Original Article *Iran J Ped Hematol Oncol. 2016, Vol6.No3,172-181*

Gene Expression and Promoter Methylation Status of VHL, Runx-3, E-cadherin, P15 and P16 Genes During EPO-Mediated Erythroid Differentiation of CD34+ Hematopoietic Stem Cells

Fatemeh Eskandari Msc[1], Hoda Pourkarim Msc [2], Mehdi Sahmani PhD [3], Mehdi Goudarzi PhD [4], Naser Mobarra PhD [5], Ali Dehghanifard PhD [6], Gholamreza Khamisipour PhD [7], Nasim Kalantari Msc [1], Hanieh Rahmani Msc[8] Mehdi Azad PhD [9,*]

1. Department of Hematology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran
2. Department of Hematology, Allied Medical School, Tehran University of Medical Sciences, Tehran, Iran
3. Department of Clinical Biochemistry, Cellular and Molecular Research Center, Qazvin, Iran
4. Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Science, Tehran, Iran
5. Stem cell Research Center, School of Medicine, Golestan University of Medical Sciences, Gorgan, Iran
6. Sarem Cell Research Center, Sarem Women's Hospital, Tehran, Iran
7. Department of Hematology, Faculty of Allied Medicine, Bushehr University of Medical Sciences, Bushehr, Iran
8. Department of Pharmacodynamics and Toxicology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
9. Department of Medical laboratory sciences, Faculty of Allied Medicine, Qazvin University of Medical Sciences, Qazvin, Iran

*Corresponding Author: Mehdi Azad. PhD. Department of Medical laboratory sciences, Faculty of Allied Medicine (Hematology), Qazvin University of Medical Sciences, Qazvin, Iran. Email Address: haematologicca@gmail.com.

Received: 28 November 2015 **Accepted:** 12 March 2016

Abstract

Background: VHL (von Hippel-Lindau), Runx-3 (Runt-related transcription factor 3), E-cadherin (Epithelial cadherin), P15 (INK4a, cyclin dependent kinase inhibitor), and P16 (INK4b) genes are essential in hematopoiesis. The aim of this study was to explore the correlation between gene expression and promoter methylation in CD34+ stem cells before and after differentiation to erythroid lineage.

Materials and Methods: CD34+ hematopoietic stem cells were separated from umbilical cord blood using MidiMacs (positive selection) system. Expanded CD34+ stem cells were differentiated into erythroid lineage with human recombinant erythropoietin (EPO). DNA extraction was done by QIAamp DNA Mini Kit. RNA was extracted using RNase Mini plus Kit. MSP (Methylation specific PCR) technique was done for methylation assay. Methylation status and expression assay was done for VHL, Runx-3, E-cadherin, P15, and P16 genes on both CD34+ stem cells and differentiated erythroid cells.

Results: The results showed that, before differentiation, P15 had comparative methylation pattern and average expression and it remained unchanged after differentiation (p=0.01). concerning P16, results revealed no methylation pattern and complete expression in absence of EPO and with EPO it changed to comparative status (p=0.01). E-cad and Runx-3 genes had relative methylation pattern and fully expression before and after differentiation but their expression after that, was increased and decreased Respectively (p=0.04). VHL gene had no significant methylation status before or after differentiation and its expression was complete (p=0.01).

Conclusion: The obtained results indicated that promoter methylation of P15, P16, VHL, Runx3 and E-cad was one of the definitive expression control mechanism of these genes.

Key words: Differentiation, EPO, Hematopoietic Stem Cells

Introduction

Studies about cell differentiation have led to access general mechanisms of regulating and specific factors of gene expression. In fact intracellular signaling and gene expression regulators are the most impressive factor for primary differentiation process in the cell (1). So the requirement for specific elements like cell cycle controlling molecules or cytokines is quite understandable. In mammalian cells DNA is methylated only at cytosine located 5' to guanosine in CpG

