Original Article

Ineffectiveness of Methylation in Rgulation of VHL, ECAD, and RUNX3 Genes in Erythroid Cells Differentiated by Erythropoietin

Mehdi Azad[1], Mehdi Goudarzi[2], Ali Dehghanifard[3], Mousa Vatanmakanian[4], Mehdi Sahmani[*5]

[1]Department of Medical laboratory sciences, Faculty of Allied Medicine, Qazvin University of Medical Sciences, Qazvin, Iran
[2]Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Science, Tehran, Iran
[3]Sarem Cell Research Center, Sarem Women's Hospital, Tehran, Iran
[4]Department of Hematology, Allied Medical School, Tehran University of Medical Sciences, Tehran, Iran
[5]Department of Clinical Biochemistry and Genetics, Cellular and Molecular Research Centre, Qazvin University of Medical Sciences, Qazvin, Iran

Abstract

Background: Vast variety of intermediate factors including cell cycle regulators, growth factors, transcription factors, and signaling pathways are involved in hematopoietic stem cell (HSC) commitment and differentiation into distinct lineages. VHL, Ecad, and RUNX3 are among these. Epigenetics is currently introduced as a potential mechanism to control the gene regulation. The aim of this study is to reveal the correlation between the expression level and methylation pattern of mentioned genes after in vitro differentiation of cord blood HSCs into erythroid lineage mediated by erythropoietin.

Materials and Methods: After isolation and expansion, the CD34+ cord blood stem cells were divided into two parts. The first part was used to extract the DNA and RNA and the second to differentiate into erythroid lineage. Methylation specific PCR (MSP) and Real-time PCR were used to determine the methylation status and expression levels of the genes, respectively.

Results: Although the significant upregulation observed for VHL and Ecad genes and a down-regulation for RUNX3 gene after differentiation, no remarkable changes were seen in methylation pattern compared with cord blood HSCs by MSP technique.

Conclusion: It is appearing that methylation pattern in promoter region has not an effective role in expression of VHL, Ecad, and RUNX3. Moreover, considering the inability of MSP method to detect subtle differences in methylation level a more sensitive method is needed to distinguish the methylation levels of these genes before and after erythroid differentiation.

Keywords: Methylation, gene expression, erythropoietin, differentiation

***Corresponding Author:** Mehdi Sahmani, Department of Clinical Biochemistry and Genetics, Cellular and Molecular Research Centre, Qazvin University of Medical Sciences, Qazvin, Iran. Tel: (+98) 283 3359501, Fax: (+98) 283 3338034, Zip code: 3419759811; Email: m.sahmani@gmail.com

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Introduction

Currently abundance of knowledge on fundamental principles of cell biology and evolutionary mechanisms of tissues especially about gene expression has been gained[1]. Differentiation of a primary stem cell into distinct cell lineages is regulated by highly complicated pathways such as cytokines, transcription factors, cell cycle regulators, proliferation, apoptosis, and a variety of signaling

pathways[2]. Three of these key regulators are briefly introduced bellow.

Von Hippel Lindau (VHL) gene encodes Von Hippel Lindau protein which consists of three subunits. VLH protein degenerates the HIF protein by ubiquitination. HIF is a specific transcription factor for EPO gene especially in renal cells in hypoxia condition. VHL is also a tumor suppressor agent and a cell cycle regulator. This factor accelerates the angiogenesis by upregulation of VEGF (Vascular Endothelial Growth Factor) and regulates the cell cycle progression by suppression of mTOR gene[2].

Runt-Related Transcription Factor 3 (RUNX-3) is involved in cell cycle regulation, and differentiation. This factor is also a tumor suppressor. Downregulation of RUNX3 is extensively reported in malignancies[3].

Calcium Dependent Adhesion E Gene (E-cad) codes for Ecad protein which has an essential role in intracellular adhesions thereby it affects the evolutionary processes and differentiation[4].

