$1,25(OH)_2D_3$ Incubation Up-Regulates HOX A9 Gene in HL-60 Cells

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ABSTRACT

There are many genes involved in vitamin D dependent differentiation of acute myeloid leukaemia cell line HL-60 cells. Involvement of HOX genes in leukaemia and differentiation is just beginning to be appreciated. In order to understand the relationship with $1,25(OH)_2D_3$ dependent differentiation of myeloid leukaemia, we studied the expression of HOX A9 gene. HL-60 cells were exposed to $1,25(OH)_2D_3$ for 24 and 72 hours. Gene expression were investigated using quantitative real-time RT-PCR (LightCycler). HOX A9 levels were found up-regulated after $1,25(OH)_2D_3$ treatment, compared to housekeeping RPS9 and HPRT genes. Our study is the first attempt to quantification of HOX A9 gene during $1,25(OH)_2D_3$ treatment, using this technology. We suggest that there is a clear relationship between differentiation induction and over-expression of HOX A9 gene in HL-60 cells. Possible correlation of high expression levels of HOX A9 gene in AML pathogenesis remains to be established.

Key Words: Gene expression, HOX A9, Vitamin D, RT-PCR.

ÖZET

$1,25(OH)_2D_3$ İnkübasyonu HL-60 Hücrelerinde HOX A9 Geninin Upregülasyonuna Yol Açmakta

Akut miyeloid lösemi hücre dizini olan HL-60 hücrelerinde vitamin D'ye bağımlı farklılaşmada rol oynayan çok sayıda gen vardır. Lösemi ve farklılaşmada HOX genlerinin rolü yeni anlaşılmaya başlamıştır. Miyeloid löseminin $1,25(OH)_2D_3$ bağımlı farklılaşmasını anlamak için bu çalışmada HOX A9 gen ekspresyonuna bakıldı. HL-60 hücreleri 24 ve 72 saat süre ile $1,25(OH)_2D_3$'le muamele edildi. Gen ekspresyonu kantitatif, gerçek zamanlı PCR ile yapıldı. $1,25(OH)_2D_3$ tedavisinden sonra RPS9 ve HPRT genlerine kıyasla HOX A9 gen düzeyleri yüksek bulundu. Bizim çalışmamız, bu teknoloji kullanılarak $1,25(OH)_2D_3$ tedavisinde HOX A9 genini ölçen ilk çalışmadır. Sonuç olarak HL-60 hücrelerinde farklılaşma ile HOX A9 geninin overekspresyonu arasında belirgin ilişki vardır. HOX A9 geninin yüksek düzeyleri ile AML patogenezi ilişkisi araştırma konusudur.

Anahtar Kelimeler: Gen ekspresyonu, HOX A9, D vitamini, PCR.

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INTRODUCTION

Acute myeloid leukaemia (AML) is a disorder characterized by a differentiation block and the concept of the differentiation therapy of AML with vitamin D analogues has aroused considerable interest. There are many genes involved in vitamin D dependent differentiation of acute myeloid leukaemia cell line HL-60 cells[1]. Ligand inducible effects on differentiation are initiated through the direct activation or repression of target genes but the whole identities of such genes have not clear.

It has been demonstrated that hematopoietic cells express many HOX (homeobox) genes. Involvement of HOX genes in leukaemia and differentiation is just beginning to be appreciated. Several lines of evidence suggest that multiple genes of the HOX A, HOX B, and HOX C appear to play a role in lymphoid cells. Furthermore, several genes, such as HOX A9, HOX A10, HOX B3, HOX B7, and HOX B8, may control myelomonocytic differentiation. Among the traits of these myelomonocytic differentiation related genes, especially the HOX A9 gene is remarkable because it is also an AML translocation partner between the chromosomes 7 and 11[2,3].

Various methods are available for detecting gene expression levels, including northern blots, differential display, S1 nuclease protection and serial analysis of gene expression (SAGE). Here in this study we investigated the role of HOX A9 gene during $1,25(OH)_2D_3$ dependent differantiation of HL-60 cells, using real-time Quantitative RT-PCR (LightCycler) which is recently developed method for the rapid and sensitive detection of gene expression.