islands. Promoter CpG islands of the genes play an important regulatory role in normal and malignant cell development (2). So methylation pattern of CpG islands was an impressive tool to understanding both normal and aberrant gene expression, in this context current study try to find out the alternation of methylation pattern of five member of tumor suppressor gene family in stem cell before and after differentiation to erythroid lineage in presence of EPO (erythropoietin), which are follows: runt-related transcription factor 3 (RUNX3), Epithelial cadherin (E-cad), Von Hippel–Lindau (VHL), P15INK4a (Cyclin dependent kinase inhibitor) and P16INK4b promoter. RUNX proteins belong to a family of metazoan transcription factors that serve as a master regulators of development. They are frequently unregulated in human cancers (3). The RUNX gene family consist of RUNX1/AML1, RUNX2 and RUNX3. All RUNX family members share the central Runt domain, through which the proteins interact with core binding factor β (CBFβ) and bind to an specific DNA sequence. The RUNX family members play pivotal role in normal development and neoplasia. RUNX3 involved in neurogenesis and thymopoiesis, and also has a tumor suppressor gene function (4). According to other studies Runx3 act as a tumor suppressor gene that inhibits the gastrointestinal tumor formation more than other cancers (5). In addition, it has significant expression in normal and malignant CD34+ hematopoietic stem cells (6). E-cadherin is a component of the adherent junctions that stabilize the architecture of the non-compact myelin region (7). In other cell types, E-cadherin has been considered as a signaling receptor that modulates intracellular signal transduction and cellular responses. E-cadherin adhesion has been demonstrated to specifically affect pluripotent stem cells (PSC) fate. E-cadherin adhesion is required to maintain pluripotency (8, 9) and increased integrin adhesion-mediated factors promote differentiation (10). P15 (INK4b) and P16 (INK4a) are members of the INK4 family of cyclin dependent kinase (CDK) inhibitors (11). P15 is a common tumor suppressor gene (12, 13) that is activated in response to transforming growth factor β1 (TGF-β1) (11). Recently, the loss of P15 expression and their aberrant gene methylation have been reported in tumors and various human neoplasia, such as laryngeal squamous cell cancer (13), leukemia (11, 14), colorectal carcinoma (15). The p16INK4a is a cyclin-dependent kinase inhibitor which can be regulated by a feedback loop with Retinoblastoma (Rb) (11). It can inhibit cyclin D-Cdk4/6 thus preventing hyperphosphorylation of RB. In this manner, p16INK4A plays a role in inhibition of G1 to S transition in the cell cycle (13). P16 hypermethylation is associated with more tumor virulence in colon cancer and provides a growth advantage because it allows the evasion of early G1 checkpoint (11). In the study conducted by Minami et al in 2003, the role of P15 and P16 in apoptosis and inducible differentiation in erythroid lineage was showed. According to the p15 and p16 location on chromosome, their deletion is coincidental in most tumors. The next gene, VHL (von Hippel-Lindau) has an effect on HIF (hypoxy indiucible factor) ubiquitination and it's degradation. HIF is a transcription factor that regulates EPO expression in stress condition like hypoxia in renal cells. Since it is a tumor suppressor gene, therefore high level of HIF in most tumors highlighted the VHL role.

The aim of current study was to assay the correlation between gene expression and promoter methylation in CD34+ stem cells before and after differentiation to erythroid lineage in presence of EPO.

Materials and Methods

Isolation and Expansion of CD34+ Stem Cells

Human cord blood units were collected from Iranian blood transfusion organization. In the first step, mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation (density 1.077 g/mL, Sigma). Then CD34+ cells were purified by means of indirect CD34 Micro Bead kit using MACS method (miltenyibiotec, USA). After that harvested cells were counted by Hemacytometer. Expansion of cells were done using Stem Span medium enriched by recombinant human FMS-like tyrosine kinas 3 (Flt3, 50 ng/mL, Gibco, USA), thrombopoietin (TPO, 50 ng/mL, Gibco, USA) and stem cell factor (SCF, 50 ng/mL, Gibco, USA) and the medium was changed every two days. the isolated cells were divided into two groups after washing with PBS buffer. These cells was used for DNA and RNA extractions related to the pre differentiation stage.

Differentiation to Erythroid lineage

Cell differentiation was done by IMDM selective differential medium that supplemented with fetal bovin serum (FBS), recombinant human erythropoietin (EPO, 100 ng/ml, Gibco, USA) and stem cell factor (SCF, 50 ng/mL, Gibco, USA). The medium was changed every two days and after about two or three consecutive times, erythroid precursors were used for differentiation confirmation by flowcytometry analysis.