Currently emerging evidences are presenting the epigenetics as an inevitable contributor in regulation of cell differentiation which affects proliferation and differentiation of many cell lineages in all the evolutionary steps. Epigenetics is defined as the alteration in gene expression as well as mitotic status without any changes in nucleotide sequences[5-7]. DNA methylation is one of the well studied epigenetic mechanisms, which can regulate the gene expression[8].

Although there are much of ambiguities on details of evolution and differentiation mechanisms, extensive studies on transcription factors, tumor suppressors, and cell cycle regulators have enlighten many aspects of the issue. The factors selected for present study have essential role in hematopoietic stem cell (HSC) differentiation, moreover considering their promoter structure these genes are suitable candidates to be evaluated regarding promoter methylation during differentiation.

Since no descriptive study has been accomplished on methylation status and expression levels of VHL, Ecad and RUNX3 genes, we provided beneficial information on this issue as well as presented the possible correlation between the methylation and expression level by comparing these two parameters in CD4+ cord blood stem cells (CB-HSCs) and erythropoietin (EPO) associated differentiated cells.

Methods

Isolation and expansion of CD34+ CB-HSCs

Cord blood bags were provided from Sarem hospital (Tehran, Iran) and Tehran Blood Transfusion Organization. The CD34+, CB-HSCs were isolated from cord blood using MACS (Monoclonal Antibody Cell Sorting) method and Indirect CD34+ MicroBead Kit. The cells were then cultured in Stem Span medium enriched by Flt3, TPO, and SCF growth factors to be expanded.

Induction of erythroid differentiation

Pure and expanded CD34+ cells were used to be differentiated into erythroid lineage by EPO.

To differentiate the CB-HSCs into erythroid lineage by EPO, 500µl of prepared IMDM medium enriched by EPO, SCF and FBS were used per 100,000 cells. EPO was added by the final concentration of 100ng/ml. the medium was replaced each 48 hours and after 7 days. The cells were evaluated regarding the successful differentiation.

Confirmation of erythroid differentiation

Using specific primers for CD71 and CD 235 genes (as erythroid specific markers) polymerase chain reaction was performed to ensure the expression of the genes. It should be noted that CD71 and CD 235 cDNAs were used in this step to evaluate the gene expression. The products were resolved on 1.5% agarose gel. We also conducted flow cytometry analysis to confirm erythroid differentiation using FITC-labeled monoclonal anti-CD71.

DNA and RNA isolation and cDNA synthesis

DNA was isolated from either CD-HSCs or differentiated cells to be used in MSP reaction using DNA extraction Kit (Qiagen) according to the manufacture procedure. Total RNA was extracted using RNase Mini Plus Kit (Qiagen) and cDNA was synthesized using cDNA synthesis kit (Fermantase).

Bisulfite treatment

DNA from pre and post differentiation stages were treated with bisulfite using EpiTect® Bisulfite Kit (Qiagen) and stored in -20°C. DNA treated with *SssI* was used as positive control in MSP reaction. Untreated DNA was applied as negative control.

Real-time PCR

cDNA from CB-HSCs and differentiated cells were used in Real-time PCR reaction. ABI 7500 device and CYBER Green were applied as Real-time PCR instrument and nucleic acid stain, respectively, and results were interpreted using Pfaffl calculations. GAPDH was selected as internal control of the genes. The primer sequences were those which previously described by the same author[9].

Methylation specific PCR

DNA from both CB-HSCs and EPO mediated differentiated cells were applied in MSP reaction to evaluate the methylation pattern. Specific primers for either methyalted or unmethylated forms were used which have been described in details previously by the same author[9].

Results

To determine successful erythroid differentiation we performed both PCR and flow cytometry analysis. Using CD71 and CD 235 cDNAs in PCR reaction revealed remarkable upregulation of these genes in EPO mediated differentiated cells, which suggests a high efficacy of EPO to differentiate the CB-HSCs into erythroid lineage *in vitro* (Figure1).

