Original Article

Correlation between Methylation and Expression Level of P15 and P16 Genes during Differentiation of Cord Blood Stem Cells into Erythroid Lineage Mediated by Erythropoietin

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Abstract

Background: Several influential factors such as transcription factors and intracellular signaling components are involved in differentiation of stem cells into a specific lineage. P15 and p16 proteins are among these factors. Accumulating evidences has introduced the epigenetic as a master regulator of these factors during lineage specification. The main objective of this study is to determine the correlation between the expression level and methylation pattern of P15 and P16 genes in erythroid lineage after in vitro differentiation by erythropoietin (EPO).

Materials and Methods: The purified and expanded CD34+ cord blood stem cells were differentiated into erythroid lineage in the presence of EPO. DNA was isolated from both cord blood stem cells and differentiated cells. The Real-Time PCR performed using cDNA and the isolated DNA was used in methylation Specific PCR (MSP) reaction for methylation pattern analysis in both pre and post differentiation stages.

Results: The study demonstrated that P15 and P16 genes have partial methylation after erythroid differentiation by EPO. The Expression of P15 gene was higher after differentiation and the expression of P16 gene had a slightly decreased level in post differentiation stage.

Conclusion: Significant increase in P15 gene expression after differentiation to erythroid lineage, suggests the remarkable efficacy of this gene in erythroid function. According to upregulation of P15 gene after differentiation despite unchanged methylation status and slight down regulation of P16 gene with slight hyper-methylation of the gene it can be suggested that although the methylation can affects the expression level of P16 gene, the P15 gene is not affected by this mechanism during erythroid differentiation mediated by EPO.

Keywords: Methylation, gene expression, stems cell, erythropoietin, differentiation

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Introduction

In vitro studies related to mechanisms of different cell types have led to decipher the general mechanisms of transcription and regulation of the gene expression[1]. Differentiation process in primitive cells is highly dependent on the control of gene expression and precise regulation of intracellular signaling that required specific controlling factors including cytokines, specific transcription factors and cell cycle regulators[2]. Two of these critical factors are cyclin dependent kinase inhibitors of 2A and 2B types, which are respectively known as P16 and P15[3].

P16 is a tumor suppressor and cell cycle control element that is capable of inducing differentiation and apoptosis in erythroid lineage[3]. Cyclin dependent kinase 4 (CDK-4) and CDK-6 proteins are potentially inhibited by P16, therefore Mouse double minute 2 homolog (MDM2) is not activated and P53 protein (which is normally decomposed by mdm2), continues to be undamaged in this condition and may subsequently inhibit the tumor progression[3]. The chromosomal location of P15 and P16 genes are adjacent to each other, therefore their deletion often occurs simultaneously in most tumors[3]. P15 gene is also a tumor suppressor protein that potentially inhibits D cyclin complex with CDK4 and CDK6 and thus controls the cell cycle at G1 point[3]. Recently there are numerous efforts to enlighten precise mechanism by which these genes are controlled during differentiation.

Epigenetics is an important topic that is introduced as one of the control pathways of gene expression and is defined as the changes in gene expression without any basic change in gene sequence[4]. According to extensive studies, epigenetics has been introduced as an undeniable principle in regulation of stem cell differentiation which is significantly capable of controlling all stages of growth and proliferation of different cells. One of the most studied mechanisms of epigenetics is the methylation of promoter region of genes which results in regulation of broad spectrum of genes[4,5].

Accumulating documents have indicated the contribution of epigenetics in cell evolution[6]. Tumor suppressor proteins which have significant role in changing the gene profile of differentiated cells can be potentially controlled by methylation[6,7].

Considering the fact that the correlation between the methylation pattern and expression level of P15 and P16 genes have not yet elucidated during differentiation, we have addressed this issue in EPO mediated differentiated cells and compared the results with CD34+ stem cells.

Methods

Isolation and expansion of CD34+ stem cells: Cord blood bags were collected from Sarem Hospital and Tehran blood transfusion organization. The CD34+ cells were purified using indirect CD34+ MicroBead Kit (Indirect CD34 MicroBead Kit, Milteny Biotech, Catalog no. 130-046-701) with MACS (Monoclonal Antibody Cell Sorting) method. The isolated cells were expanded using stem span medium which is enriched by growth factors Flt3, TPO and SCF. Isolated cells were washed with PBS buffer before the induction of differentiation.