Erythroid Differentiation Confirmation

To confirm erythroid differentiation, flowcytometry was done by using monoclonal antibody against erythroid lineage differentiation marker (CD71) and PCR by using primers for CD235 and CD71 markers.

DNA and RNA Isolation

DNA and RNA extraction was done using Qiagen kit. RNA integrity was assayed by agarose gel electrophoresis.

DNA and RNA Modification for MSP and Real Time PCR Assay

DNA modification was processed using Qiagen EpiTect® Bisulfite kit . SssI methylase (New England Biolabs, Catalog no. M0226S) was used to prepare positive control for MSP technique. Subsequently, cDNA synthesis was performed using fermentase kit.

Gene expression Assay by Real Time PCR

Expression of target genes (VHL, E-cad, P15 , P16, Runx3) was assessed by Real time PCR before and after differentiation step by using Real Time PCR kit (ABI) and SYBER GREEN as fluorescent dye. GAPDH primer was used as internal control in this step.

MSP Reaction to Assess the Methylation Pattern of Genes

Methylation specific PCR was performed to evaluate the methylation status of the genes using bisulfite treated DNA of pre and post differentiated cells. MSP reaction was conducted for all the five genes using EpiTect MSP kit (EpiTect Bisulfite Kit, Qiagen, Cat no. 59104) according to the its protocol.

Statistical Analysis

Statistical analysis was performed using SPSS (V.16) software and $p < 0.05$ was considered significant.

Results

Erythroid Differentiation Confirmation Using Flowcytometry and PCR

The purity of isolated cells from umbilical cord blood after expansion was done using anti-CD34 monocolonal antibody and Flowcytometry system. Phycoerythrin was used as fluorescent dye for anti-CD34 labeling in this step. Flowcytometry result for differentiated cells in presence of erythropoietin was shown in Figure I. Results showed 78% of CD71 expression. In addition, PCR results indicated the desirable erythroid differentiation (Figure II). There was no product in C1 Column, and no expression of CD71 and CD235. The result showed positive PCR reaction

for CD71 and CD235 in column C2. MSP results for undifferentiated cells was shown in table I and illustrated that the size of DNA for studied genes was approximately the same. Figure III showed the MSP Results after electrophoresis on 1.5% agarose gel. DNA bands were related to PCR products for methylated and unmethylated status of studied genes. M (red) was for reaction with methylated primers and the U (blue) was for unmethylated ones. Since the results showed ,with methylated and unmethylated primers for *Runx3, E-cad,* and *P15* genes; So a relative methylation and expression was predictable in this step. Two other genes, *P16* and *VHL*, reacted with only unmethylated primers so this indicated the unmethylation pattern for their promoters, they also had complete expression in this step. MSP results for differentiated cells in presence of EPO showed relative methylation status for *E-cad, Runx3, P15* and *P16* genes because they had a reaction with both methylated and unmethylated primers and this eveidence also indicated relative expression in this step (p=0.04) , (p=0.01) and (p=0.01) respectively . The results showed VHL gene had no methylation pattern and fully expression status (p=0.01) (Figure IV).

Real Time PCR Results for Gene Expression

Relative expression of *VHL, E-cad, Runx3, P15* and *P16* genes for differentiated and undifferentiated cells in presence of EPO was done by using of Real Time PCR kit (ABI,USA) and thermo cycler (Step One model, ABI,USA) system. Figure V revealed the comparative CT (cycle threshold) values for genes before and after differentiation to erythroid lineage in a presence of EPO. Normalized values and calibrated ratio of gene expression was displayed in figures VI and VII respectively. Quantitative Real Time PCR Results showed a relative gene expression after calibration stage. According to these results, expression level of studied genes after differentiation step to Erythroid lineage in presence of EPO were respectively 1, 0, 125, 25 and were higher than before differentiation step.