The flow cytometry results also indicated that CD71 surface marker is well expressed on EPO associated differentiated cells. These results showed 81% expression of CD71 surface marker on differentiated cells compared with CB-HSCs (Figure 2).

VHL, Ecad, were upregulated and RUNX3 gene was downregulated after erythroid differentiation

Using Real–time PCR method we observed significant upregulation for VHL and Ecad and a downregulation for RUNX3 gene after differentiation by EPO. VHL, Ecad, and RUNX3 genes changed respectively 7, 3.5, and 0.25 fold in differentiated cells compared with CB-HSCs. Figure 3 and figure 4 indicate normalized values and calibrated values for gene expression, respectively.

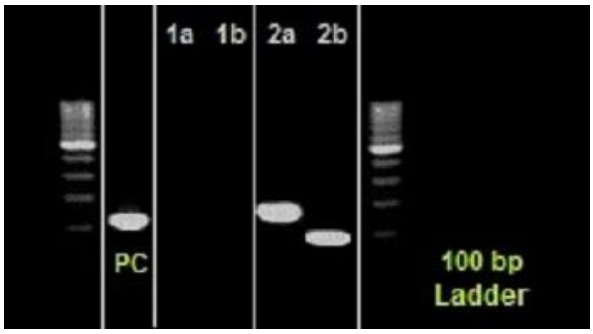

Figure 1. PCR analysis of CD71 and CD 235 cDNAs from CB-HSCs and differentiated cells. The patterns revealed sharp bands for the products related to differentiated cells. 1a and 1b lines indicate cDNAs from CB-HSCs. 2a and 2b lines indicate cDNAs from differentiated cells.

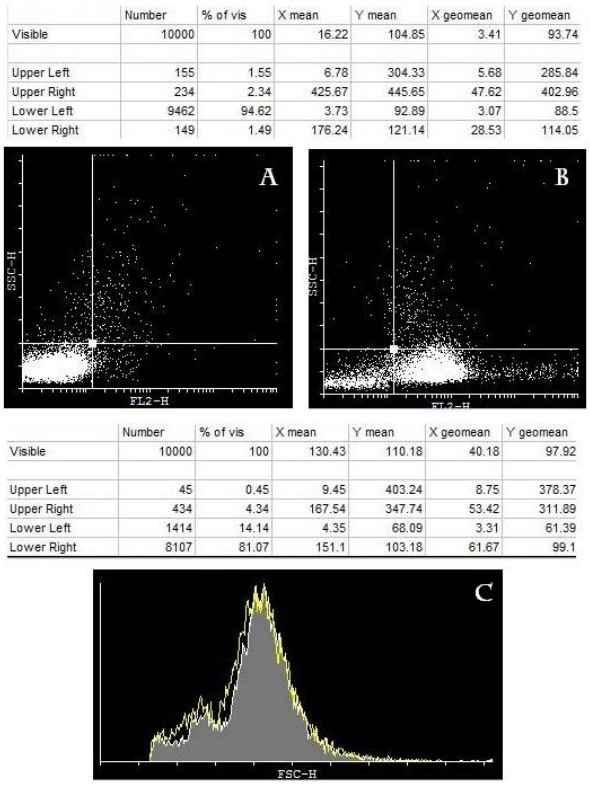

Figure 2. CD71 surface marker analysis by flow cytometry. (A) Staining of differentiated cells with isotype matched control labeled to FITC. (B) CD71 expression after EPO mediated erythroid differentiation in cells conjugated to FITC. (C) Histogram showing 81% expression of CD71 surface marker on differentiated cells.

Methylation Specific PCR results

After bisulfite treatment MSP reaction was performed and the products were resolved on a 1.5% agarose gel. Product size for the three genes was according to bellow.