Induction of erythroid differentiation: One part of expanded cells was used to isolate the DNA and RNA, and the second part cultured in the presence of EPO. In order to induction of stem cells differentiation by EPO, 500µl of prepared Iscove's Modified Dulbecco's Medium (IMDM) enriched by Stem Cell Factor (SCF) and Fetal Bovine Serum (FBS) and EPO (with final concentration of 100ng/ml) was used per 100,000 cells. The medium was replaced every two days. The mentioned cells were examined regarding the levels of successful erythroid differentiation after three or four successive replacement.

Table 1: Oligonucleotide sequences used in Real-time PCR reaction.

Oligo name	Sequence
P15-F	GGGAAAGAAGGGAAGAGTGTCGTT
P15-R	GCATGCCCTTGTTCTCCTCG
P16-F	GGGGGACCAGAGGCAGT
P16-R	GGTTGTGGCGGGGGCAGTT

F: Forward primer, R: Reverse primer

Confirmation of Erythroid differentiation: PCR reaction was performed using CD71 and CD235 cDNAs (as erythroid markers) and specific primers. Flow cytometry analysis was performed as a confirmative method using FITC-labled anti CD71 monoclonal antibody to identify CD71 surface marker. For the positive control the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene to ensure the presence of products.

DNA and RNA isolation from the expanded cells: DNA was extracted from both CD-HSCs and differentiated cells to be used in MSP reaction using DNA extraction Kit (QIAamp DNA Mini Kit, Qiagen, Catalog no. 51104) according to the manufacturer´s procedure. Total RNA was extracted using RNase Mini Plus Kit (RNeasy Mini Kit, Qiagen, Catalog no. 74104) and cDNA was synthesized using cDNA synthesis kit (QuantiTect Reverse Transcription Kit, Qiagen, Catalog no. 205311). DNA and RNA were stored at -20°C for further analysis.

Bisulfite treatment: At this stage, the stored DNA from the previous stage was processed using Epi Tect® Bisulfite kit of Qiagen Company (EpiTect Bisulfite Kit, Qiagen, Catalog no. 59104) and transferred to the -20°C freezer. SssI methylase (New England Biolabs, Catalog no. M0226S) was used to prepare the positive control for MSP technique.

Measurements of gene expression using Real-time PCR: Real-time PCR was performed separately for each gene using cDNAs of differentiated and undifferentiated cells. The data were interpreted using Pfaffl calculations. At this stage, GAPDH was used as internal control. ABI 7500 device and CYBER Green were applied as Real-time PCR instrument and nucleic acid stain, respectively. The sequence of the primers is indicated in table 1.

Methylation specific PCR: Next, MSP technique (EpiTect MSP Kit, Qiagen, Catalog no. 59305) was conducted for both groups separately using processed DNA from CD34+ and differentiated cells. The sequence of the primers used for MSP in this project

Table 2: The primer characteristics for P15 and P16 genes used in MSP reaction.

Gene	Product size (bp)	Cycles	Annealing Temp (°C)	Form*	5′ to 3′ Sequences
P15	151	38	50	UF	TGT GAT GTG TTT GTA TTT TGT GGT T
				UR	CCA TAC AAT AAC CAA ACA ACC AA
	150	38	60	MF	GGTTC GTA TTT TGC GGT T
				MR	CGT ACA ATA ACC GAA CGA CCG A
P16	249	35	60	UF	TTA TTA GAG GGT GGG GTG GAT TGT
				UR	CAA CCC CAA ACC ACA ACC
	237	35	65	MF	ATA A
				MR	TTA TTA GAG GGT TCT GAT CGC
					GAC CCC GAA CCG CGA CCG TAA

*UF: Unmethylated Forward, UR: Unmethylated Reverse, MF: Methylated Forward, MR; Methylated reverse

has been published by the same author earlier[3]. Details of primers and PCR conditions used in this step are listed in table 2.

Results

As previously mentioned, PCR was used to confirm the differentiation of stem cells into erythroid lineage. Banding patterns of specific erythroid differentiation markers in PCR indicated a favorable differentiation towards red blood cell precursors (Figure 1).

Analysis of CD71 surface marker by flow cytometry confirmed the successful erythroid differentiation in the cells differentiated by EPO. Using fluorescent isothiocyanate-labled murine anti CD71 monoclonal antibody (IgG1), flow cytometry results indicated 80% successful differentiation. Partec device and Cyflogic software were used to test analysis and data interpretation, respectively (Figure 2).

Real-time PCR results for P15 gene showed increased expression of this gene after differentiation. Expression of P15 gene in EPO dependent differentiated cells was approximately 8 times increased more than before differentiation.

