superfamily. Expression of PML-RAR alpha in heterogeneous myeloid cell lines increases ATRA sensitivity and ATRA-induced differentiation of APL cells is strictly dependent on the presence of PML-RAR alpha translocation which occurs between chromosomes 15 and 17[5,6]. Some gene products have been reported to be sensitive to differentiation inducer agents, but little is known about the molecular mechanism of this sensitivity. We have shown that up-regulation of CXCR4 gene in vitamin D dependent differentiation of promyelocytic leukaemia cell line HL-60 at 72 hours of cultures, using cDNA array technology[7]. This finding directed us to investigate CXCR4 gene expression during the ATRA treatment in five APL patients. Two samples from each patients were compared to each other: Primary diagnostic sample and a sample taken at remission. Quantitative real-time PCR (LightCycler) has been used for quantification, which is a recently developed method for the rapid and sensitive detection of gene expression.

MATERIALS and METHODS

Patient Samples

Five APL patients have t(15;17) and treated with ATRA (45 mg/m^2, daily). Among these five patients, four were adult (average age: 40) and one was childhood age. Diagnosis of APL was based on FAB's criteria's. All of the patients were positive for PML-RAR alpha fusion transcripts detected by RT-PCR (Table 1). The sample of the child was obtained in the Paediatric Haematology/Oncology-Our Children's Leukaemia Foundation, Medical Faculty, University of İstanbul. The samples of the adult patient's were obtained in Adult Haematology-Oncology Section, Department of Internal Medicine, Medical Faculty, University of İstanbul, all the treatments have been performed between 1998-2001.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Mononuclear cells from bone marrow aspirates were isolated by ficoll-hypaque centrifugation. Total RNA was extracted by using guanidinium thiocyanate-phenol-chloroform extraction method as previously described. The quality of the RNA was controlled by agarose gel electrophoresis. cDNA was synthesised from 1 µg of total RNA by using random hexamers[8]. The accession number, chromosomal locations and sequences of gene-specific primers were shown in Table 2.

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

Patient	Age/sex	BP	WBC (10^9/L)	Hb (g/dL)	Blast count at diagnosis	S (m)	Ratio**
1	19 (F)	bcr1/2	7000	10	18%	14+	0.07
2	69 (F)	bcr1/2	2600	10	50%	16+	0.55
3	44 (M)	bcr1/2	2500	5.1	95%	47+	8.4
4	29 (M)	bcr3	39.500	6.7	80%	12+	0.3
5	2 (M)	bcr1/2	10.000	8	20%	n.a.	0.06

BP: Breakpoint, WBC: White blood cells, Hb: Hemoglobin, S (m): Survival (months), n.a.: Data is not available.

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

$$\text{**Ratio: } \frac{\text{Observed expression of the CXCR4 gene in ATRA treated patient samples/ Observed expression of the housekeeping RPS9 gene in ATRA treated patient samples}}{\text{Observed expression of the CXCR4 gene in nontreated patient samples/ Observed expression of the housekeeping RPS9 gene in nontreated patient samples}}$$

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). 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 "LightCycler", a rapid thermal cycling instrument of Roche (Roche Diagnostics GmbH, Germany) in capillary glass tubes. The amplification program consisted of 1 cycle of 95°C with a 60-second hold, followed by 45 cycles of 95°C with a 10-second hold, annealing temperature at 55°C with a 5-second hold, 72°C with a 20-second hold. Amplification was followed by melting curve analysis using the program run for one cycle at 95°C with a 0-second hold, 65°C with a 10-second hold, and 95°C with a 0-second hold at the step acquisition mode. A negative control without cDNA template was run with every assay to assess the overall specificity. Each assay included duplicate reactions for each dilution and was repeated. Standard curves were obtained by using of 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 kinetic approach using the LightCycler software. The levels of housekeeping gene RPS9 were used as internal controls for normalisation of RNA quantity and quality differences in all samples. Gene expression differences calculated following formula:

$$\text{Ratio: } \frac{\text{Observed expression of the CXCR4 gene in ATRA treated patient samples/ Observed expression of the housekeeping RPS9 gene in ATRA treated patient samples}}{\text{Observed expression of the CXCR4 gene in nontreated patient samples/}}$$

Table 2. Primer sequences of the studied genes

Genes	Chromosomal location	Accession number (GenBank)	Primer sequences
RPS9 housekeeping gene	19q13.4	U 14971	CGTCTCGACCAAGAGCTGA GGTCCTTCTCATCAAGCGTC
CXCR4 (stromal cell derived factor 1 receptor)	2q21	D 10924	CCGCTTCTACCCCAATGACT GCGAAGAAAGCCAGGATGAG

Observed expression of the housekeeping RPS9 gene in nontreated patient samples

RESULTS

The expression of CXCR4 gene was determined by real-time quantitative PCR (LightCycler). Diagnostic samples were compared to ATRA treated patient's samples in five APL patients (Table 1). Blast amounts were less then 5% in remission bone marrow samples. Ratios obtained from observed gene expression levels of ATRA-treated samples divided to observed gene expression levels of nontreated samples. Observed expression ratios were 0.07, 0.55, 8.4, 0.3 and 0.06 for patients 1 to 5 respectively.