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Tabel I: Size of DNA before and after methylation

Gene	Methylated Size	Un-methylated Size
VHL	158bp	165bp
Runx3	130bp	130bp
E-cad	168bp	178bp
P16	150bp	151bp
P15	148bp	154bp

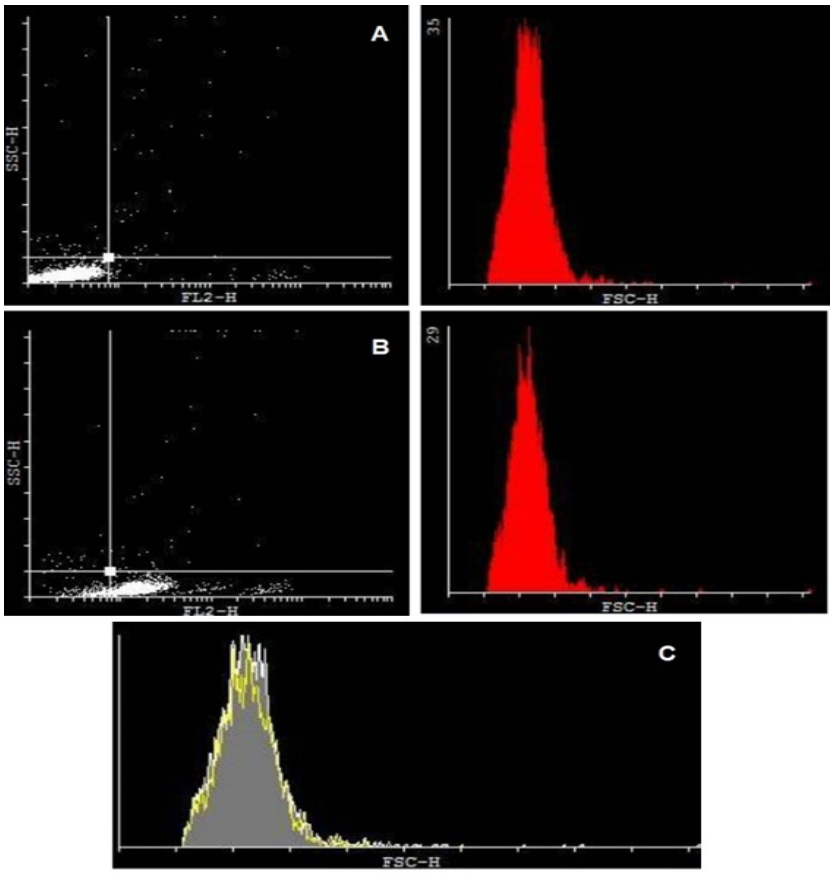

Figure 1. Transferrin receptor (CD71) expression in presence of erythropoietin. (A) related to Isotype control. (B) $CD71^{+}$ Erythroid differentiated cell. PE (phycoerythrin) dye is used for antibody labeling in this step. (C) %78 of primary cells differentiated to erythroid lineage

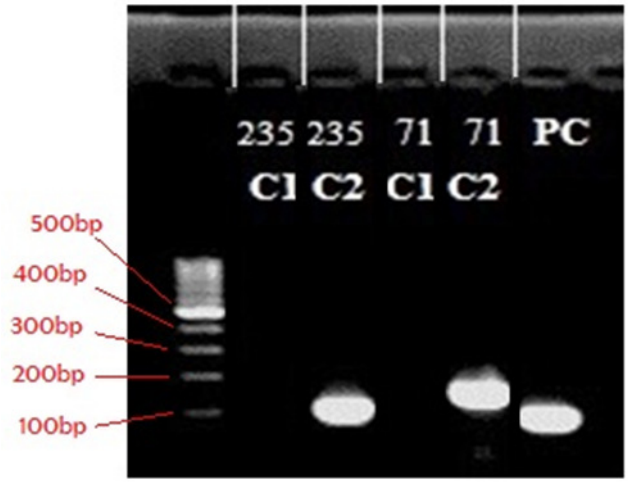

Figure 2. C1 Columns showed CD71 and CD235 expression in undifferentiated cells and C2 columns was in differentiated cells for these two markers.