The results for P16 gene indicated slight down regulation after EPO mediated differentiation. The expression level for this gene in CD34+ cells was approximately 2 times more than that in post differentiation stage. The normalized values and calibrated ratios for P15 and P16 genes are indicated in figure 3 and figure 4, respectively.

However the MSP results for P15 gene showed partial methylation pattern before and after differentiation without visible changes, the results for P16 revealed intense unmethylated pattern before differentiation (Figure 5) and a partial methylation after differentiation by EPO (Figure 6).

Discussion

Epigenetics and its control mechanisms lead to changes in gene expression without any changes in sequences[8]. However, epigenetics is not a general process in control of gene expression and it only affects certain genes[9]. Moreover different epigenetic mechanisms can be involved in control of all stages of cell growth and differentiation[10]. Based on

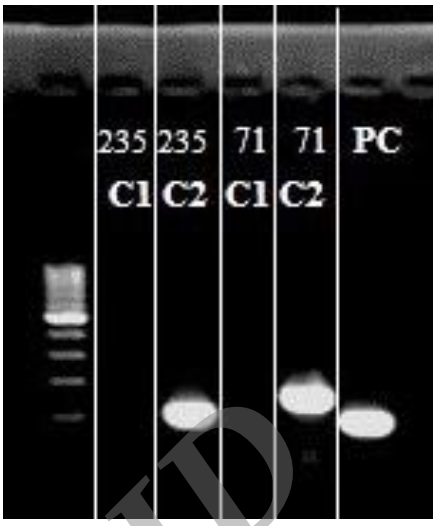
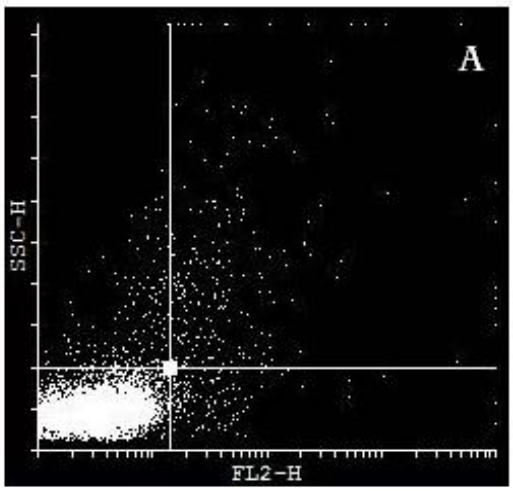


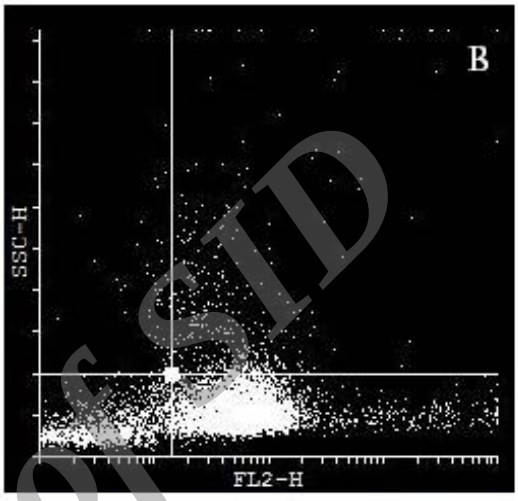
Figure 1. The C1 wells demonstrate the CD235 and CD71 cDNAs from undifferentiated cells. Absence of bands in both of the wells indicates weak expression of CD71 and CD235 in pre-differentiation stage. C2 wells represent the CD235 and CD71 cDNAs from EPO mediated differentiated cells which are sharply visible. PC represents positive control in which GAPDH gene was used as housekeeping gene.

numerous studies on formative and evolutional mechanisms, the differentiation into different cell types is highly dependent on precise control of gene expression and various components such as signaling pathways, transcription factors and tumor suppressor proteins. Two examples of these intermediate factors are P16 INK4a and P15 INK4b which are respectively known as P16 and P15. The main role of these factors is control of cell cycle in G1 point[3,11]. Transcription and tumor suppressor factors are two intermediate components associated with differentiation mechanisms, which have been evaluated in the present study. Having enough CpG islands in their promoter regions, these genes are hypothetically can be controlled by methylation[12-14]. In addition to the role of P15 and P16 in the CD34+ stem cell differentiation, other criterion for selecting of these genes is the potential association between the promoter methylation and their expression levels.