CXCR4 gene ratios were under-expressed in two cases (one, five) with blast counts at diagnosis 18%, 20%, respectively, but only moderately under-expressed in cases two and four, where the blast count was at diagnosis 50% and 80%. It was over-expressed in case three where the blast count was 95%. Gene specific amplifications of the CXCR4 and RPS9 genes were demonstrated with melting curves and agarose gel picture (Figures 1,2).

DISCUSSION

In the present study we analysed the CXCR4 gene expression in five ATRA treated APL patients who carry t(15;17) by real-time quantitative PCR (LightCycler). The real-time (kinetic) RT-PCR using LightCycler with SYBR Green I dye detection and product verification by melting curve analysis is a rapid and sensitive method to validate the gene expression[9]. To our knowledge, this technology was used for the first time in the quantification of the CXCR4 gene expression during the ATRA treatment of APL patients.

We have previously reported that CXCR4 gene is upregulated by 1.25(OH)2D3 during myeloid differentiation in leukaemia cell line HL 60, using cDNA array technology[7]. Similarly, Loetscher et al have observed the same results, using northern blotting[10]. These observations directed us to investigate the CXCR4 gene expression differences in ATRA treatment. In experiments we chose APL t(15;17) patients because of their high sensitivity to ATRA treatment. In our experiment, CXCR4 gene ratios were found under-exp-

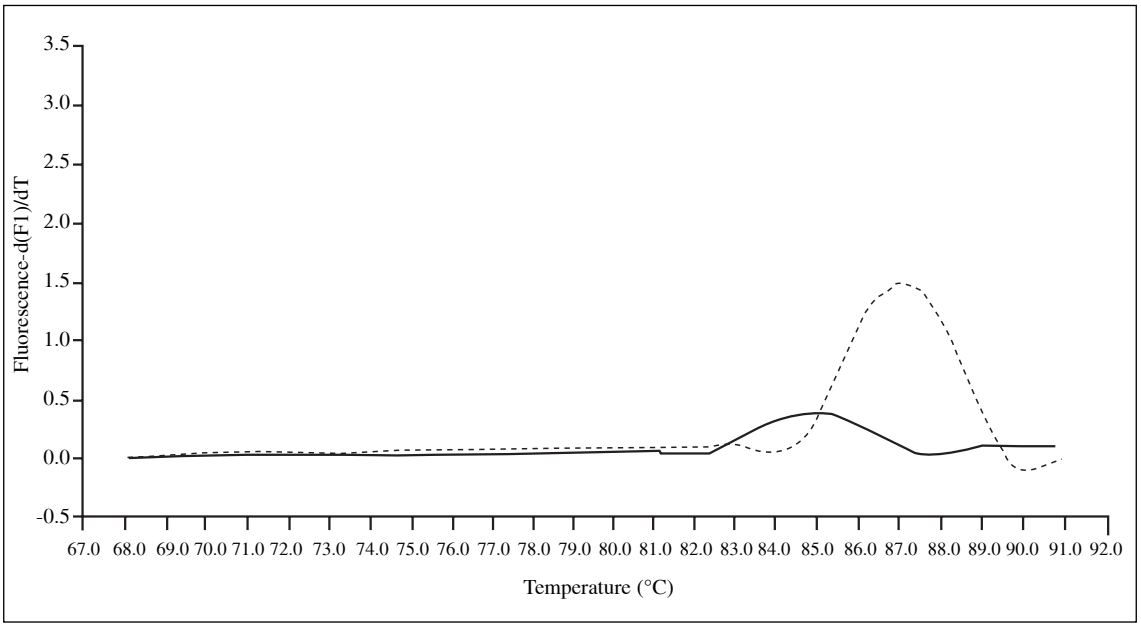

Figure 1. Spesific amplifications of CXCR4, and RPS9 genes. Melting curve analysis demonstrating the gradual reduction in fluorescence as temperature increases. The falls off at 85°C for CXCR4, 87°C for RPS9 indicates the spesific products that melts at this temperature.