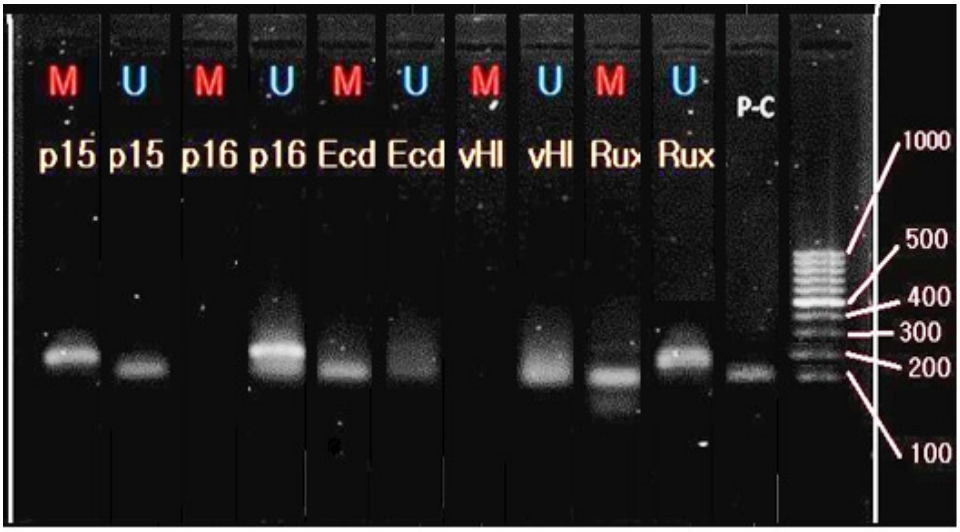

Figure 3. MSP results for differentiated cells. M (red) was for reaction with methylated primers and the U (blue) was for unmethylated ones.

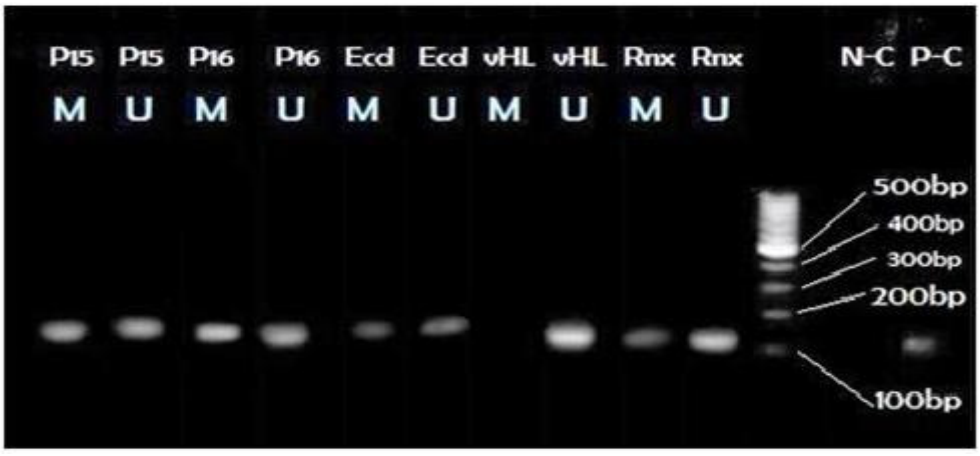

Figure 4. MSP results for studied gens in differentiated cells in presence of EPO

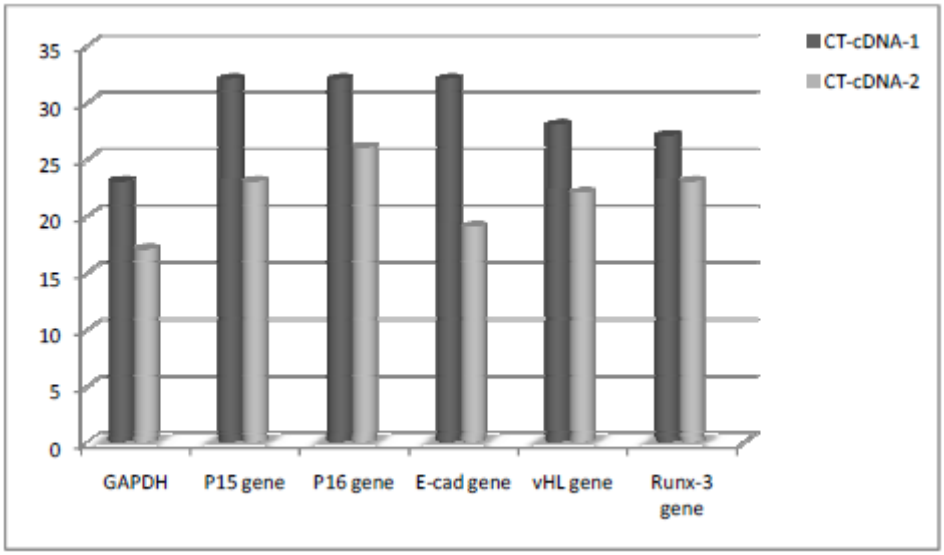

Figure 5. CT values from Real-Time PCR, before and after the differentiation to Erythroid lineage in presence of EPO