According to the results of present study, the genes in the research were evaluated regarding two aspects: I) expression level of P15 and P16 genes in cord blood stem cells was determined before and after differentiation into erythroid lineage. II) The methylation pattern in promoter region of these genes was determined to set up a rational relation between the achieved results. According to broad studies, the

	Number	% of vis	X mean	Y mean	X geomean	Y geomean
Visible	10000	100	16.22	104.85	3.41	93.74
Upper Left	165	1.65	7.17	312.71	5.99	291.83
Upper Right	224	2.24	444.07	445.79	50.41	402.98
Lower Left	9487	94.87	3.76	92.97	3.08	88.57
Lower Right	124	1.24	209.03	121.43	33.14	113.52





	Number	% of vis	X mean	Y mean	X geomean	Y geomean
Visible	10000	100	130.43	110.18	40.18	97.92
Upper Left	52	0.52	9.97	401.09	9.25	375.21
Upper Right	427	4.27	170.07	347.09	54.65	311.22
Lower Left	1483	14.82	4.78	69.91	3.53	62.82
Lower Right	8038	80.38	152.28	103.14	62.48	99.08

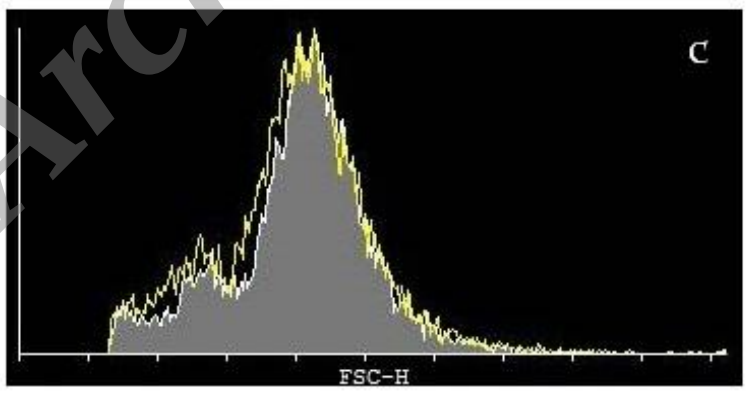


Figure 2. Transferrin receptor (CD71) surface marker expression analysis by flow cytometry. (A) Staining of differentiated cells with isotype matched control labeled to FITC. (B) CD71 expression after EPO mediated erythroid differentiation on cells conjugated to FITC. (C) Histogram showing 80% differentiated cells regarding to expression of CD71 surface marker.

methylation of promoters with CpG rich regions results in lack of expression in that gene. In contrast, hypo-methylation can allow the gene to be more transcribed. However, it is possible that in some cases the promoter of the genes may have partial methylation which leads to partial expression of the

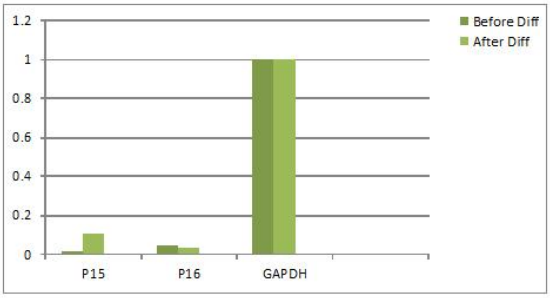


Figure 3. Normalized values of gene expression for P15 and P16 genes before and after EPO mediated erythroid differentiation.

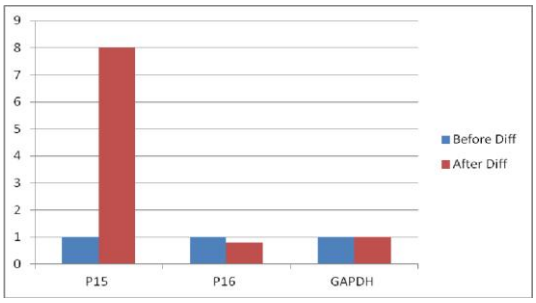


Figure 4. Calibrated ratios of gene expression for P15 and P16 genes before and after EPO mediated erythroid differentiation.

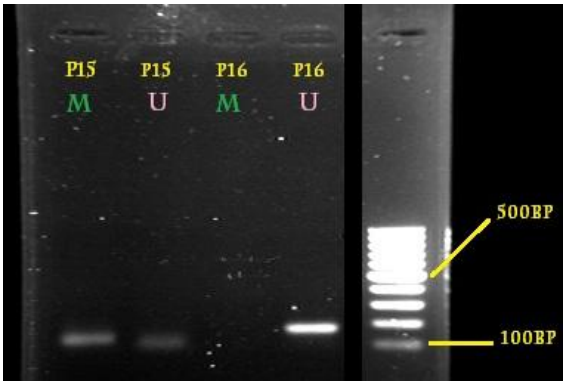


Figure 5. Methylation specific PCR results for P15 and P16 genes before differentiation. The wells indicated by the letter M (Green) are related to the reactions in which primers specific to the methylated genes have been used, and the wells marked with the letter U (Pink) is related to the reactions in which the target gene primers were used in unmethylated state.