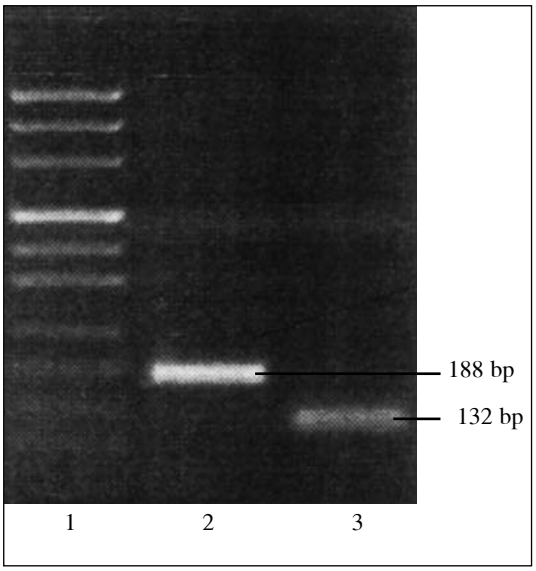

Figure 2. Reactions were analyzed by agarose gel electrophoresis and revealed single amplification products of the predicted sizes (Lane 1, Molecular weight marker; Lane 2, CXCR4 gene 188 bp: Lane 3, RPS9 housekeeping gene 132 bp).

ressed in two cases (one, five) with blast counts at diagnosis 18%, 20%, respectively but only moderately under expressed in cases two and four where the blast count was at diagnosis 50% and 80%. Moreover, it was over-expressed where the blast count was at diagnosis 95%. These findings indicate that ATRA treatment might be effective in CXCR4 gene expression related to amount of the blast population.

Relationship between expression differences of CXCR4 gene and ATRA dependent differantiation is important for myeloid malignancy process as well as normal hematopoiesis. In the literature, it has been concluded that CXCR4 is expressed on CD34+ including more primitive, pluripotent progenitors, and may therefore play a role in the hematopoietic stem cells. CXCR4 expression is critical for the biological effects of SDF-1. SDF-1 is preferentially active in myelomonocytic blasts as a result of differentiation-related expression of its receptor CXCR4. The exact role of SDF is not very clear. It has been suggested that SDF-1 activates distinct signalling pathways that may mediate cell growth, migration and transcriptional activation and may contribute to leukaemic marrow infiltration, by increased CXCR4 expression[2,11]. Gupta et al used the inducible HL-60 cell line as a model system for comparative analysis of CXCR4 expression. They demonstrate that CXCR4 expression undergoes complex regulation at multiple checkpoints, with the likely involvement of different G-proteins for signal transduction during cellular differentiation and following activation with SDF-1[12].

In the literature, studies about the differentiation effect of the ATRA treatment and expression differences of many genes have been published, including integrins. It has been suggested that ATRA increased the expression of integrin alpha-5 beta-1 on hepatocarcinoma cells[13]. Also, in vitro treatment with ATRA consistently increased the expression of beta integrins (CD11a/LFA-1 and CD11b/Mac-1) in fresh APL promyelocytes[14]. Zhang et al were investigated the expression profiles of 514 immunologically relevant genes in naive and SP2/0 tumor-specific activated mouse T-cell populations using array technology. They found the CXCR4 was among the significantly affected genes[15]. To identify changes in the gene expression patterns of promyelocytic leukaemia cells during differentiation, Lee et al were compared the gene expression profiles in NB4 and HL-60 cells with and without ATRA treatment using a cDNA microarray. NB4 cells, an acute promyelocytic leukaemia cell line, have the t(15;17) translocation and differentiate in response to ATRA, whereas HL-60 cells lack this chromosomal translocation. Interestingly, they did not find any common gene expression profiles regulated by ATRA in NB4 and HL-60 cells, even though the granulocytic differentiation induced by ATRA was observed in both cell lines. It has been suggested that the molecular mechanisms and genes involved in ATRA-induced differentiation of APL cells may be different and cell type specific[16]. In this respect, the concordance of our observation between this study and our previously reported CXCR4 gene upregulation in HL-60 cells was remarkable[7].

CXCR4 play important roles in the immunopathogenesis of HIV-1 infection[17]. Besides, the interplay between CXCR4 and SDF-l alpha in eosinophils potentially plays an important role in the accumulation of these cells at the allergic inflammatory sites[18]. In the light of these data, it should be taken into consideration that CXCR4 expression differences observed can be

depend on not only to ATRA treatment but also other reasons.

This study is the first attempt to quantify the CXCR4 gene during ATRA treatment. Similar studies may help to understand the exact role of this gene. Our real-time (kinetic) RT-PCR using LightCycler with SYBR Green I dye detection method is a rapid, sensitive and reliable approach for this aim.

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