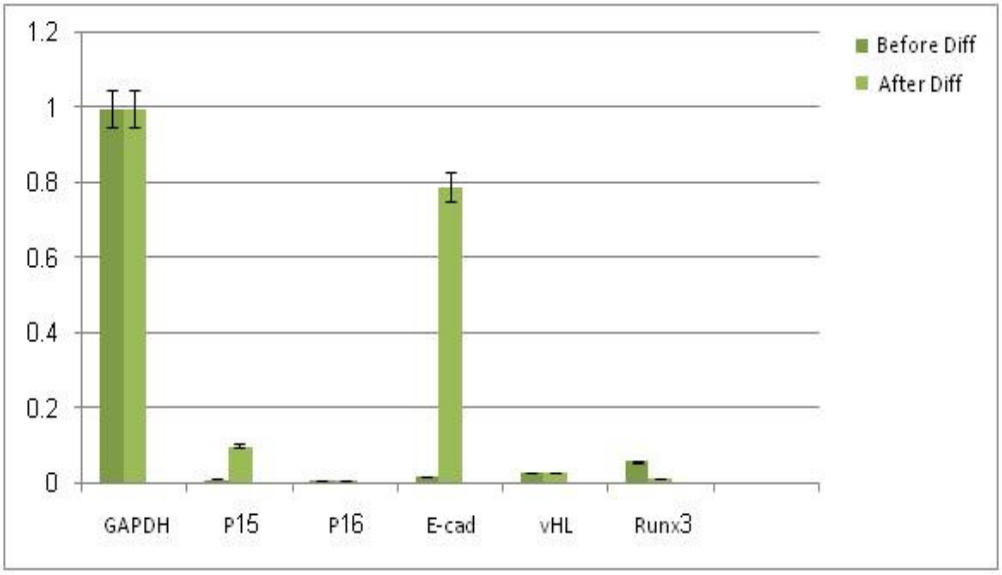

Figure 6. Normalized values of gene expression before and after the differentiation

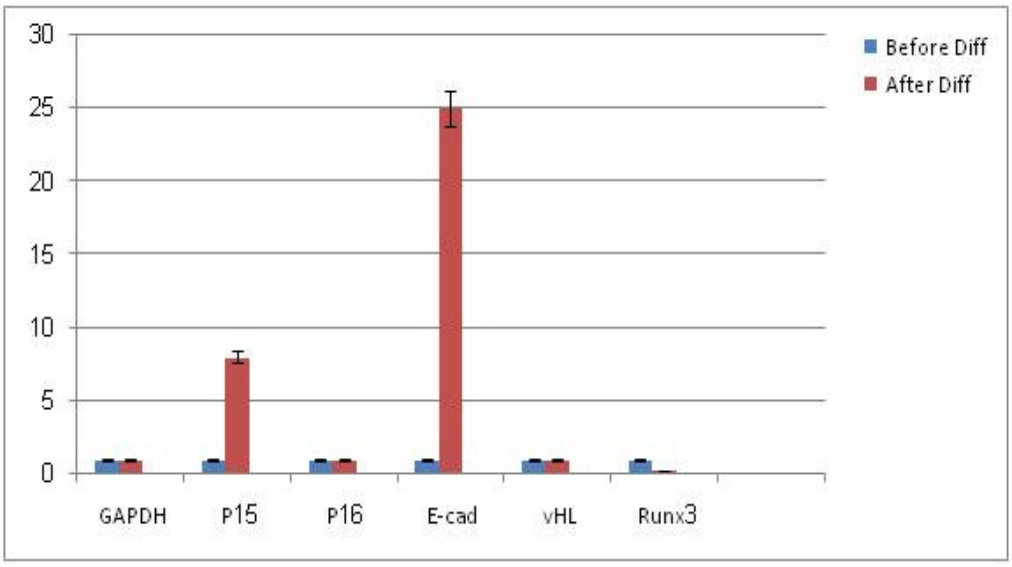

Figure 7. Calibrated ratio of gene expression before to after the differentiation