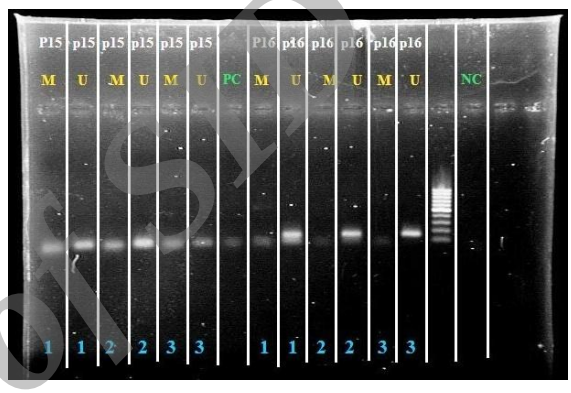


Figure 6. Methylation specific PCR results for P15 and P16 genes after EPO mediated erythroid differentiation. NC and PC (green) respectively represent negative and positive controls. The wells marked by letter M are related to the reactions in which primers specific to the methylated genes were used. And the wells marked by letter U (blue) are related to the reactions in which primers specific to the unmethylated genes were used. Rows 1, 2 and 3 (blue) represent three different annealing temperatures used in PCR reaction. The partial amplification patterns for both U and M forms suggest the partial methylation status.

gene as a predictable result of the condition. Nevertheless there is a complicated association among the gene expression level and methylation as well as many other epigenetic and non-epigenetic mechanisms that control the gene transcription[4,5]. Therefore, methylation status of genes cannot always be predicted through the expression level of the gene and there is multitude of controversy about this correlation, since the regulation of a particular gene is not exclusively dependent on one factor and there are numerous factors involved in such outcome in the cell[15,16].

As previously mentioned, MSP results for P15 gene in post-differentiation stage showed partial methylation and Real-time PCR results indicated an increased expression of the gene in post-differentiation stage, which is 8 times of the expression level in pre-differentiation stage. These results recommend the methylation as ineffective mechanism to regulate this gene[3].

It needs to be mentioned that According to the upregulation of P15 gene in differentiated cells and unchanged methylation pattern it can be deciphered that many other potential factors may be involved in this process which required further studies. There are always a group of different mechanisms involved in expression level of a gene that even may not be aligned together but ultimately what determines the level of gene expression is the sum of all pathways involved in controlling of gene expression. However the methylation is a potential mechanism to control special genes. Thus, when all the CpG islands in a particular allele are methylated, gene silencing is more probable than when methylation congestion in

promoter is lower. Taken together, it can be concluded that MSP method may not able to distinguish subtle differences in gene methylation level but also it can determine the presence or absence of methylation in larger amounts[3].

Lack of methylation in P16 gene before differentiation changed to partial methylation status in post-differentiation stage and this can justify the decreased expression of this gene. The results of gene expression analysis indicated a reduction in the expression compared with the pre-differentiation stage. These results suggest that methylation may be a possible mechanism involved in down regulation of P16 in post-differentiation stage because following the partial methylation in promoters, gene expression is slightly decreased. We assumed that P15 gene expression can potentially be affected by methylation but contrary to this impression, it is not much affected by this mechanism or may affected slightly which cannot be detected by MSP method. According to our results, expression of P16 gene can be controlled by methylation, in cord blood CD34+ stem cells and EPO associated differentiated cells. However more investigation is recommended to shed the light into precise mechanism controlling these two genes before and after erythroid differentiation.

Conclusion

Significant increased expression of P15 gene in EPO mediated differentiated cells suggests a more important role of this gene in differentiated or erythroid committed precursor cells. Moreover, considering the unchanged methylation pattern of P15 gene before and after erythroid differentiation of cord blood stem cells the upregulation of this gene in post-differentiation stage cannot be attributed to methylation mechanism. This can be attributed to non-methylation mechanisms for regulation of this gene or inability of MSP method to distinguish the subtle differences in methylation pattern of genes. However we demonstrated that down regulation of P16 gene can be due to hyper-methylation after erythroid differentiation. More studies are needed to elucidate the exact mechanisms controlling the gene expression during erythroid differentiation and also the crosstalk between these mechanisms.

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