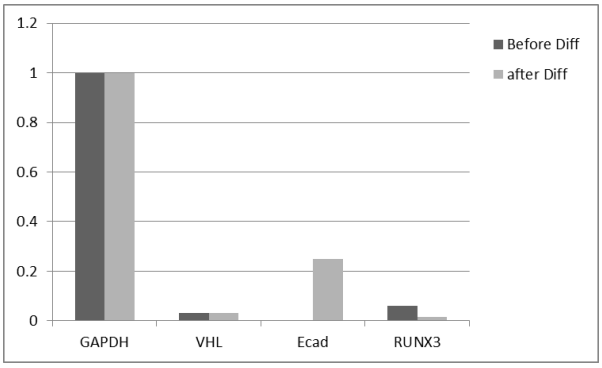

Figure 3. Normalized values of gene expression for VHL, Ecad and RUNX3 genes before and after EPO mediated erythroid differentiation.

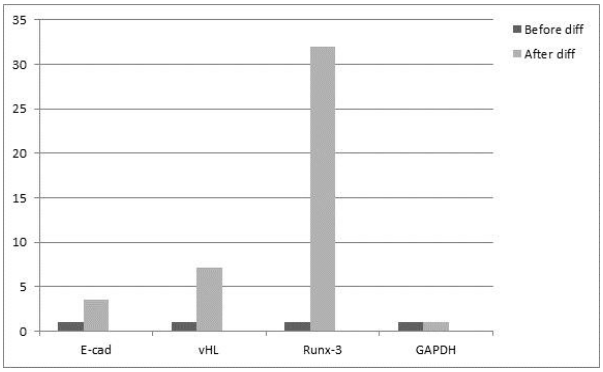

Figure 4. Calibrated ratios of gene expression for VHL, Ecad and RUNX3 genes before and after EPO mediated erythroid differentiation.

1) Methylated vHL Gene – 158 bp
2) Unmethylated vHL Gene – 165 bp
3) Methylated RUNX3 Gene – 130 bp
4) Unmethylated RUNX3 Gene – 130 bp
5) Methylated E-Cad Gene – 168 bp
6) Unmethylated E-Cad Gene – 178 bp

Ecad and RUNX3 genes showed partial methylation pattern in differentiated cells which were the same as the patterns in CB-HSCs that has been previously described[9].

The results for VHL genes revealed no amplified of methyalted primers and a well amplified pattern for unmethylated primers (Figure 5).

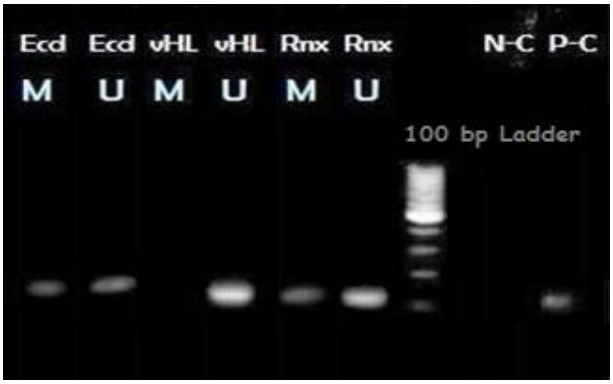

Figure 5. Methylation specific PCR results indicate the patterns for methyalted and unmethylated forms of VHL, Ecad and RUNX3 genes. The wells marked by U letter represent the reaction in which unmethylated primers have been applied and those which marked with M letter represent the reactions with methyalted primers. N-C and P-C indicate the negative and positive control, respectively.

Discussion

Epigenetics has been introduced as one of the most critical regulator for gene expression and is defined as the mechanisms by which the gene expression is affected but the sequence remain unchanged. It should be noted that not all the genes are regulated via epigenetic elements but some genes have a particular structure that allows them to be controlled by these mechanisms. The most delighted epigenetic mechanism is DNA methylation. Many vital processes including cell and tissue evolution, proliferation, apoptosis, and differentiation have been reported to be regulated through DNA methylation[10]. Among these processes, hematopoietic stem cell differentiation is an ideal model to study the epigenetic mechanisms[11]. In this study we have addressed this issue for VHL, Ecad and RUNX3 genes as factors contributing in HSC differentiation.