Discussion

In 2010, Hung et al has investigated the methylation pattern of mouse hematopoietic stem cell meanwhile of their differentiation (16). Their target genes were a group of genes with an important role in differentiation to CFU-L and CFU-GEMM lineages. According to this study, increased methylation of CD34+ stem cells can improve their lymphoid differentiation. in field of differentiated mouse stem cells to erythroid lineage, studies about pattern of methylation showed that, most of the CD34+ stem cell genes have no methylation status before or after differentiation (17). Sorin armeand was the first one who mentioned the role of E-cad in hematopoesis (18). In the study which was done in 2004, the result revealed that there is meaningful relation between methylation pattern of E-cad and clinical symptoms in patients with multiple myeloma and monoclonal gammopathies (19). In another study in 2008, the result showed that patients with hypermethylation in E-cad promoter have poor prognosis of disease. (9). In this study, methylation pattern of E-cad was investigated in cord blood CD34+ hematopoietic stem cells, before and after differentiation to Erythroid lineage. The result of presented study showed that E-cad have relative methylation before and after of differentiation in target cells. In another words, its expression in the status of promoter methylation was reduced to some extent, but it was still expressed. Real time PCR results confirmed the important role of E-cad in metabolism of CD34+ stem cells before differentiation. After differentiation to Erythroid lineage in presence of EPO, E-cad expression was 125 times higher than before differentiation ,which can indicated its more important role after differentiation. P15 and P16 were other studied genes which were tumor suppressor (20), and previous studies were mostly about the comparison of methylation pattern of these genes in malignant tissue with healthy ones. Herman et al in 1996, showed that hypermethylation of P15 promoter region lead to decrease in expression of that and this pathway was one of the P15 expression regulatory mechanism (21). In another study in 1998, p15 hypermethylation in MDS (Myelodysplastic Syndrome) patients related to poor prognosis and increase the

probability of shifting to ALL (Acute Lymphoid Leukemia) (22). James et al obtained the important results in their study in 1997. These significant results showed that the decrease of p15 and p16 expression was the most important factor in malignancies. In current study the methylation status of P15 gene was relative before and after differentiation. P16 gene had no methylation pattern in CD34+ stem cells before differentiation but after differentiation in presence of EPO, it changed and had relative methylation pattern. In this study P15 and P16 expression level was higher than CD34+ cells. VHL gene was studied by Herman for the first time and his work showed that in the most malignancies with promoter methylation, remarkable reduction in its expression was happened, too (21). There was a lot of evidence which showed that with methylation of VHL leads to defective expression of this gene and decreased function in human tumors. (23, 24). No study was done about expression status and methylation pattern of VHL in stem cells specially hematopoietic type before and after differentiation to erythroid lineage. In this study showed the methylation pattern of the gene to some extent. In fact, no methylation was seen for VHL before or after differentiation in presence of EPO. Real time PCR results indicated that VHL expression in CD34+ hematopoietic stem cells were 7.2 times higher than erythroid differentiated cells (25). In 2003, Kalev et al showed that RUNX3 was necessary for hematopoietic cells production (26). Bac et al study in 2004 showed the important role of Runx3 in TGF-B signaling. The study of Li et al revealed the effect of RUNX3 hypermethylation in malignancy (27). In other several studies, the role of Runx3 hypermethylation in colon cancer and melanoma pathogenesis was described (28, 29). The result of this study for Runx-3 hypermethylation status in CD34+ hematopoietic stem cells before and after differentiation to erythroid lineage in presence of EPO, was the same, and had relative methylation pattern in promoter region. From the point of expression view its expression was 4 times higher in comparison to erythroid progenitors.

Conclusion

The results that obtained from this study indicated that promoter methylation of p15, p16, VHL, Runx3 and E-cad was one of the definitive expression control mechanism of these genes. According to these five genes expression in all steps including before and after differentiation, the obtained results revealed the important role of these factors in CD34+ stem cells metabolism before and specified role in RBC committed progenitors after the differentiation. In the expression level of these genes, not only an acceptable similarity was seen but also some contradiction was existed. It may because of that this study was done by MSP techniques, which can show only the quantitative methylation not the qualitative one. These differences and other factors that involved in expression, all together, were the reason for the conflict between expression and MSP results.

Conflict of interest

There was no conflict of interest.

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