MATERIALS and METHODS

Cell Culture and $1,25(OH)_2D_3$ Treatment

Human myeloid HL-60 leukaemia cells were treated for 24 and 72 hours with $1,25(OH)_2D_3$ in isopropanol (5×10^{-8} M) in Iscove's modified Dulbecco's medium (IMDM; Sigma Diagnostics, St. Louis, MO, USA) with 10% fetal calf serum (FCS; Bioclear, Wilts, UK). Cells were harvested and RNA was extracted.

RNA Isolation and DNase I Treatment

Total RNA was isolated from specimens by using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. All RNA samples were treated with DNase I (Boehringer Mannheim, Mannheim, Germany) to remove the genomic DNA contamination in the preparations, according to the instructions of the manufacturer. 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.

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). Work was always carried out on desktop coolers (pre-cooled to 4°C). DNA Master SYBR Green 1 mix (Roche, Mannheim, Germany) was used and the master mixtures were prepared exactly as the recommendations of the manufacturer, except the concentrations of Mg, primers and volume of cDNA. 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 gene-specific primers were shown in Table 1. 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

Table 1. Primer sequences of the studied genes

Genes	Chromosomal location	Accession number (GenBank)	Primer sequences
RPS9 (Housekeeping)	19q13.4	U14971	CGTCTCGACCAAGAGCTGA
			GGTCCTTCTCATCAAGCGTC
HPRT hypoxantine	Xq26.1	NM 00194.1	ggCAgTATAATCCAAAgATggTCA
phosphoribosyltransferase 1			gTCTggCTTATATCCAACACTTCgT
(Housekeeping)			
HOXA homeobox A9	7p15	NM 002142.2	CCCAgCAgCCAACTggCTTCATgCgC
			CACTCgTCTTTTgCTCggTCTTTg

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 levels of housekeeping genes RPS9 and HPRT were used as an internal control for normalisation of RNA quantity and quality differences in all samples. A software tool was used for to obtain ratios, named relative expression software tool (REST), and two groups were compared: Treated HL-60 cells to nontreated HL-60 cells[4]. Ratios were calculated using the following formula:

$$\text{Ratio} = \frac{(E_{target})^{\,\wedge\,\text{Crossing Point target (MEAN control-MEAN sample)}}}{(E_{reference})^{\,\wedge\,\text{Crossing Point reference (MEAN control- MEAN sample)}}}$$

RESULTS and DISCUSSION

HOX A9 levels were found up-regulated as 131072-fold at 24 hours and 4096-fold at 72 hours after 1,25(OH)$_2$D$_3$ treatment, compared to housekeeping gene RPS9 levels. Similar results were found as up-regulated 4095-fold at 24 hours and 8192-fold at 72 hours compared to housekeeping gene HPRT. These findings were in parallel to previous studies which suggested that HOX A9 may control myelomonocytic differentiation[2].

We have previously demonstrated more than 40 genes were differentially expressed include down-regulated C-MYC after 1,25(OH)$_2$D$_3$ treatment of HL-60 cells, on a cDNA array study which contains of scanning 406 genes in human haematology genome[5]. Pan et al were studied the HOX B4 and C-MYC relation during the vitamin D treatment of the HL-60 cells. They were established that HOX B4 levels are regulated by 1,25(OH)$_2$D$_3$ and reveal that HOX B4 participates in the down-regulation of C-MYC expression[6]. Taking together, differentiation induction during 1,25(OH)$_2$D$_3$ treatment plays an important role in several gene expression pathways and the genes of the HOX family may be involved in the regulation of the C-MYC expression.

To our knowledge, our study is the first attempt to quantification of HOX A9 gene during 1,25(OH)$_2$D$_3$ treatment 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 HOX A9 gene. Based on our findings this method should be considered as an important choice of the HOX A9 gene expression analysis. We suggest that there is a clear relationship between differentiation induction and overexpression of HOX A9 gene in HL-60 cells. Possible correlation of high

expression levels of HOX A9 gene in AML pathogenesis remains to be established. Further gene expression studies may also help to understand the exact role of this gene in hematopoiesis.

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