A body of valuable concepts on tissue formation and gene regulation has been presented so far, but many of precise mechanisms by which stem cells differentiate into specific lineages have yet to be fully determined. Currently it has been deciphered that stem cell commitment into specialized cell lineages is mainly controlled via gene expression level which in turn can be regulated by transcription factors, cell cycle regulators, and signaling pathways[12-15].

Three of these regulators are VHL, Ecad, and RUNX3 that assumed to control hematopoietic stem cell differentiation into erythroid lineage have been evaluated in present study. Having sufficient CpG islands in their promoter region, these factors are potentially supposed to be regulated by DNA methylation during erythroid differentiation. VHL protein is a regulator for self-renewing capacity and is also a tumor suppressor. The VHL gene deletion has

been reported in a wide spectrum of malignancies[16]. Runx3 is also a tumor suppressor agent and a transcription factor which controls the differentiation process of many cells. Runx3 has an essential role in cell cycle regulation by binding to specific promoter sequence of cell cycle regulator genes[17]. This factor has also a well-known role in differentiation of hematopoietic stem cells into thymocytes[10]. Ecad protein is an intracellular adhesion molecule which can thereby affects cell differentiation[18]. Ecad role in hematopiesis was introduced and confirmed in 1995[10]. The factors have a key role in regulating the HSC proliferation and self-renewing capacity, erythroblastic island formation in bone marrow, and HSC multi lineage differentiation[10]. These genes have a special promoter structure which may allow them to be controlled by epigenetic mechanisms (like methylation) without sequence alteration. In fact CpG islands rich promoter can be transcriptionally active or silent which is potentially determined by methylation[10]. As mentioned earlier, another criterion for selecting these genes is their putative role in HSC lineage specification.

Present study has covered the expression levels and methylation status of VHL, Ecad, and RUNX3 genes and a possible correlation between these two parameters after EPO mediated erythroid differentiation. We utilized EPO to differentiate the CB-HSCs into erythroid lineage in vitro and the cells were differentiated successfully, which confirms the efficacy of EPO during erythroid differentiation. High level expression of CD71 surface marker detected on differentiated cells substantiated the effectiveness of EPO not only in upregulation of CD71 gene (as a determinant of erythroid lineage) in transcriptional level, but also in translational stage of the CD71 mRNA.

Expression of VHL gene was in a high level in both pre and post differentiation stages which suggests the critical role of this gene in both stages. Ecad has a significant promotion of expression level in differentiated cells which suggests a much more important function of this factor in differentiated cells compared with CB-HSCs. The results for RUNX3 genes showed a decreased expression after differentiation by EPO.

We revealed the unsteady expression level for all the three genes before and after differentiation while the methylation status remained unchanged. These results suggest that although all the three genes have a potential of being regulated by promoter methylation but it is appear that methylation is not a potent mechanism to control these genes during erythroid differentiation. It is valuable to note considering that the MSP method is not able to disclose subtle differences in methylation quantity it is possible that there may be a little change in the DNA methylation which have not been detected via this method. Conduction of a well-established quantitative analysis for methylation status (eg. Bisulfite sequencing) is recommended to discover the real correlation between the methylation status and gene expression. We can also assume that other probable mechanisms such as loss of heterogeneity, protein mislocalization, and histone modification may be involved in controlling these genes during erythroid differentiation that required further investigations.

Conclusion

Significant upregulation of VHL and Ecad genes and a comparative downregulation for RUNX3 gene in erythroid lineage compared with CB-HSCs along with the unchanged methylation pattern suggest other controlling mechanisms or incapability of MSP method to detect the subtle changes in DNA methylation.

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